Pathophysiological and platelet anti-aggregatory effects of nitric oxide

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Thesis Summary

Background

The phenomenon of platelet hyper-aggregability, documented in several cardiovascular disease states, is a factor that predisposes subjects towards pathological thrombotic events. Moreover, decreased platelet responsiveness to exogenously donated nitric oxide, implying reduced sensitivity to both nitric oxide donors and to endogenous sources of nitric oxide, with the resultant failure to regulate platelet function, may also contribute to the pathological complications associated with coronary artery disease (CAD).

The series of studies described herein were designed to firstly examine the phenomenon of platelet hyper-aggregability and attenuated platelet responsiveness to donors of nitric oxide in a series of patient cohorts. The first series of experiments was also designed to examine what role superoxide plays within each phenomenon. In doing so a whole blood superoxide detection method was established. Following on from these initial investigations, a study was then undertaken to examine potential determinants of reduced platelet responsiveness to nitric oxide. Finally, the thesis examined the anti-aggregatory and vasomotor effects of acute and chronic nitrate pharmacotherapy in a cohort of subjects with mild to moderate stable angina pectoris (SAP). As the final investigation was performed in a randomized fashion, an examination of the effects of nitrate pharmacotherapy on the phenomenon of reduced platelet responsiveness to nitric oxide was performed, along with an examination of the phenomenon of nitrate tolerance development following chronic nitrate exposure. An examination of the incidence of possible platelet hyper-aggregability upon acute nitrate withdrawal, as a means of explaining the phenomenon of "rebound" ischaemia, was also performed.

Studies examining the phenomena of platelet hyper-aggregability and hypo-responsiveness to donors of nitric oxide; involvement of superoxide

Background

It has previously been demonstrated that platelets from patients with SAP are hyper-aggregable to ADP and hypo-responsive to the anti-aggregatory effects of the nitric oxide donors nitroglycerine and sodium nitroprusside. The mechanism/s behind platelet hyper-aggregability towards ADP and hypo-responsiveness to nitric oxide can be viewed simplistically as an imbalance between effects of those agents that govern platelet activation and of those that inhibit platelet function. Superoxide, a known scavenger of nitric oxide, may therefore play some part in both phenomena. The current study examined further the phenomena of platelet hyper-aggregability towards ADP and hypo-responsiveness to donors of nitric oxide in a series of samples from normal volunteers and patients with SAP or acute coronary syndromes (ACS). The current study also examined the role of superoxide within each phenomenon. In order to do this the current investigation was separated into three distinct, but yet related studies.

Study #1

Platelets from patients with SAP or an ACS were demonstrated to be hyper-aggregable in response to ADP and hypo-responsive to the anti-aggregatory effects of SNP or NTG. Investigating the role of superoxide within the phenomenon of platelet hyper-aggregability, the addition of superoxide dismutase (SOD)/catalase to a whole blood sample obtained from either a SAP or ACS subject, significantly inhibited the extent of ADP-induced platelet aggregation. Moreover, this effect was only observed in those samples obtained from patients and not in samples from normal volunteers (NV), suggesting a role for superoxide in the phenomenon of platelet hyper-aggregability.

Administration of SOD/catalase to whole blood samples was then demonstrated to have no overall effect on the extent of platelet responsiveness to SNP, in samples from either patients or NVs. However, when subjects were categorized according to history of angina (SAP/ACS versus NV) a significant inverse relationship was observed between baseline SNP responsiveness and the degree of change in SNP responsiveness post SOD/Catalase administration, a relationship that was absent from the NV cohort. The relationship between baseline SNP responsiveness and the extent of change in SNP responsiveness post

administration of SOD/catalase was shown to be bidirectional within the angina pectoris cohort. i.e. a restoration of platelet responsiveness to SNP was observed post SOD/catalase administration in those subjects with a "poor" initial SNP responsiveness, with deterioration in SNP responsiveness post SOD/catalase administration within those subjects with "good" baseline SNP responsiveness. From these results it was concluded that superoxide plays a critical role in not only subjects with an attenuated responsiveness to SNP, but also in those patients with a normal SNP response.

Study #2

As initial observations suggested a role for superoxide in the regulation of platelet aggregation in samples from patients, along with evidence of its involvement in the phenomenon of platelet hypo-responsiveness to nitric oxide, a method of detecting and quantifying the extent of whole blood superoxide was developed. Utilizing lucigenin-derived chemiluminescence (LDCL) superoxide was detected prior to and post induction of platelet aggregation in whole blood samples. Furthermore, the extent and rate of superoxide generation post induction of platelet aggregation was shown to correlate with the extent of platelet aggregation. A lag period between the induction of platelet aggregation and the commencement of the aggregation-associated generation of superoxide, was also observed and found to be a function of the concentration of platelet agonist used and the extent of platelet aggregation detectable prior to the aggregation-associated increase in LDCL.

Study #3

In whole blood samples obtained from a series of subjects that included NVs and patients with either SAP or ACS, the extent of baseline and aggregation-associated LDCL was found to be significantly greater within the cohorts of ACS patients compared to that of SAP or NVs. The extent of baseline or aggregation-associated LDCL was not related to the extent of platelet aggregation and platelet responsiveness to nitric oxide across all the subject cohorts examined. When the extent of LDCL (baseline or aggregation-associated) was expressed relative to platelet aggregation/SNP responsiveness, blood from ACS patients were shown to release significantly greater amounts of superoxide per unit aggregation compared to their SAP counterparts.

Conclusions

- Platelets from patients with SAP or ACS are hyper-aggregable to ADP (1μM) and hypo-responsive to the anti-aggregatory effects of SNP (10μM) compared to normal volunteers.

- Addition of SOD/catalase significantly inhibits the extent of platelet aggregation in whole blood samples obtained from SAP and ACS patients, an effect that was observed in patients only. The extent of inhibition of aggregation post superoxide scavenging was demonstrated not to be a function of the extent of platelet aggregation, suggesting that superoxide production stimulates platelet aggregation in all patients with acute/chronic ischaemic heart disease, but is not necessarily a major determinant of the extent of individual hyper-aggregability.

- Mean platelet responsiveness to SNP remained unaffected by the addition of SOD/catalase across all subject cohorts examined. However, baseline platelet responsiveness to SNP within those subjects with a history of angina pectoris was shown to be inversely correlated with the degree of change in SNP responsiveness post administration of SOD/catalase, a relationship that was absent from the NV cohort. These results may imply a role for superoxide in the phenomenon of platelet hypo-responsiveness to donors of nitric oxide.

- Utilizing lucigenin-derived chemiluminescence, superoxide was detected in an unstimulated whole blood sample. Induction of platelet aggregation initiated further release of superoxide in a platelet aggregation dependent fashion.

- The extent of baseline and aggregation-associated LDCL were found to be significantly greater within ACS patients compared to those obtained from SAP and NVs, emphasizing the potential for release of superoxide at the sites of vascular injury where platelet aggregation plays an integral part in thrombus formation.

- No significant relationship was found between the extent of platelet aggregation/platelet responsiveness to SNP compared to either the extent of baseline or aggregation-associated LDCL. These results suggest that the mechanism/s behind both phenomena are

more complex than an excess superoxide scavenging available nitric oxide equals platelet hyper-aggregability and/or platelet hypo-responsiveness to donors of nitric oxide.

Studies examining the determinants of platelet hypo-responsiveness to donors of nitric oxide

Background

Having confirmed that platelets from patients with SAP and ACS are hypo-responsive to the anti-aggregatory effects of nitric oxide, the second study was designed to examine the possible clinical determinants of platelet hypo-responsiveness to donors of nitric oxide in a number of subject cohorts that included NVs, non-ischaemic patients (NIP), SAP and ACS patients.

Study

Platelets from patients with SAP or an ACS were demonstrated to be hyper-aggregable towards ADP and hypo-responsive to the anti-aggregating effects of SNP compared to a series of control subjects that included NVs and patients who were initially hospitalized for unspecified chest pain but subsequently found to be free of CAD (NIP). When examining differences in the extent of platelet aggregation across the subject cohorts, a gender-dependent differential anti-aggregatory was observed between patients receiving/not receiving aspirin. The extent of platelet responsiveness to SNP was related to the extent of platelet aggregation but not found to be related to age or the number of coronary risk factors.

Utilizing univariate and multivariate analysis, presence of ACS, as distinct from SAP or NVs, was associated with significant attenuation of platelet responsiveness to nitric oxide. Pharmacotherapy with either perhexiline or statins was associated with an improvement in platelet responsiveness to nitric oxide, an effect that was absent with the use of various other anti-anginal medications. Furthermore, platelet responsiveness to SNP within those subjects with triple vessel disease reflected a response that was similar to that observed within the ACS subject cohort. These results imply a link between the severity of ischaemia and the extent of platelet responsiveness to donors of nitric oxide at least in the SAP population.

Conclusions

- By multivariate analysis, ACS was a determinant of significantly reduced responsiveness to the anti-aggregatory effects of nitric oxide.

- The extent of nitric oxide resistance was not correlated with any classical risk factors for CAD, but rather improvement in responsiveness may be observed with the use of perhexiline or statins.

- A retrospective observation was made regarding a gender-dependent differential anti-aggregatory effect between patients receiving/not receiving aspirin, with female patients apparently hypo-responsive to aspirin.

Studies evaluating the anti-aggregatory and arterial vasomotor effects of prophylactic nitrate pharmacotherapy

Background

Organic nitrates along with nitric oxide exert potent anti-aggregatory effects *in vivo*, effects that have also been demonstrated at an *ex vivo* level, generally with the use of high dose nitrate regimens. The current study was designed to investigate the anti-aggregatory effects of two commonly utilized nitrate preparations, SR isosorbide 5-mononitrate (ISMN) and transdermal-nitroglycerine (TD-NTG). Platelet responsiveness to nitric oxide donors and the effects on platelet aggregability following acute nitrate withdrawal were also examined during both acute and chronic nitrate exposure in a cohort of SAP patients.

Study

In a randomized crossover study of ISMN (120mg) versus TD-NTG (15mg/24hrs), neither nitrate regimen was shown to induce any significant change in platelet response to ADP, NTG or SNP as measured *ex vivo*, or in the levels of superoxide as detected by LDCL. Furthermore, there was no evidence of platelet hyper-aggregability during the "nitrate-free" periods. Utilizing applanation tonometry, both nitrate preparations were demonstrated to markedly reduce the augmentation index AI(x) at 4 and 8-hrs post acute dosing, an effect that persisted during chronic dosing but with evidence of partial tolerance development.

Conclusions

- Neither nitrate regimen examined in the current study was demonstrated to induce any detectable change in platelet aggregation, probably reflecting a lack of sensitivity of the technology used, rather than a lack of effect of each nitrate *in vivo*.

- Neither nitrate regimen was demonstrated to alter ADP-induced platelet aggregation, nor platelet responsiveness to either NTG or SNP, during chronic pharmacotherapy or nitrate-free periods, providing evidence for safety of both nitrate regimens.

- Both nitrate regimens were demonstrated to induce similar effects on pulse wave reflection, a process that is subject to attenuation during acute/chronic nitrate exposure, but which does not exhibit the "zero-hour" phenomenon.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the university library, being available for loan and photocopying.

Andrew S Holmes
(*January 2003*)

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Cheers

Publications and Presentations

Publications

Willoughby S.R., S. Stewart, Y.Y. Chirkov, J.A. Kennedy, **A.S. Holmes** and J.D. Horowitz, 2002, Beneficial clinical effects of perhexiline in patients with stable angina pectoris and acute coronary syndromes are associated with potentiation of platelet responsiveness to nitric oxide, European Heart Journal, 23, 1946-1954.

Chirkov Y.Y., **A.S. Holmes**, S.R Willoughby, S. Stewart and J.D. Horowitz, 2002, Association of aortic stenosis with platelet hyper-aggregability and impaired responsiveness to nitric oxide, Am J Cardiol, 90, 551-4.

Chirkov Y.Y., **A.S. Holmes**, S.R. Willoughby, S. Stewart, R.D. Wuttke, P.R. Sage and J.D. Horowitz, 2001, Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets, Journal of the American College of Cardiology, 37, 1851-7.

Worthley M.I., **A.S. Holmes**, C.J. Zeitz, Y.Y. Chirkov and J.D. Horowitz, 2001, Effects of diabetes mellitus and associated glycemic control on platelet function in unstable angina pectoris, Heart, Lung and Circulation, 10, A104.

Worthley M.I., **A.S. Holmes**, C.J. Zeitz, Y.Y. Chirkov and J.D. Horowitz, 2001, Glycemic control and platelet function in diabetic patients with threatened myocardial infarction. Proceedings of A.P.P.S, 32(2) Suppl 2 47P.

Worthley M.I., **A.S. Holmes**, C.J. Zeitz, Y.Y. Chirkov and J.D. Horowitz, 2001, Glycemic control and platelet function in diabetes patients with threatened myocardial infarction. Circulation, 104, II-378.

Chirkov Y.Y., **A.S. Holmes**, L.P. Chirkova and J.D. Horowitz, 2000, Nitrate resistance in platelets from patients with stable angina pectoris, Circulation, 102, e87.

Holmes A.S., M.I. Worthley, Y.Y. Chirkov and J.D. Horowitz, 2000, Do products of nitric oxide synthase cause platelet hyper-aggregability in patients with angina pectoris? Heart, Lung and Circulation, 9, A157.

Chirkov Y.Y., **A.S. Holmes**, L.P. Chirkova and J.D. Horowitz, 1999, Nitrate resistance in platelets from patients with stable angina pectoris, Circulation, 100, 129-134.

Holmes A.S., Y.Y. Chirkov and J.D. Horowitz, 1999, Increased superoxide concentration as a possible basis for platelet hyper-aggregability and nitric oxide resistance in patients with ischaemic heart disease, European Heart Journal, 20, 544.

Chirkov Y.Y., **A.S. Holmes**, R.D. Wuttke, S.R. Willoughby, S. Stewart, P.R. Sage and J.D. Horowitz, 1999, Determinants of platelet responsiveness to nitric oxide donors in the presence and absence of ischaemic heart disease, European Heart Journal, 20, 545.

Holmes A.S., Y.Y. Chirkov and J.D. Horowitz, 1999, Superoxide generation as a basis for platelet hyper-aggregability and nitric oxide resistance in patients with myocardial ischaemia. Australian and New Zealand Journal of Medicine, 29, 6.

Presentations

Abstract title: Platelet nitric oxide resistance in patients with congestive heart failure is ameliorated with ACE inhibition. Chirkov Y.Y., **A.S. Holmes**, J.M. Stepien and R.A. Anderson, World Congress of Cardiology, Sydney, 2002.

Abstract title: Aortic stenosis: Evidence of valvular endothelial dysfunction. Chirkov Y.Y., **A.S. Holmes**, S. Chandy, R. Kanna and J.D. Horowitz, World Congress of Cardiology, Sydney, 2002.

Abstract title: Aortic stenosis is associated with platelet hyper-aggregability and impaired responsiveness to nitric oxide. Chirkov Y.Y., **A.S. Holmes**, S.R. Willoughby, S. Stewart and J.D. Horowitz, World Congress of Cardiology, Sydney, 2002.

Abstract title: Is the coronary slow flow phenomenon associated with platelet hyper-aggregability or hypo-responsiveness to nitric oxide, **Holmes A.S.**, Y.Y. Chirkov, J.D. Horowitz and J.F. Beltrame, World Congress of Cardiology, Sydney, 2002.

Abstract title: Platelet hyper-aggregability and diminished platelet responsiveness to nitric oxide in patients with aortic stenosis. Chirkov Y.Y., **A.S. Holmes**, S.R. Willoughby, S. Stewart, J.D. Horowitz, American Heart Association meeting Anaheim, 2001 and the European Society of Cardiology Meeting, Stockholm 2001.

Abstract title: Platelet hyper-aggregability and diminished platelet responsiveness to nitric oxide in patients with aortic stenosis. Chirkov Y.Y., **A.S. Holmes**, S.R. Willoughby, S. Stewart and J.D. Horowitz JD, American Heart Association meeting Anaheim, 2001 and the European Society of Cardiology Meeting, Stockholm 2001.

Abstract title: Perhexiline improves platelet responsiveness to nitric oxide in patients with acute coronary syndromes. Chirkov Y.Y., **A.S. Holmes**, J.M. Stepien and J.D. Horowitz, American Heart Association meeting Anaheim, 2001 and the European Society of Cardiology Meeting, Stockholm 2001.

Abstract title: Effects of diabetes mellitus and associated glycemic control on platelet function in unstable angina pectoris. Worthley M.I., **A.S. Holmes**, C.J. Zeitz, Y.Y. Chirkov and J.D. Horowitz, American Heart Association meeting Anaheim, 2001.

Abstract title: Do products of nitric oxide synthase cause platelet hyper-aggregability in patients with angina pectoris? **Holmes A.S.**, M.I. Worthley, Y.Y. Chirkov and J.D. Horowitz, Australian and New Zealand Cardiac Society Meeting, Melbourne, 2000.

Abstract title: Superoxide generation as a basis for platelet hyper-aggregability and nitric oxide resistance in patients with myocardial ischaemia. **Holmes A.S.**, Y.Y. Chirkov and J.D. Horowitz, Australian and New Zealand Cardiac Society, Wellington, 1999.

Abstract title: Determinants of platelet responsiveness to nitric oxide donors in the presence and absence of ischaemic heart disease. **Holmes AS.**, Y.Y. Chirkov, R.D. Wuttke, S.R. Willoughby, S. Stewart, P.R. Sage and J.D. Horowitz, Australian and New Zealand Cardiac Society, Wellington, 1999.

Abstract title: Increased superoxide concentration as a possible basis for platelet hyper-aggregability and nitric oxide resistance in patients with ischaemic heart disease. **Holmes AS.**, Y.Y. Chirkov and J.D. Horowitz, European Society of Cardiology, Barcelona, 1999.

Abstract title: Determinants of platelet responsiveness to nitric oxide donors in the presence and absence of ischaemic heart disease, Chirkov Y.Y., **A.S. Holmes**, R.D. Wuttke, S.R. Willoughby, S. Stewart, P.R. Sage and J.D. Horowitz, European Society of Cardiology, Barcelona, 1999.

Chapter 1

Introduction: Pathophysiological and platelet anti-aggregatory effects of nitric oxide

Chapter 1: Overview

This chapter firstly describes the discovery, production and regulation of the potent platelet anti-aggregatory agent, nitric oxide. The chapter then examines normal platelet function with attention being given to the role of nitric oxide. This chapter will then discuss various models/methods that are used to assess both platelet and endothelial function.

Having discussed a number of phenomena that surround normal vascular function, this chapter will then examine various disturbances of endothelial/platelet function in relation to ischaemic heart disease. Particular attention will be paid to the phenomenon of "endothelial dysfunction" and pathological platelet aggregation. Within this context this chapter will then examine the utility limitations of nitrate pharmacotherapy, both at the vascular and platelet level, with specific reference being given to the phenomena of both nitrate tolerance induction and nitrate/nitric oxide resistance.

This chapter will then outline the rationale for undertaking a series of studies that involve elucidation of potential mechanisms, determinants and clinical implications of platelet hyper-aggregability, hypo-responsiveness to nitric oxide and nitrate tolerance induction in subjects with acute and chronic ischaemic heart disease.

Table 1.1 Abbreviations used in this chapter

Abb	Definition	Abb	Definition	Abb	Definition
1,2-GDN	*1,2-Glyceryl dinitrate*	HEARTS	*Healing and Early Afterload Reducing Therapy*	PHOX	*Phagocyte oxidase*
1,3-GDN	*1,3-Glyceryl dinitrate*	HMG-CoA	*3-Hydroxy 3-methylglutaryl coenzyme A*	PIP_2	*Phosphotidylinositol 4,5-bisphosphate*
4S	*Scandinavian Simvastatin Survival Study*	HOPE	*Heart Outcomes Prevention Evaluation study*	PKC	*Protein kinase C*
5-HT	*5-hydroxytriptamine*	ICAM-1	*Intracellular adhesion molecule-1*	PLA_2	*Phospholipase A_2*
AA	*Arachidonic acid*	IHD	*Ischaemic heart disease*	PLC	*Phospholipase C*
ACE	*Angiotensin converting enzyme*	IMPACT-II	*Integrilin to Minimize Platelet Aggregation and Coronary Thrombosis-II*	PRISM	*Platelet Receptor Inhibition in Ischaemic Syndrome Management*
ADMA	*Asymmetric dimethyl-arginine*	INF-γ	*Interferon-gamma*	PRISM-PLUS	*Platelet Receptor Inhibition in Ischaemic Syndrome Management-in patients limited by Unstable Signs and Symptoms*
ADP	*Adenosine di-phosphate*	IL-1	*Interleukin-1*	PTCA	*Percutaneous transluminal coronary angioplasty*
Ang II	*Angiotensin II*	iNOS	*Inducible nitric oxide synthase*	PURSUIT	*Platelet IIb/IIIa Underpinning the Receptor for Suppression of Unstable Ischaemia Trial*
ATRB	*Angiotensin receptor blocker*	IP_3	*Inositol 1,4,5 trisphosphate*	RESTORE	*Randomized Efficacy Study of Tirofiban for Outcomes and Restenosis*
BH_4	*Tetrahydrobiopterin*	ISIS	*International Study of Infarct Survival*	RISC	*Research on Instability in Coronary artery disease*
CAD	*Coronary artery disease*	ISDN	*Isosorbide di-nitrate*	RGD	*Arginine-glycine-aspartic acid*
cAMP	*Cyclic adenosine monophosphate*	ISMN	*Isosorbide mono-nitrate*	RT-PCR	*Reverse transcriptase-polymerase chain reaction*
CFV	*Cyclical flow variations*	*L*-NAME	*L-N-nitro-L-arginine methyl ester*	ROS	*Reactive oxygen species*
cGMP	*Cyclic guanosine monophosphate*	*L*-NMMA	*L-N^G-monomethyl-L-arginine*	RPFA	*Rapid platelet function analyzer*
cGMP-PK-1	*Cyclic guanosine monophosphate-protein kinase-1*	MCP-1	*Monocyte chemoattractant protein-1*	SALT	*Swedish Aspirin Low-dose Trial*
CHF	*Congestive heart failure*	NMA	*N-monomethyl arginine*	SAPAT	*Swedish Angina Pectoris Aspirin Trial*
COX	*Cyclooxygenase*	Mox-1	*Monocyte oxidase-1*	SAVE	*Survival and Ventricular Enlargement study*
CPA	*Cone and plate(let) analyzer*	mt ALDH	*Mitochondrial aldehyde dehydrogenase*	SIN-1	*3-morpholinosydnonimine*
CPR	*Cytochrome P_{450} reductase*	NAC	*N-acetyl cysteine*	SMILE	*Survival of Myocardial Infarction Long-Term Evaluation*
CSA	*Clot signature analyzer*	NADPH	*Nicotinamide adenine dinucleotide phosphate*	SNAP	*S-nitroso-N-acetyl penicillamine*
DAG	*Diacylglycerol*	NIDDM	*Non-insulin dependent diabetes mellitus*	SNC	*S-nitroso-cysteine*
DAVIT	*Danish Verapamil Infarction Trial*	nNOS	*Neuronal type nitric oxide synthase*	SNP	*Sodium nitroprusside*
DPI	*Diphenyliodonium*	NO+	*Nitrosonium cation*	SOD	*Superoxide dismutase*
DTS	*Dense tubular system*	NO-	*Nitroxyl anion*	TIMI	*Thrombolysis in Myocardial Infarction*
EDRF	*Endothelial derived relaxing factor*	NOS	*Nitric oxide synthase*	TNF-α	*Tumor necrosis factor-α*
ECG	*Electrocardiogram*	NTG	*Nitroglycerine*	TREND	*Trial on Reversing Endothelial dysfunction*
eNOS	*Endothelial derived nitric oxide synthase*	O_2^-	*Superoxide*	TXA_2	*Thromboxane A_2*
EPIC	*Evaluation of c7E3Fab in the Prevention of Ischaemic Complications*	OCS	*Open canalicular system*	VASP	*Vasodilator stimulated phosphoprotein*
EPILOG	*Evaluation in PTCA to Improve Long-term Outcome with abciximab GPIIb/IIIa*	ODQ	*1H-[1,2,4] oxodiazolo [4,3–α] quinaxalin-1*	VSMC	*Vascular smooth muscle cell*
EPISENT	*Evaluation of Platelet IIb/IIIa Inhibitor for Stenting Trial*	OH^-	*Hydroxyl radical*	vWF	*Von Willebrand factor*
ET-1	*Endothelin-1*	OPUS-TIMI	*Orbofiban in Patients with Unstable Coronary Syndromes- Thrombolysis in Myocardial Infarction*		
FAD	*Flavine adenine dinucleotide*	ox-LDL	*Oxidized low density lipoprotein*		
fMLP	*N-formyl-L-methionyl-L-leucyl-phenylalanine*	PDE	*Phosphodiesterase*		
FMN	*Flavin mononucleotide*	PDGF	*Platelet derived growth factor*		
GISSI	*Gruppo Italino per lo Studio della Sopravvivenza nell' Infarto micocardico*	PET	*Positron emission tomography*		
G-protein	*GTP binding protein*	PAF	*Platelet activating factor*		
GPIIb/IIIa	*Glycoprotein IIb/IIIa*	PGD_2	*Prostaglandin D_2*		
GSH	*Glutathione*	PGE_1	*Prostaglandin E_1*		
GSNO	*s-nitroso-glutathione*	PGG_2	*Prostaglandin G_2*		
GTPase	*Guanosine triphosphatease*	PGH_2	*Prostaglandin H_2*		
H_2O_2	*Hydrogen peroxide*	PGI_2	*Prostaglandin I_2*		

Abb = Abbreviation

Section A:

Normal Vascular Physiology-

Nitric Oxide

[A.1] Endothelial-derived relaxing factor and nitric oxide

[A.1.1] Discovery of EDRF

Furchgott and Zawadzki, were the first investigators to recognize that vascular relaxation induced by acetylcholine was dependent on the presence of the vascular endothelium. Exposure of muscarinic receptors on endothelial cells to acetylcholine stimulated the release of a potent vasodilator initially termed endothelial-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980).

[A.1.2] Identification of endothelium derived relaxing factor as nitric oxide or a nitric oxide compound

Following the identification and initial characterization of EDRF, it was observed that EDRF and nitric oxide possessed similar biological and chemical properties, leading to the proposal that EDRF was indeed nitric oxide. Utilizing porcine aortic endothelial cells Palmer *et al* (1987), demonstrated that bradykinin, in a concentration-dependent fashion, stimulated the release of nitric oxide in amounts sufficient to account for the relaxation attributed to EDRF. These observations were also confirmed by Ignarro *et al* (1987a/b) and by Radomski *et al* (1987b), who also demonstrated that nitric oxide functioned as a potent inhibitor of platelet aggregation, similar to EDRF.

Despite the above evidence indicating that EDRF was nitric oxide, controversy over its chemical identity still exists. Relaxation generated following EDRF addition to canine coronary artery segments differed significantly between the proximal and distal arteries. Contrasting this, nitric oxide evoked the same degree of relaxation irrespective of coronary location (Hoeffner *et al.*, 1989). By adding either nitric oxide or EDRF over a series of smooth muscle preparations obtained from the vasculature, tracheal, gastrointestinal and uterine, Shikano *et al* (1987), demonstrated a differential selectivity between the relaxant effects of EDRF and nitric oxide. Moreover, studies utilizing cultured bovine aortic

endothelial cells demonstrated that the amount of nitric oxide released under basal conditions, or in response to either bradykinin or the calcium ionophore A23187, is insufficient to account for the vasorelaxant activities of EDRF that is derived from the same source (Myers *et al.*, 1990). It was concluded that the biological efficacy of EDRF resembled that of *S*-nitroso-*L*-cysteine or other *S*-nitroso-thiols rather than that of nitric oxide (Myers *et al.*, 1990). Nitrosothiol physiology is discussed further in section A.1.5.

[A.1.3] Reactivity

Nitric oxide is a relatively stable radical that readily diffuses across cellular membranes to interact with its intracellular target. Nitric oxide has an single electron within the $2p$-π anti-bonding orbital, but unlike other oxygen based radicals that either readily donate or accept electrons to form stable adducts, nitric oxide can react specifically with transition metals such as iron in heme-containing proteins (guanylate cyclase and haemoglobin) (Stamler *et al.*, 1992c; Wink *et al.*, 1996).

As nitric oxide has an unpaired electron it rapidly reacts with other radical species that include the hydroxyl radical, nitrogen dioxide, alkyl, alkoxy and alkyl peroxide radicals (Stamler *et al.*, 1992c). Lately much research interest has been focused on the reaction of nitric oxide with the oxygen free radical superoxide, resulting in the formation of peroxynitrite (Butler *et al.*, 1995). Peroxynitrite is a powerful oxidant that has been implicated in a number of protein oxidation reactions including oxidation of sulfhydryl groups (Loscalzo and Welch, 1995). Formation of peroxynitrite from the scavenging of nitric oxide by superoxide has been suggested to serve as a central mechanism behind "endothelial dysfunction," a phenomenon thought directly responsible for the initiation of various cardiovascular risk factors. A discussion on the role of peroxynitrite in the phenomenon of endothelial dysfunction and its role in platelet hypersensitivity is covered within this chapter (section C.3.2.3 and C.12.1.4 respectively). For a detailed review discussing the involvement of peroxynitrite in various cardiac disease states see Ronson *et al* (1999).

[A.1.4] Nitroxyl/nitrosonium

Nitric oxide can also exist in redox isoform states of the nitrosonium cation (NO^+) and the nitroxyl anion (NO^-), each of which has different biological roles/function. The oxidized form of nitric oxide, the nitrosonium cation, is the key species in the process of nitrosation. A

process in which the NO^+ group is often transferred to a sulphur or nitrogen atom through intermediate carriers including *S*-nitrosothiols an *N*-nitrosamines (Hughes, 1999). One such example of this nitrosation was postulated by Jia *et al* (1996), in which a cysteine located within the β-subunit of *S*-nitroso-haemoglobin is nitrosylated in the lung.

The nitroxyl anion has been shown to be produced by nitric oxide synthase (NOS) itself (Pufahl *et al.*, 1995; Schmidt *et al.*, 1996) along with being generated from azide by peroxidase (Tatarko and Bumpus, 1997), by the decomposition of *S*-nitrosothiols in the presence of thiols (Arnelle and Stamler, 1995), by the decomposition of peroxynitrite (Khan *et al.*, 2000), despite evidence to the contrary (Martinez *et al.*, 2000; Merenyi *et al.*, 2000), and by the reduction of nitric oxide by ferricytochrome *c* (Sharpe and Cooper, 1998). A few research groups have demonstrated that the nitroxyl anion is a potent relaxant of vascular smooth muscle preparations, that is accompanied by an increase in cGMP content (Fukuto *et al.*, 1992; Li *et al.*, 1999b), implying a potential transformation of the nitroxyl anion into nitric oxide. Further to this Nelli *et al* (2000), have demonstrated that copper ions have the ability to promote the rapid oxidation of nitroxyl to nitric oxide. In a series of parallel studies utilizing Angeli's salt (a donator of nitroxyl anion) and *S*-nitroso-*N*-acetyl-*D*, *L*-penicillamine (SNAP) that liberates nitric oxide in the presence of copper ions, Nelli *et al* (2001), demonstrated an enhancement in rat aorta relaxation to both agents in the presence of copper sulphate. However, this phenomenon was said to occur because of a reduction in the destruction of nitric oxide by superoxide rather than an enhanced nitric oxide generation. However, relaxation to Angeli's salt was blocked by proadifen, suggesting a role for cytochrome P_{450}.

[A.1.5] S-nitrosothiols

Nitric oxide as well as its redox isoforms, nitrosonium cation and the nitroxyl anion, or an oxidized derivative N_2O_3, react readily in the presence of oxygen with sulphydryl containing proteins to form nitroso-thiol adducts. These include *S*-nitroso-*N*-acetyl-*D*, *L*-penicillamine (SNAP), *S*-nitroso-glutathione (GSNO) and *S*-nitroso-cysteine (SNC) (Butler *et al.*, 1995). Following on from the observations of Myers *et al* (1990), Stamler *et al* (1992a), determined that concentrations of free nitric oxide in plasma were significantly less than those of *S*-nitroso-thiols and that nitric oxide also circulates as an adduct of serum albumin and other proteins. This discovery gave rise to the hypothesis that *S*-nitroso-thiols serve as the primary

redox form of nitric oxide in plasma, such that it functions as a stable reservoir allowing maintenance and delivery of nitric oxide to various tissues (Stamler *et al.*, 1992b). For recent reviews on the chemical properties, biological function and clinical potential of *S*-nitroso-thiols see Gaston and Hogg (Gaston, 1999; Hogg, 2000).

[A.2] Biochemistry

[A.2.1] Synthesis of nitric oxide

Investigations further characterizing EDRF's function suggested that the amino acid *L*-arginine was the substrate responsible for nitric oxide generation within vascular endothelial cells (Palmer *et al.*, 1988a; Palmer *et al.*, 1988b). Generation of nitric oxide from porcine aortic endothelial cells induced by bradykinin and the calcium ionophore A23187 was increased when the endothelial cells were co-cultured with *L*-arginine. This enhancement of nitric oxide production was absent when the endothelial cells were co-cultured with the sterically inactive *D*-arginine, suggesting that *L*-arginine serves as the substrate for the enzyme(s) catalyzing nitric oxide production. Studies using ^{15}N-labelled *L*-arginine then supported this hypothesis (Palmer *et al.*, 1988a), with specific enzyme systems responsible for nitric oxide production being identified soon after.

[A.2.2] Nitric Oxide Synthase

To date three isoforms of nitric oxide synthase (NOS) have been identified as being an enzyme responsible for catalyzing the conversion of *L*-arginine to *L*-citrulline, all of which contain regions homologous to cytochrome P_{450} reductase (Bredt *et al.*, 1991). These have been designated Type I (neuronal; nNOS), type II (inducible; iNOS) and type III (endothelial derived; eNOS) respectively (Forstermann *et al.*, 1994). Distribution/localization and factors that influence the regulation of these NOS isoforms and hence nitric oxide formation are discussed below.

All three NOS isoforms catalyze a five-electron oxidation of one of two terminal guanidino nitrogens of *L*-arginine to form the N^{ω}-Hydroxyl-*L*-Arginine intermediate; further oxidation results in the formation of *L*-citrulline and nitric oxide. Multiple co-factors are required for NOS function, including nicotinamide adenine dinucleotide phosphate (NADPH), flavine adenine dinucleotide (FAD), flavin mononucleotide (FMN) iron protoporphyrin IX (heme),

and tetrahydrobiopterin (BH_4), with the flavins and biopterin being bound to all three isoforms (Forstermann *et al.*, 1994; Gross and Wolin, 1995; Lloyd-Jones and Bloch, 1996).

NOS are composed of two distinct functional domains, which together provide the machinery required for nitric oxide production. The C-terminal end contains the NADPH/FAD and FMN fragments representing the reductase region of NOS, whilst the N-terminal region represents the oxygenase domain and contains binding sites for heme, *L*-arginine and BH_4 (Griffith and Stuehr, 1995).

Initial investigations describing the function of NOS determined that eNOS is activated when calmodulin binds reversibly in response to elevated Ca^{2+} (Busse and Mulsch, 1990; Mayer *et al.*, 1989; Mulsch *et al.*, 1989). Abu-Soud and Stuehr (1993), whilst examining nNOS function concluded that the calmodulin binding region acts as a hinge between the C-terminal and N-terminal domains. Calmodulin binding aligns the oxygenase and reductase domains of NOS and allows electron transfer from FMN to heme thereby facilitating electron transfer that leads to nitric oxide formation.

iNOS induced in cytokine activated macrophages was also shown to readily transfer NADPH-derived electrons onto heme via FMN due to its constitutively bound calmodulin (Abu-Soud and Stuehr, 1993). This suggests that unlike nNOS and eNOS, the oxygenase and reductase domains of iNOS are permanently aligned allowing constant flow of electrons from NADPH to heme and thus explaining the high-output of nitric oxide from this isoform (Alderton *et al.*, 2001; Griffith and Stuehr, 1995).

[A.2.2.1] Co-factors

As a regulatory mechanism controlling the generation of nitric oxide, the presence of the co-factors heme, BH_4 and the substrate *L*-arginine are required for successful monomer dimerization. In the absence of heme, monomers of all three isoforms of NOS are unable to bind BH_4 and *L*-arginine and fail to catalyze the production of nitric oxide. Addition of BH_4 to an assay mixture of nNOS was demonstrated to increase the affinity of the NOS isoform to *L*-arginine. BH_4 and *L*-arginine were shown to stabilize the NOS dimer and prevent its monomerization (Kotsonis *et al.*, 2000).

In the absence of or in low concentrations of the substrate *L*-arginine or co-factors such as BH_4, NOS is capable of catalyzing the uncoupled reduction of molecular oxygen to produce superoxide. Initially, porcine nNOS was shown to generate the ROS hydrogen peroxide in the presence of low concentrations of *L*-arginine (Heinzel *et al.*, 1992). The observation of a release of ROS from the NOS enzyme was then confirmed in nNOS transfected human kidney cells, where superoxide was shown to combine with nitric oxide to form peroxynitrite (Xia *et al.*, 1996). Miller *et al* (1997), whilst examining the function of nNOS suggested that superoxide generation originates both from the oxygenase and the reductase domains of the enzyme in a Ca^{2+}/calmodulin dependent mechanism, an observation that was later confirmed by Vasquez-Vivar *et al* (1999). Depletion of cytosolic *L*-arginine in macrophages was also demonstrated to trigger superoxide formation from iNOS (Xia and Zweier, 1997).

Pre-incubation of iNOS with diphenyleneiodonium (DPI), a flavoprotein inhibitor, abolished the superoxide signal generated in an *L*-arginine deficient system, suggesting that superoxide generation occurs at the flavin binding site in the reductase domain in iNOS (Xia *et al.*, 1998a).

In contrast to nNOS and iNOS, superoxide generation from eNOS appears to be governed by an alternative mechanism. Both Vasquez-Vivar *et al* (1998), and Xia *et al* (1998b), demonstrated that eNOS generates superoxide upon activation in systems that are deficient in BH_4 rather than *L*-arginine. Pretreatment of eNOS with imidazole or cyanide which binds to the heme region of NOS prevents superoxide generation, suggesting that superoxide derived from eNOS originates from the oxygenase domain rather than the reductase domain of NOS (Xia *et al.*, 1998b). Reasons for these isoform specific differences in the regulation of superoxide generation remain unclear.

Conversely BH_4 has been suggested to inhibit the generation of superoxide from NOS by stimulating the formation of a heme-peroxo species that facilitates the oxidation of *L*-arginine to generate N^{ω}-hydroxy-*L*-arginine (Vasquez-Vivar *et al.*, 1999), but also has been suggested to be a direct scavenger of superoxide (Kotsonis *et al.*, 2000; Vasquez-Vivar *et al.*, 1998; Xia *et al.*, 1998b). Xu, addressing the question of whether NOS catalyzes the synthesis of superoxide suggested the direct interactions among NADPH, FAD, FMN, BH_4 and calmodulin were responsible for superoxide formation (Xu, 2000).

What if any, physiological role superoxide has following generation from eNOS has been addressed: - superoxide was shown to up-regulate the production of TNFα in U937 transfected cells in a mechanism that is independent of nitric oxide that is formed from eNOS (Wang *et al.*, 2000). Determination of whether this phenomenon occurs *in vivo* has yet to be established.

[A.3] Vascular and perivascular distribution of nitric oxide, nitric oxide congeners and agents affecting nitric oxide's action

[A.3.1] Nitric Oxide Synthase

[A.3.1.1] Endothelial nitric oxide synthase (eNOS)

Endothelial NOS was initially characterized as a membrane associated protein that was then identified in cultured and native bovine aortic endothelial cells (Pollock *et al.*, 1991). It was also initially isolated within a cytosolic fraction (Forstermann *et al.*, 1991). Utilizing a monoclonal antibody directed against eNOS, endothelial cells isolated from the macro/micro vascular beds of both arterial and venous samples, were shown to contain eNOS, with the enzyme being associated closely with the plasma membrane and membranes of cytoplasmic vesicles (Pollock *et al.*, 1993).

[A.3.1.1.1] Platelet eNOS

Radomski *et al* (1990), were the first to identify that platelets contain NOS which was activated following induction of platelet aggregation. Collagen (0.01–3.0μg/ml) induced platelet aggregation was potentiated following NOS inhibition by N^G-mono-methyl-*L*-arginine (*L*-NAME) and inhibited by the NOS substrate *L*-arginine, confirming that platelets themselves contained NOS, that when activated modulates platelet function. Identification of platelet eNOS was subsequently confirmed by many groups (Chen and Mehta, 1996; Mehta *et al.*, 1995; Sase and Michel, 1995; Wallerath *et al.*, 1997) as was the activation of platelet eNOS following induction of platelet aggregation (Berkels *et al.*, 1997).

[A.3.1.1.2] eNOS Regulation

As endothelial nitric oxide is a physiologically significant vasodilator and inhibitor of platelet aggregation, as well as having a multitude of other effects, the up and down-regulation of eNOS has important consequences for vascular homeostasis. Studies initially characterizing

eNOS from bovine aortic endothelial cells, showed a marked increase in eNOS protein expression following exposure of the cultured endothelial cells to 24-hrs of shear stress (Nishida *et al.*, 1992). Shear stress induced eNOS expression in both and human aortic endothelial cells was also demonstrated by Uematsu *et al* (1995), who illustrated an accompanied enhancement in nitric oxide generation. Interestingly, activation of eNOS by fluid shear stress has also been shown to occur by a mechanism that does not require an intracellular increase in free Ca^{2+}, previously assumed to be integral to enzyme system activation (Fisslthaler *et al.*, 2000).

TNF-α, present in atherosclerotic lesions (Barath *et al.*, 1990), was demonstrated to decrease the extent of eNOS expression in human umbilical vein endothelial cells in a concentration and time dependent fashion through increasing the rate of mRNA degradation (Yoshizumi *et al.*, 1993). In contrast, transforming growth factor-β_1 (TGF-β_1), also present in human atherosclerotic lesions (Nikol *et al.*, 1992), known to inhibit endothelial cell proliferation and regeneration (Frater-Schroder *et al.*, 1986), that can also be released from aggregating platelets (Heimark *et al.*, 1986), increases eNOS mRNA protein content and nitric oxide generation in a concentration-dependent fashion (Inoue *et al.*, 1995).

Oxidized low-density lipoprotein (ox-LDL) regulates eNOS expression and hence nitric oxide production by causing a time-dependent decrease in eNOS mRNA levels via post-transcriptional mRNA degradation (Liao *et al.*, 1995). Contrasting this observation, Hirata *et al* (1995), demonstrated that low concentrations of ox-LDL may up-regulate eNOS mRNA and protein levels isolated from bovine aortic endothelial cells following 24-hrs of exposure. The discrepancy between these two studies can be explained by the concentrations of ox-LDL used, with large concentrations of ox-LDL (100 μg/mL) being shown to decrease eNOS mRNA levels at 24-hrs (Hirata *et al.*, 1995).

[A.3.1.2] Inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS)

A discussion on the distribution/regulation/function of both iNOS and nNOS falls outside the scope of the current study. However, for a comprehensive review of the literature concerning the functional significance of iNOS and or nNOS see the following reviews by Bredt (1999),

Hecker *et al* (1999), Rao (2000), Taylor and Geller (2000), Wang *et al* (1999), and Wort *et al* (2001).

[A.3.2] Superoxide

In the vasculature, identification of the primary sources of ROS has recently shifted from research centering on xanthine oxidase, to a specific membrane-associated enzyme system (NAD(P)H oxidase) that catalyzes the one electron reduction of molecular oxygen to superoxide using either NADPH or NADH as the electron donor as indicated in the chemical reaction below.

$$O_2 + e^- \rightarrow O_2^{\cdot -}$$

$$2O_2 + NADPH \rightarrow 2O_2^{\cdot -} + NADP + H^-$$

[A.3.2.1] Components of NAD(P)H oxidase

The most extensively examined NAD(P)H enzyme system has been that of the neutrophil, where reactive oxygen species (ROS) stemming from superoxide, play a critical role in host defense against microbial infection (Babior, 1999; DeLeo and Quinn, 1996; Hampton *et al.*, 1998). The core phagocyte NADPH oxidase enzyme located within the membranes of the secretory vesicles and specific granules, is a membrane-spanning flavocytochrome b_{558}, composed of a complex of two sub-units $p22^{phox}$ and $gp91^{phox}$ (*phox* = phagocyte oxidase) (Babior, 1999; Jones *et al.*, 2000). The $gp91^{phox}$ contains the binding sites for heme, FAD and NADPH, all necessary components required to facilitate electron transfer from NADPH to molecular oxygen to form superoxide.

The neutrophil NADPH oxidase enzyme system also consists of three more sub-units, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ that exist as a complex in the cytosol at rest. Phosphorylation of $p47^{phox}$ or activation by arachidonic acid results in the migration of the cytosolic complex to the plasma membrane whereupon it binds to the cytochrome b_{558} to form the active oxidase (Ago *et al.*, 1999). Cross *et al* (2000), determined that the $p40^{phox}$ functions to promote the enzyme activation by increasing the affinity of $p47^{phox}$ for the flavocytochrome b_{558}. Activation of the enzyme complex also requires the involvement of two guanine nucleotide binding proteins (G-proteins), Rac 2 and Rap1A. Genetic defects in any one of the major sub-

units of the enzyme system results in a loss of enzyme function predisposing subjects to severe microbial infection, a syndrome known as chronic granulomatous disease (Segal *et al.*, 2000). For a schematic representation of the assembled phagocyte NAD(P)H oxidase enzyme system see Figure 1.1.

[A.3.2.2] Distribution and function of non-phagocyte NAD(P)H oxidase

Forms of NAD(P)H oxidase have now been identified in several non-phagocytic cell types including fibroblasts (Meier *et al.*, 1993; Pagano *et al.*, 1997), vascular smooth muscle cells (Fukui *et al.*, 1995; Gorlach *et al.*, 2000), and endothelial cells (Gorlach *et al.*, 2000; Jones *et al.*, 1996), suggesting an additional role for ROS generated from NAD(P)H apart from microbial defense. The amount of ROS generated in the enzyme systems of the above mentioned cell types is significantly lower, with slower kinetics compared to that of the burst-like production observed from activated neutrophils (Gorlach *et al.*, 2000). Explanations for the observed differences in levels and rates of ROS production have been speculated to include different sub-units (Suh *et al.*, 1999), reduced levels of expression (Gorlach *et al.*, 2000) or different sub-unit locations (Bayraktutan *et al.*, 2000).

Following the notion of sub-unit differences between phagocyte and non-phagocyte NAD(P)H oxidase enzyme systems, Suh *et al* (1999), identified a homologue of $gp91^{phox}$, mox-1 that is present in VSMC's found in the colon, prostate and uterus of human subjects. It is said that mox-1 functions in VSMC's the same way $gp91^{phox}$ functions in phagocytes, though it preferentially utilizes NADH rather than NADPH as the substrate (Griendling *et al.*, 1994). Shiose *et al* (2001), described the cloning of human cDNA that encodes a novel NAD(P)H oxidase which exhibits partial homology to the phagocyte $gp91^{phox}$. Designated NOX 4, it contains the binding sites for heme, FAD and NAD(P)H required for superoxide generation. However, unlike $gp91^{phox}$, NOX 4 expression is restricted to adult and fetal kidneys, suggesting the existence of specific NAD(P)H oxidase with tissue specific functions (Shiose *et al.*, 2001). Further to this, a series of novel $gp91^{phox}$ homologues have been identified in non-phagocyte cells (Nox 1, Nox 3, Nox 5) (Lambeth *et al.*, 2000; Lassegue *et al.*, 2001; Szocs *et al.*, 2002). In a more recent investigation Sorescu *et al* (2002), demonstrated that non-atherosclerotic and atherosclerotic human coronary arteries show clear evidence of constant superoxide production throughout the intima, media and adventitia tissue. Within an atherosclerotic specimen the most intense superoxide staining was observed

within the shoulder region of a lesion with the level of expression of $gp91^{phox}$ and $p22^{phox}$ correlating strongly with lesion severity. For a schematic representation of an assembled NAD(P)H oxidase enzyme system located within a VSMC see Figure 1.1.

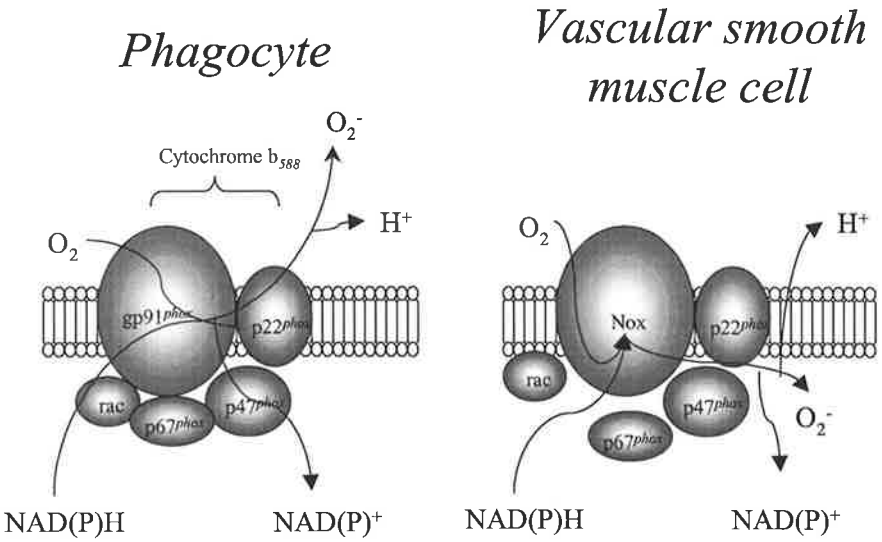


Figure 1.1 Assembled phagocyte and non-phagocyte NAD(P)H oxidase enzyme systems

Structural components of the assembled (activated) neutrophil (left) and vascular smooth muscle cell (right) NAD(P)H oxidase (Griendling et al., 2000). Upon activation the cytosolic components $p67^{phox}$ and $p47^{phox}$ are translocated from the cytosol to the membrane surface whereupon they interact with cytochrome b_{588} ($gp91^{phox}$ and $p22^{phox}$ for neutrophil NAD(P)H oxidase) to facilitate enzyme activation and the electron transfer from NAD(P)H to molecular oxygen to produce superoxide. Nox (originally referred to as Mox and identified by Suh et al., 1999) functions as a $gp91^{phox}$ like sub-unit within vascular smooth muscle cells. This figure was adapted from (Griendling et al., 2000).

Normal Platelet Function: Role of Nitric Oxide

Platelet Physiology

[A.4] Platelet Ultra-structure

Platelets are small (0.5 – 1.0μm thick, and 1.5 – 2.5μm in diameter) discoid anuclear cells formed from megakaryocytes by fragmentation within the bone marrow (Hourani and Cusack, 1991).

Bundles of microtubules under the platelet surface membrane and an extensive network of short actin filaments, maintain the discoid shape of a resting platelet. Rearrangement of these

bundles of microtubules, short actin filaments and myosin is essential in the mechanism of platelet shape change (section A.7) (Blockmans *et al.*, 1995).

Platelets have a plasma membrane rich in glycoproteins that serve as anchors and agonist/antagonist specific receptors (Hourani and Cusack, 1991). Numerous organelles are dispersed within the platelet cytoplasm including mitochondria, lysosomes, peroxisomes and glycogen particles. Depending on the level of platelet activation, the contents of two major types of secretory granules are released.

[A.4.1] α-Granules

α-Granules, which are the most prominent in size and number, are rich in platelet activating factor 4 (PAF-4), β-thromboglobulin, platelet derived growth factor (PDGF), fibrinogen, fibronectin, thrombospondin, plasminogen activator inhibitor-I, P-selectin and von Willebrand factor (vWf) (Blockmans *et al.*, 1995; Hourani and Cusack, 1991), all of which play a vital role in either platelet activation, aggregation and leukocyte recruitment. α–Granules also contain glycoprotein (GP) IIb/IIIa (Cramer *et al.*, 1990).

[A.4.2] Dense body granules

Dense body granules contain Ca^{2+}, ADP and serotonin (5-HT), the latter two are weak agonists that are released upon platelet activation, thereby promoting recruitment of more platelets to a developing thrombus and affecting vascular tone (Hourani and Cusack, 1991). Dense body granules have been shown to also contain adhesion molecules originally localized within the α-granules, including P-Selectin (Israels *et al.*, 1992), GPIIb/IIIa and GPIb-IX-V (Youssefian *et al.*, 1997), suggesting an inter-relationship between the organelles. Dense body granules also release Ca^{2+}, which becomes sequestered to the dense tubular system (DTS), upon platelet activation. The DTS is the equivalent of the smooth endoplasmic reticulum of other cells and is the site where enzymes involved in prostaglandin synthesis are located (Blockmans *et al.*, 1995) (see section A.10.1). The DTS lies in close contact with the channels of the Open Canalicular System (OCS), a network of channels leading from the interior of the platelet to the outer surface membrane. Upon platelet activation granule contents are released into the surrounding area through this OCS.

The specific sequence of platelet adhesion, activation, shape change, aggregation and release of the contents of the secretory granules that recruit more platelets to platelet aggregates, occurs prior to the development of a potentially occlusive thrombus. Described in the following selections are some of the pathways and mechanisms by which platelets form these potentially occlusive thrombi.

[A.5] Platelet Adhesion

When a blood vessel is damaged exposing the sub-endothelial microfilaments to platelets, the formation of a haemostatic plug occurs in order to minimize blood loss (Blockmans *et al.*, 1995). Platelet adhesion, the process that describes the phenomenon of platelet to non-platelet attachment, occurs prior to platelet activation/aggregation/granule secretion and is mediated by the interaction of the platelet surface membrane receptor complex GPIb-IX-V and the insoluble von Willebrand factor (vWf) (Gralnick *et al.*, 1996).

[A.5.1] GPIb/IX/V receptor complex

Glycoprotein Ib (GPIb) forms a complex with GPIX and GPV on the platelet outer surface membrane, generating the principal receptor for the sub-endothelium vWf and therefore facilitating the anchoring of platelets to the sites of vascular injury (Andrews *et al.*, 1999b). This GPIb-IX-V/vWf interaction is said to only occur under flow conditions of high shear stress such as that occurs in the regions of advanced atherosclerotic lesions (Clemetson, 1997; Ikeda *et al.*, 1991; Kroll *et al.*, 1996). vWf binds to platelet GPIb with the resultant interaction leading to protein kinase C activation, increases in intra-cellular Ca^{2+} (see section A.8) (Kroll *et al.*, 1991), facilitating the release of stored ADP from the dense granules (Moake *et al.*, 1988). At low shear, platelet adhesion to the endothelium occurs via alternative integrin complexes that include GPIa-IIa, GPIV and GP VI all of which are readily expressed on the outer platelet surface membrane (Watson and Gibbins, 1998).

Along with binding vWf within the N-terminal domain of GPIbα other regions, the GPIb-IX-V receptor complex has the ability to bind to the potent platelet agonists α-thrombin (Andrews *et al.*, 1999b) and filamin (Clemetson, 1997). Subjects with the genetic disorder Bernard-Soulier syndrome have a low or absent platelet surface expression GPIb-IX-V and consequently have a reduced responsiveness to thrombin (Lopez *et al.*, 1998). The exact significance of these interactions remains to be determined, but may facilitate further

anchoring and platelet spreading, when GPIb-IX-V interacts with filamin (Clemetson, 1997) and further platelet activation and recruitment of platelets to form platelet aggregates, when GPIb-IX-V interacts with α-thrombin (Andrews *et al.*, 1999b).

[A.5.2] Von Willebrand factor (vWf)

Von Willebrand factor (vWf) is a large multimeric glycoprotein that is synthesized exclusively in endothelial cells and megakaryocytes and is found in platelet α-granules, plasma and the sub-endothelium (Blockmans *et al.*, 1995; Weiss, 1991). Along with other functions such as delivery of Factor VIII (Lip and Blann, 1997), vWf plays a critical role in platelet adhesion to the sub-endothelium via its interaction with GPIb and platelet aggregate formation post shape change of the GPIIb/IIIa receptor (see section A.9.1) (Savage *et al.*, 1992).

[A.5.2.1] vWf and ischaemic heart disease

Several studies to date have illustrated an association between vWf levels and the presence of ischaemic heart disease (IHD) (Haines *et al.*, 1983). In a number of prospective investigations, high concentrations of vWf were demonstrated to be an independent risk factor for subsequent re-infarction/death (Jansson *et al.*, 1991; Thompson *et al.*, 1995), athero-thrombotic events (Cortellaro *et al.*, 1992), or restenosis post percutaneous transluminal coronary angioplasty (PTCA) (Montalescot *et al.*, 1995), presumably reflecting a level of platelet activation now known to play an integral part in these pathologies.

Abnormal levels of vWf have also been demonstrated to be associated with four of the major risk factors for atherosclerosis. Specifically levels of vWf are significantly higher in patients with hypertension (Blann and Waite, 1996), diabetes (Jager *et al.*, 1999), hypercholesterolaemia (Blann *et al.*, 1999; Seligman *et al.*, 2000) and in smokers (Blann *et al.*, 1997).

[A.6] Mechanisms of Platelet Activation

Following anchoring of platelets to the sub-endothelium, platelets are activated by a large variety of pharmacological and physiological stimuli, which exert their effects through specific platelet plasma membrane receptors.

[A.6.1] Strong/Weak Agonists

The interaction of an agonist and its specific receptor, some of which are described below, causes a rapid rise in intracellular Ca^{2+}, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) formation (see section A.8). Platelet shape change, GPIIb/IIIa receptor exposure, formation of platelet aggregates and granule secretion results. Some platelet agonists, e.g. thrombin and collagen, induce all of the above responses and therefore have been denoted as "strong" agonists (Holmsen, 1989). However, ADP and 5-HT stimulate shape change and platelet aggregate formation, without the liberation of the contents of the secretory granules. These are denoted as "weak" agonists. Acting synergistically with the constituents of the dense granules and α-granules (ADP, 5-HT, prostaglandins and thromboxanes liberated from arachidonate), high concentrations of weak agonists produce platelet responses similar to those observed with strong agonists (Holmsen, 1989). The interaction of platelet agonists with their specific receptors is described in the following sections.

[A.6.2] Thrombin Receptor

The serine protease thrombin is a powerful platelet agonist that readily induces platelet shape change, release of granule contents and activation of GPIIb/IIIa to facilitate the formation of platelet aggregates. Thrombin mobilizes P-selectin and the CD-40 ligand to the platelet outer membrane whereupon the ligands serve as an attachment point for leukocytes (Henn *et al.*, 1998).

Thrombin signaling on cellular surfaces is mediated through the G-protein coupled protease-activated receptor (PAR) (Coughlin, 1999). Of the four PARS that have been identified, PAR1 and PAR4 have been localized on human blood platelets (Kahn *et al.*, 1998). In a series of experiments evaluating the roles of PAR1 and PAR4 in thrombin induced platelet aggregation, monoclonal antibodies directed against PAR1 blocked platelet activation at low concentrations but not high concentrations. In contrast, antibodies directed against PAR4 were demonstrated to have no effect on platelet activation by thrombin, suggesting that PAR1 mediates platelet activation at low concentrations and PAR4 at high concentrations (Kahn *et al.*, 1999). PAR4 has also been demonstrated to be activated by the neutrophil granule protease cathepsin G (Sambrano *et al.*, 2000), suggesting a mechanism by which platelets and neutrophils interact at the sites of vascular injury or in the peripheral circulation.

PAR1 and PAR4 have been demonstrated to have distinct molecular signaling characteristics. Activation of PAR1 triggers a rapid rise in intra-platelet Ca^{2+}, whereas activation of PAR4 triggers a significantly prolonged release of Ca^{2+} (Shapiro *et al.*, 2000), implying a mechanism by which thrombin can specify the tempo of platelet activation.

Thrombin, recognizes a specific region (Arg41-Ser 42 for PAR1) in the amino-terminal domain of the receptor (Coughlin, 2000). Thrombin cleaves the sequence at this site unmasking a new amino terminus beginning with the sequence SFLLRN. This then functions as a tethered ligand that docks with the body of the receptor initiating the trans-membrane signaling via G-protein dependent processes (Vu *et al.*, 1991). Furman *et al* (1998b), demonstrated that the tethered ligand causes platelet surface expression of P-selectin (reflecting α-granule secretion), activation of the GPIIb/IIIa receptor complex and binding of fibrinogen to the complex forming platelet aggregates. Thrombin has also been shown to cleave PAR4 at ARG-47/Gly-48 resulting in the production of a GYPGQV tethered ligand that binds to the PAR4 receptor (Xu *et al.*, 1998).

[A.6.3] Adenosine di-phosphate receptor

Despite evidence for quite sometime that ADP activates platelets (Born, 1962; Gaarder *et al.*, 1961), the precise molecular mechanisms behind this activation remained obscure. ADP induces a number of platelet intra-cellular events, including a rapid Ca^{2+} influx (Sage *et al.*, 1989; Sage *et al.*, 1990), mobilization of intra-cellular Ca^{2+} stores (Hallam and Rink, 1985) and inhibition of adenylate cyclase (Hall and Hourani, 1994), the biochemistry of which will be explained below.

Initial experiments attempting to identify the ADP receptor suggested that ADP works via more than one receptor. The thienopyridines, ticlopidine and clopidogrel, have been shown to be specific ADP receptor antagonists (see section C.15). However, these compounds were shown not to block ADP-induced shape change or intra-cellular Ca^{2+} mobilization (Gachet *et al.*, 1995). Initial experiments on platelets from patients with a poor responsiveness to ADP (Nurden *et al.*, 1995), led to the proposal of a two-receptor model (Gachet *et al.*, 1997; Hourani and Hall, 1994) of ADP-induced aggregation. A model that has now been updated too a three receptor model (Daniel *et al.*, 1998).

Cellular receptors through which extra-cellular purine nucleotides elicit a physiological response have been classified as P2 receptors and are divided into two functional subtypes. P2X receptors are ligand-gated ion-channels and P2Y receptors are G-protein coupled receptors (Fredholm *et al.*, 1994). Within human platelets, the $P2X_1$, $P2Y_1$ and now the $P2Y_{12}$ have been identified (Barnard and Simon, 2001), all having a role in initiating the signal transduction required for ADP-induced platelet aggregation. Initially Mackenzie *et al* (1996), identified a $P2X_1$ receptor in human platelets that mediates a rapid influx of Ca^{2+} upon ADP binding.

Daniel *et al* (1998), and Jin *et al* (1998), have provided evidence for the existence of two separate G-protein coupled ADP receptors, one linked to the inhibition of adenylyl cyclase, originally designated $P2T_{AC}$ or P_{2T} but now known as $P2Y_{12}$, and another receptor that is coupled to the direct activation of phospholipase C ($P2Y_1$). The $P2Y_1$ receptor alone has been demonstrated to mediate ADP-induced platelet shape change. However, signaling events from both the $P2Y_1$ and the $P2Y_{12}$ have also been demonstrated to be essential for ADP-induced platelet aggregation (Jin and Kunapuli, 1998). More recently Goto *et al* (2002), investigating the functional significance of the $P2Y_{12}$ receptor, demonstrated that it was involved in mediating platelet activation following exposure of platelets to conditions of shear with the binding of vWF to GPIbα. A schematic representation of the platelet ADP receptors is shown in Figure 1.2 and has been reviewed recently by a number of investigators (Hoylaerts *et al.*, 2000; Cattaneo and Gachet, 2001; Gachet, 2001).

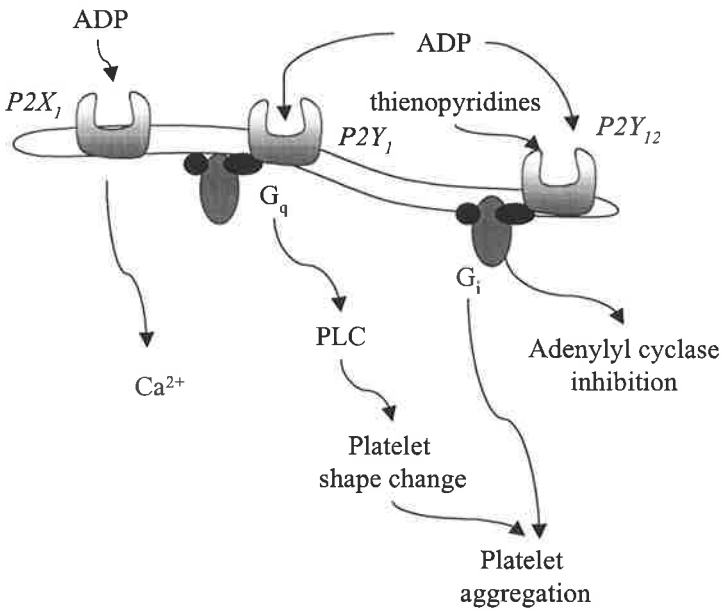


Figure 1.2 Proposed model of the platelet ADP receptor mediated aggregation

Three purinergic receptors on the platelet surface contribute separately to the complex process of ADP-induced platelet aggregation. The $P2X_1$ receptor is responsible for rapid influx of Ca^{2+} into the platelet cytosol. The $P2Y_1$ receptor is thought to be responsible for the mobilization of calcium from internal stores required for aggregation. The $P2Y_{12}$ is the site at which the thienopyridines function to inhibit platelet aggregation.

[A.6.4] Thromboxane A_2 Receptors

As described in section C.13.2, and shown in Figure 1.7, platelets have the ability to convert arachidonic acid to the potent platelet agonists prostaglandin G_2 (PGG_2), prostaglandin H_2 (PGH_2) and thromboxane A_2 (TxA_2), which amplify signals from such platelet activators as thrombin, collagen, ADP, and PAF (FitzGerald, 1991).

Analogues of TxA_2 and PGH_2 have indistinguishable effects on platelets and vascular tissue, suggesting a common receptor. Despite the suggestion of the existence of separate platelet endoperoxidase and TxA_2 receptors (Carmo *et al.*, 1985), most research has centered on the identification/characterization of the TxA_2/PGH_2 receptor.

Studies utilizing TxA_2/PGH_2 agonists and antagonists, and the assessment of their rank-order of potency, indicated that there are at least two tissue specific classes of TxA_2/PGH_2 receptors in both platelets and vascular smooth muscle cells. Saussy *et al* (1986), utilizing a competitive TxA_2/PGH_2 antagonist was one of the first to localize the TxA_2/PGH_2 receptor in

human platelet membrane. Brass *et al* (1987), demonstrated that the TxA_2 receptor is linked via a G-protein to phospholipase C (PL-C). Utilizing a monoclonal antibody that recognizes G_q, Shenker *et al* (1991), demonstrated that pretreatment of platelet membranes with this antibody caused a marked inhibition of TxA_2 receptor stimulated GTPase activity, reconfirming the results of Brass *et al* (1987).

Studies utilizing the radiolabelled TxA_2 analogue ^{125}I BOP, provided further evidence for the existence of two separate classes of TxA_2 receptors in platelets, by demonstrating sites with both low and high affinity for this ligand (Dorn, 1989). Takahara *et al* (1990), utilizing a novel TxA_2/PGH_2 receptor antagonist (GR32191), discovered the existence of two separate receptor systems that mediated separate effects. Dissociation of [^{3}H] GR32191 from human platelets demonstrated two separate binding sites, one that was rapidly dissociated, and a site that was bound irreversibly. The site at which [^{3}H] GR32191 was shown to bind irreversibly controlled phospholipase activation and hence platelet aggregation. However, platelet shape change and Ca^{2+} mobilization remained unaffected. These results were then further delineated by Dorn and DeJesus, who demonstrated that platelet shape change and aggregation were independently coupled to separate TxA_2/PGH_2 receptor subtypes (Dorn and DeJesus, 1991).

Two isoforms of the human TxA_2 receptor have been identified, one from the placenta (TPα) and the other identified originally in the endothelium (TPβ), both of which have been detected in human platelets utilizing polymerase chain reaction (PCR) (Hirata *et al.*, 1996). These two isoforms have been shown to have the same agonist-induced activation of PL-C but distinct effects on adenylyl cyclase activity. TPα was shown to activate adenylyl cyclase whilst TPβ inhibited its formation (Hirata *et al.*, 1996). Habib *et al* (1999), utilizing isoform specific monoclonal antibodies, found that TPβ protein could not be detected, suggesting that TPβ was expressed at very low levels in human platelets with the TPα being the predominant TxA_2 receptor. Upon agonist induced activation the $G_{\alpha q}$ and other G-proteins (G_{11}) (Kinsella *et al* 1997) and ($G_{\alpha 13}$) (Djellas *et al* 1999) that is dissociated from the TxA_2 receptors, is required to initiate phosphorylation of PLC or platelet shape change.

As a counter-regulatory mechanism against uncontrolled platelet activation, most platelet agonists induce cAMP activation, which inhibits platelet activation/aggregation. It has been demonstrated that cAMP induces protein kinase A-dependent phosphorylation of the $G\alpha_{13}$

protein both *in vitro* and *in vivo* (Manganello *et al.*, 1999). Thus this provides a counter-regulatory mechanism to platelet activation/aggregation through inhibition of the TxA_2 mediated second messenger signaling system.

[A.6.4.1] Clinical significance

Dorn *et al* (1990), observed that patients with an acute myocardial infarction (MI) have an increased number of platelet TxA_2/PGH_2 receptors compared to control subjects without a history of IHD. These patients also exhibited an increase in the maximal velocity of U44619 (a TxA_2 mimetic) induced platelet aggregation, the magnitude of which positively correlated with the platelet TxA_2/PGH_2 receptor numbers. Transient elevations in excretion of TxB_2 (stable metabolite of TxA_2) have been observed in patients with ischaemic heart disease (IHD) and in patients following thrombolysis (Fitzgerald *et al.*, 1988; Fitzgerald *et al.*, 1986; Kerins *et al.*, 1989). However, the most convincing evidence for the functional importance of TxA_2 in the pathogenesis of ACS is provided by the observation that aspirin, an inhibitor of TxA_2/PGH_2 formation and hence an inhibitor of platelet aggregation, shows a benefit in both unstable angina pectoris (UAP) (The RISC Group, 1990) and acute MI patients (ISIS-2, 1988). See also section C.13 for a further discussion on aspirin's primary and secondary event protection properties.

[A.6.5] Other Receptors

A number of other pro-aggregants including adrenaline, collagen, serotonin and platelet-activating factor (PAF) act independently or synergistically with other platelet agonists to illicit platelet activation. For a series of reviews summarizing their involvement in the phenomenon of platelet activation/aggregation see the following reviews by Alberio and Dale (1999), Blockmans *et al* (1995), Hourani and Cusack (1991), Moroi and Jung (1997), Siess (1989), Watson and Gibbins (1998).

[A.7] Platelet Shape Change

One of the first responses of platelets to stimuli is a rapid change of shape from a flat smooth ellipsoid form to a spherical form containing pseudopods that project out of the platelet membrane surface to give a "spiny sphere" configuration (Holmsen, 1989). A number of intra-platelet biochemical steps are required prior to the rearrangement of the microtubular

bundles and short actin filaments that govern platelet shape change. Below is a discussion summarizing some of these processes.

[A.8] Biochemistry of Platelet Activation and Aggregation

Platelet activation is a complex process with many signal-transducing and effector mechanisms being involved. Following binding of an agonist to a cell membrane bound receptor, platelets become activated through a series of intracellular second messenger systems whose actions are governed by a number of heterotrimeric guanine nucleotide binding regulatory proteins (G-proteins). To date it is known that platelets have three functionally distinct G-proteins, with many different sub-types for each functional class. G_P couples are thought to stimulate phosphoinositide-specific phospholipase C, whereas G_S and G_i couples are thought to activate and inhibit adenylyl cyclase, respectively (Brass *et al.*, 1993). Each of these G-proteins comprise α-β-γ sub-units in which the α-sub-unit forms the guanine nucleotide binding site, with the βγ portions helping to anchor the G-protein to the cellular membrane (Kroll and Schafer, 1989). The β-γ G-protein sub-units also mediate the activation of phospholipase A_2 and the ion gated channels within a platelet. Upon receptor binding, a $G_{P\alpha}$-GTP sub-unit dissociates from the heterotrimeric sub-unit and activates the phosphoinositide-signaling pathway common to many cellular systems (Kroll and Schafer, 1989). A schematic representation of the platelet phosphoinositide-signaling pathway is shown in Figure 1.3.

[A.8.1] Biochemical Platelet Activation

Two intracellular biochemical pathways play a central role in platelet activation by most but not all agonists. The α-GTP sub-unit activates PL-C which catalyses the breakdown of phosphotidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Blockmans *et al.*, 1995; Kroll and Schafer, 1989; Siess, 1989). Rittenhouse (1983), reconfirming the results of Billah *et al* (1980), was able to demonstrate in human platelets and in the presence of Ca^{2+}, that PL-C was the enzyme responsible for the hydrolysis of PIP_2. Products of PIP_2, DAG and IP_3, then take divergent pathways affecting platelet activation.

DAG activates Protein Kinase C (PKC) and promotes its translocation from the cytoplasm to the platelet membrane. IP_3 binds to a specific IP_3 receptor on the dense tubular system

promoting mobilization of stored Ca^{2+} and influx of external Ca^{2+}, raising the cytosolic free calcium concentration through the platelet ion channels. IP_3 was demonstrated to dose dependently induce the rapid release of stored Ca^{2+} (O'Rourke *et al.*, 1985). Fast release of Ca^{2+} from Ca^{2+} stores in platelets occurs post agonist stimulation but is also followed by a partial back Ca^{2+} sequestration and extrusion from the platelets via a Na^{+}/Ca^{2+} exchanger (Valant and Haynes, 1993) or plasma membrane Ca^{2+}-ATPase (Rosado and Sage, 2000). Calcium mobilization resulting from its release then leads to the activation of phospholipase A_2.

Billah *et al* (1980) and Rittenhouse-Simmons (1981) were the first to demonstrate PLA_2 activity within platelets. Platelets were then discovered to contain multiple isoforms of PLA_2, which vary in their specific activity for different phospholipid substrates (Chang *et al.*, 1987). PLA_2 hydrolyses phosphatidylcholine and phoshatidylethanolamine with the consequent liberation of arachidonic acid (AA). This is then converted by cyclo-oxygenase to the prostaglandin cyclic endoperoxides, PGG_2 and PGH_2. Thromboxane synthase then rapidly converts PGH_2 to TxA_2. For a schematic representation of the biochemical pathways involved in platelet activation see Figure 1.3.

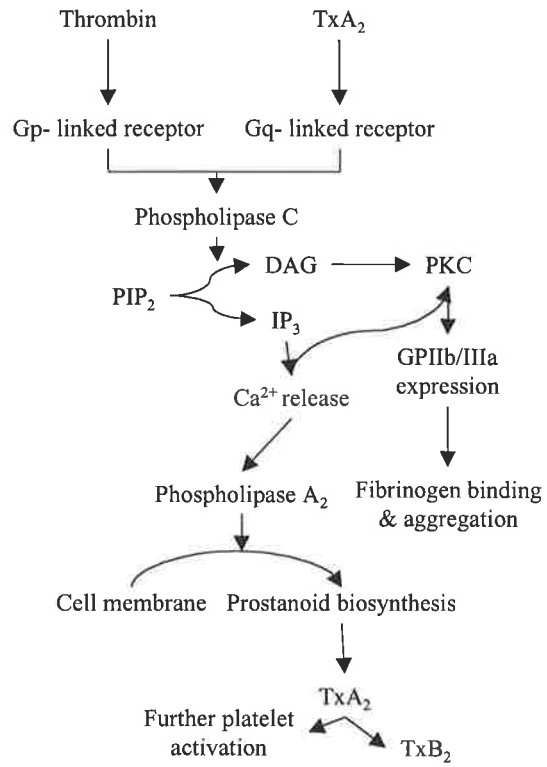


Figure 1.3 Agonist induced platelet activation

Via agonist specific platelet receptors, activation of phospholipase C results in the production of DAG and IP_3 from PIP_2. DAG activates protein kinase C that phosphorylates intra-platelet proteins resulting in granule secretion and expression of GPIIb/IIIa. IP_3 induces the release of Ca^{2+} from the dense tubular system that activates phospholipase A_2. Phospholipase A_2 cleaves arachidonic acid from the cell surface membrane phospholipids to generate thromboxane A_2 through the actions of cyclo-oxygenase and lipoxygenase. DAG, diacylglycerol; PIP_2, Phosphotidylinositol 4,5-bisphosphate; IP_3, Inositol 1,4,5-trisphosphate; TxA_2, Thromboxane A_2. This figure was adapted from (Body, 1996).

[A.9] Platelet Aggregation

Platelet aggregation is the process by which activated platelets adhere to each other, an event that is mediated by binding of adhesive proteins such as fibrinogen and vWf, to the platelet glycoprotein IIb/IIIa (GPIIb/IIIa) receptor.

[A.9.1] Glycoprotein IIb/IIIa (GPIIb/IIIa)

The platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) consists of a two-chain α sub-unit bound non-covalently to a single-chain β sub-unit, with each sub-unit spanning the platelet membrane (Shattil *et al.*, 1998). Initial studies utilizing monoclonal antibodies that block platelet aggregation by preventing a platelet-fibrinogen interaction indicated that there were approximately 40 000 to 80 000 GPIIb/IIIa receptors per platelet (Coller *et al.*, 1983; Wagner

et al., 1996). Platelet ultra-structural studies further revealed the presence of a pool of GPIIb/IIIa within the α-granule, the membranes of the open canicular system (Cramer *et al.*, 1990) and the dense granule membranes of platelets (Youssefian *et al.*, 1997). Upon exposure to platelet agonists these pools of GPIIb/IIIa are translocated to the platelet outer surface membrane where they bind ligands that include fibrinogen, fibrin (formed from thrombin cleavage of fibrinogen (Parise, 1999)), vWf and others, resulting in platelet cross-linking and the initial formation of platelet aggregates (Coller, 1995).

[A.9.2] Recognition Sequences

Several extra-cellular binding domains for various plasma adhesive proteins within the GPIIb/IIIa receptor have been characterized with much clinical interest being focused on one binding domain in particular (see section C.14).

[A.9.3] Arg-Gly-Asp (RGD)

Haverstick *et al* (1985), investigating the role of the cell-binding domain of fibronectin in the interactions of fibronectin with platelets, found that small peptides containing the arginine-glycine-aspartic acid (RGD) sequence completely inhibited thrombin induced platelet aggregation. Peptides containing this RGD sequence were also shown to be effective at inhibiting adhesion of platelets on fibrinogen and vWf substrates. Utilizing monoclonal antibodies that were previously shown to immunoprecipitate both GPIIb and GPIIIa (Coller *et al.*, 1983), Plow *et al* (1985), inhibited the binding of fibrinogen, fibronectin, vWf and thrombospondin to platelets by > 85%, suggesting a related mechanism of binding of these adhesive proteins to activated platelets.

A tetrapeptide analogue of the cell attachment site for fibronectin (Arg-Gly-Asp-Ser) was shown to inhibit platelet aggregation induced by ADP, collagen or thrombin (Gartner and Bennett, 1985). These peptides were then demonstrated to prevent fibrinogen binding to ADP stimulated platelets further suggesting commonality in the platelet-binding site of various adhesive proteins. These initial studies implied that platelets have a receptor capable of recognizing the RGD sequence found in various adhesive proteins. This hypothesis was then confirmed by Pytela *et al* (1986), who demonstrated that platelet GPIIb/IIIa was responsible for the recognition of the RGD sequence found in fibrinogen, fibronectin, and vitronectin.

Parker and Gralnick (1986) then demonstrated that the platelet GPIIb/IIIa receptor complex was also the major binding site for vWf released from an activated platelet.

As these initial studies into the mechanisms behind platelet aggregation found that peptides containing the RGD sequence were potent inhibitors of platelet aggregation (irrespective of the agonist used to induce platelet activation) several RGD containing synthetic peptides have been developed for potential clinical use (see section C.14).

[A.10] Mechanism of Platelet Inhibition

Activation of platelets is counter-regulated by biochemical processes that attenuate or prevent agonist-induced platelet activation/aggregation. These processes are mostly governed by the actions of cyclic AMP and cyclic GMP, both of which are induced by various prostanoids and nitric oxide/metabolites respectively.

[A.10.1] Prostanoids

Of all the prostanoids that can be derived from arachidonic acid through the actions of cycloxygenase, only three products, prostaglandin I_2 (PGI_2), prostaglandin D_2 (PGD_2) and prostaglandin E_1 (PGE_1) demonstrate potent anti-aggregatory effects on platelets, with much of the research interest focusing on the former.

[A.10.1.1] Prostacyclin (PGI_2)

PGI_2 (prostacyclin) is one of the most potent endogenous inhibitors of platelet aggregation and therefore is a critically important regulator of platelet function. PGI_2 is thirty times more active than PGE_1 and 10 times more active than PGD_2 at inhibiting the extent of platelet aggregation (Whittle *et al.*, 1985). As described in section C.13.2, PGI_2 is synthesized from arachidonic acid and is primarily derived from the vascular endothelium and released in response to stimulation by bradykinin, thrombin, histamine, ATP and TxA_2 (Body, 1996).

Much of prostacyclin's effect on platelets was originally described in the 1970's. Moncada *et al* (1976), were the first to describe that blood vessel microsomes from pig or rabbit aorta contain an enzyme that transforms PG endoperoxide to an unstable product that relaxes some blood vessels and prevents platelet aggregation. Arachidonic acid injected into the ear vein of rabbits, resulted in sudden death and was partly due to the formation of occlusive thrombi in

the microvascular bed of the lung. PGI_2 infused intravenously, reduced the mortality of rabbits given AA (1.5mg/kg) (Bayer *et al.*, 1979). Similar anti-thrombotic and *ex vivo* anti-aggregatory effects of PGI_2 were then reconfirmed in other models of thrombus formation (Ubatuba *et al.*, 1979). The efficacy of PGI_2 in preventing coronary artery (circumflex or left anterior descending) occlusion was shown to correlate with the degree of *ex vivo* inhibition of ADP-induced platelet aggregation (platelet-rich plasma samples) (Aiken *et al.*, 1979).

The direct clinical utility of PGI_2 is limited. PGI_2 is chemically and metabolically unstable, being rapidly metabolized *in vivo* to the pharmacologically inactive 6-keto-$PDG_{1\alpha}$ (Oliva and Nicosia, 1987). Given PGI_2 has been shown to be a powerful anti-platelet agent, several analogues of PGI_2 have been synthesized including cicaprost, iloprost and carbacyclin (Armstrong, 1996), with iloprost demonstrating a significant anti-aggregatory effect in subjects with hypercholesterolaemia (Oliva *et al.*, 1989).

[A.10.1.2] Cyclic adenosine 3'5'-monophosphate (cAMP)

The inhibitory effect of PGI_2, PGE_1 and PGD_2 on platelets was shown to be correlated with an activation of the adenylate cyclase system leading to a rise in intra-platelet cAMP (Gorman *et al.*, 1977; Tateson *et al.*, 1977; Whittle *et al.*, 1985).

PGI_2/PGE_1, PGD_2 as described above, exert their anti-platelet effects by binding to a G_S-protein linked receptor that is coupled to adenylyl cyclase. Adenylyl cyclase then catalyzes the conversion of ATP to cAMP, which in turn activates cAMP-dependent protein kinases (Body, 1996; Kroll and Schafer, 1989).

cAMP itself decreases thrombin binding to platelets (Lerea *et al.*, 1987) and inhibits PLC mediated DAG and IP_3 formation, integral to platelet activation (Knight and Scrutton, 1984). Pre-incubation of platelets with PGD_2 or PGE_1 with the resultant stimulation of adenylyl cyclase and cAMP formation, inhibits the subsequent Ca^{2+} response generated following exposure of platelets to a number of agonists (Feinstein *et al.*, 1983; Zavoico and Feinstein, 1984). cAMP dependent protein kinases phosphorylate the vasodilator-stimulated phosphoprotein (VASP) in a mechanism of action that is shared with the cGMP dependent pathway of inhibition of platelet aggregation. Phosphorylated VASP then promotes Ca^{2+} retention in a mechanism that is not well understood (Body, 1996).

Evidence exists suggesting that inhibition of platelet aggregation via the cAMP dependent pathway may also involve a Ca^{2+} independent pathway. Originally proposed by Pannocchia and Hardisty and developed further by Manganello *et al* (1999), cAMP was demonstrated to phosphorylate the TxA_2 receptor effectively preventing further platelet activation (Pannocchia and Hardisty, 1985).

A fundamental difference between the actions of prostacyclin and another anti-platelet agent nitric oxide, is that prostacyclin acts only to inhibit already activated platelets and therefore has no effect on platelet adhesion (Body, 1996). This mechanism of action is in contrast to nitric oxide, which inhibits both adhesion and aggregation of activated platelets.

[A.11] Nitric oxide

Numerous studies to date have demonstrated that platelet aggregation is significantly inhibited by the actions of nitric oxide. In a series of elegant studies performed by Radomski and Moncada, endogenous nitric oxide, released from porcine endothelium following treatment with bradykinin, inhibited human platelet aggregation (Radomski *et al.*, 1987c; Radomski *et al.*, 1987d). Nitric oxide was then shown to be a potent inhibitor of platelet adhesion under flow conditions (de Graaf *et al.*, 1992), with nitric oxide released from activated platelets serving to inhibit further recruitment of platelets to a growing thrombus (Freedman *et al.*, 1997). Many other studies to date have also demonstrated considerable anti-platelet effects of nitric oxide (Gries *et al.*, 1998; Korbut *et al.*, 1995; Langford *et al.*, 1996; Michelson *et al.*, 1996b; Nong *et al.*, 1997; Yao *et al.*, 1992; Yoshimoto *et al.*, 1999).

Given that the biological effectiveness of organic nitrates is mediated principally via nitric oxide (Geiger *et al.*, 1992) (see section D.1.3), an understanding of its mechanism of action within a platelet is essential.

[A.11.1] Mechanism

Nitric oxide binds to the heme iron complex of soluble guanylate cyclase resulting in a deformation of the porphyrin ring (Ignarro *et al.*, 1999). This change in conformational shape results in increased enzyme activity that intern catalyzes the formation of cyclic guanosine 3', 5'-monophosphate (cGMP) from guanosine triphosphate. Confirmation that the anti-platelet

properties of nitric oxide are mediated primarily via a guanylate cyclase/cGMP dependent process, came from a study in which the guanylate cyclase inhibitor 1H-[1,2,4] oxodiazolo [4,3-α] quinaxalin-1 (ODQ) was demonstrated to attenuate the anti-aggregatory action of nitric oxide (Moro *et al.*, 1996). It was also noted that ODQ did not affect the action of prostacyclin or that of a cGMP mimetic, implying a cGMP dependent mechanism was primarily responsible for the inhibition of platelet aggregation by nitric oxide (Moro *et al.*, 1996).

cGMP in turn increases the activity of cGMP-protein kinases (cGMP-PK). Along with having a number of effects that include an interaction with phosphoinositide 3-kinase and the thromboxane receptor (see A.12), cGMP-PK phosphorylate the vasodilator-stimulated phosphoprotein (VASP) (Butt *et al.*, 1994; Wang *et al.*, 1998).

As described in the above sections, platelet activation results from phospholipase C activation, production of DAG and IP_3 eliciting the release of Ca^{2+} from intracellular stores. This then induces phospholipase A_2 production, protein kinase C activation promoting granule secretion and fibrinogen receptor expression. Nitric oxide stimulation of cGMP-PK inhibits agonist induced production of both DAG and IP_3 along with enhancing sequestration of Ca^{2+} within the intracellular stores, thereby effectively inhibiting platelet activation (Body, 1996). In a more recent study examining the effects of nitric oxide on platelets, Trepakova *et al* (1999), demonstrated that nitric oxide inhibits platelet cation influx indirectly, by promoting sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase dependent refilling of the Ca^{2+} stores. Other effects of cGMP also include the inhibition of cAMP phosphodiesterase activity, thereby increasing the amount of available cAMP (Maurice and Haslam, 1990).

In an interesting investigation examining the intravascular significance of the nitric oxide/cGMP-PK signaling pathway *in vivo*, Massberg *et al* (1999), utilizing cGMP-PK-deficient mice, demonstrated that platelet cGMP-PK but not endothelial or smooth muscle cGMP-PK, was essential to prevent intravascular adhesion and aggregation of platelets after ischaemia. Results obtained by Massberg *et al* (1999) and Horstrup *et al* (1994), led to the postulation that cGMP-PK dependent phosphorylation of VASP was essential to prevent platelet activation. However, deletion of the *VASP* gene was demonstrated not to significantly alter mice platelet function, but rather to inhibit the release of Ca^{2+} from IP_3 sensitive stores in both wild type and VASP-deficient platelets (Aszodi *et al.*, 1999; Hauser *et al.*, 1999).

These results therefore suggested that one of the major mechanisms by which cGMP-PK functions may be via limiting the release of Ca^{2+} from IP_3 sensitive stores.

Wang *et al* (1998), investigating the role of nitric oxide in preventing TxA_2 stimulation of platelets, demonstrated that cGMP, via cGMP-PK, prevents TxA_2 receptors from coupling to and activating their cognate G proteins via phosphorylation at an unidentified region of the receptor. *In vivo* phosphorylation of TxA_2 receptors by cGMP-PK was observed when using ^{32}P-labeled platelets that were exposed to 8-Br-cGMP, further indicating that the TxA_2 receptor itself serves as a substrate for cGMP-PK (Wang *et al.*, 1998). For a schematic diagram delineating the pathway of nitric oxide's function in platelets see Figure 1.4.

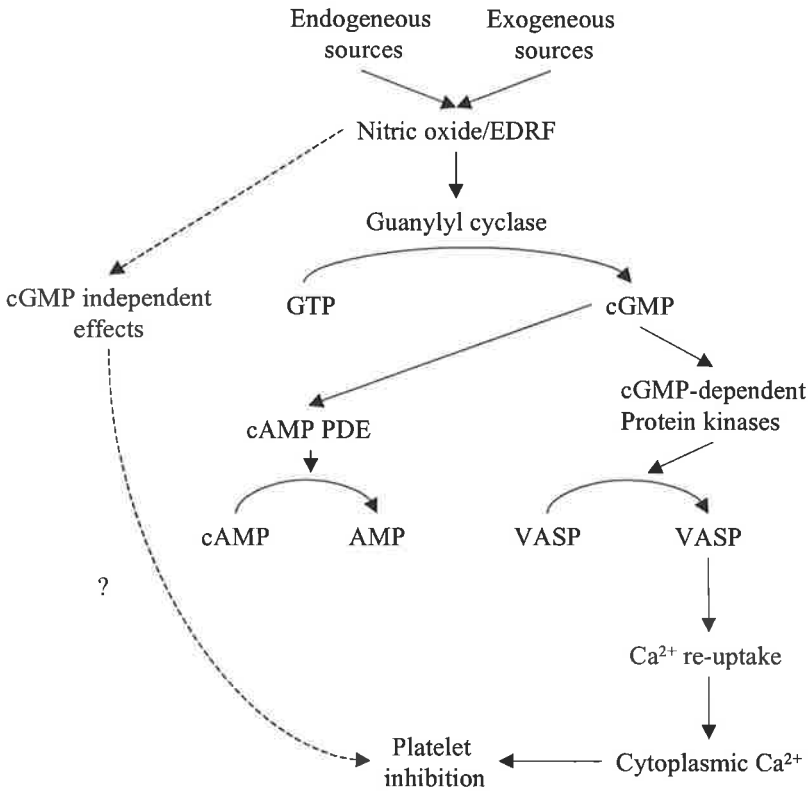


Figure 1.4 Biochemical mechanism of platelet inhibition by nitric oxide and/or endothelial derived relaxing factor

Nitric oxide or EDRF via guanylate cyclase and GTP stimulates the production of cGMP. This in turn activates cGMP-dependent protein kinases that activate VASP, promoting Ca^{2+} re-uptake within the platelet leading to inhibition of platelet adhesion/activation/aggregation. GTP, guanosine tri-phosphate; cGMP, cyclic guanosine mono-phosphate; PDE, phosphodiesterase; VASP, vasodilator-stimulated phosphoprotein. This figure was adapted from Body 1996 (Body, 1996).

[A.11.1.1] cGMP independent effects of nitric oxide

Initially thought to function solely via a cGMP dependent mechanism, it is now known that nitric oxide may inhibit platelet function via mechanisms that are independent of cGMP. A number of examples in endothelial cells, myocytes, and neutrophils, have shown nitric oxide functioning independently of cGMP (Heller *et al.*, 1999b; Sandirasegarane and Diamond, 1999; Ward *et al.*, 2000). However, at a platelet level only a few studies have demonstrated a cGMP independent anti-aggregatory effect of nitric oxide. Brune and Lapetina, whilst investigating the anti-aggregatory properties of sodium nitroprusside (SNP), found that SNP increased ADP-ribosylation of a 34kDa protein, independent of its effects on guanylate cyclase and the production of cGMP (Brune and Lapetina, 1989). Pawloski *et al* (1998), utilizing *S*-nitroso-haemoglobin demonstrated that both cell-free and intra-erythrocytic *S*-nitroso-haemoglobin inhibited platelet aggregation, an effect that was dependent on the oxidation state of the heme within the hemoglobin and independent of intra-platelet cGMP. Martinez *et al* (2001), investigating the role of matrix metaloproteinase-2 (MMP-2) in platelet activation/adhesion demonstrated a synergistic inhibition of platelet adhesion to fibrinogen when phenanthroline (MMP-2 inhibitor) and SNAP were added to a platelet suspension prior to thrombin administration. This inhibition of platelet function was independent of cGMP with no enhanced platelet cGMP generation being detected.

Potential mechanism/s behind cGMP-independent inhibition of platelet function by nitric oxide are unknown at present. However, Boulos *et al* (2000) whilst investigating the effects of peroxynitrite (generated from the interaction of nitric oxide and superoxide) on platelet function, demonstrated that it readily diffuses into the platelet cytosol and inhibits arachidonic acid-induced platelet aggregation by direct nitration of the COX tyrosine residues.

[A.12] Effects of S-nitrosothiols on platelet function

As outlined briefly in section A.1.5, *S*-nitrosothiols are stable nitric oxide derivatives of thiols, which have been shown to mimic some of the biological properties of authentic nitric oxide (Gruetter *et al.*, 1980; Ignarro and Gruetter, 1980). In the presence of copper ions, *S*-nitrosothiols rapidly release nitric oxide with the concomitant oxidation of the remaining thiol to its corresponding disulfide (Williams, 1996). Utilizing *S*-nitroso derivatives of *N*-acetyl penicillamine, cysteine, and β-*D*-thioglucose, Mellion *et al* (1983), demonstrated a

concentration dependent inhibition of platelet aggregation that was also accompanied by a marked increase in intra-platelet cGMP levels. Moreover, the addition of methemoglobin, a scavenger of nitric oxide, partially reversed the anti-aggregatory effects, attenuated the accumulation of cGMP, and inhibited the activation of guanylate cyclase by *S*-nitrosothiols (Mellion *et al.*, 1983).

Howard *et al* (1998), examining the transport of *L*-arginine across human platelets determined that platelets contain an *S*-nitroso-glutathione/glutathione disulphide/Cu^{2+} dependent regulatory mechanism for successful uptake of *L*-arginine. *L*-arginine transport into platelets remained unaffected by either authentic nitric oxide or nitrosonium cation, but was almost 4.7-fold greater with the combination of *S*-nitroso-glutathione plus Cu^{2+}.

More recently Tsikas *et al* (1999), whilst examining the effect of *S*-nitroso-cysteine (SNC) on human platelet aggregation and cGMP formation, demonstrated that SNC potently inhibited both collagen and arachidonic acid-induced platelet aggregation. The addition of ODQ was demonstrated to inhibit SNC-induced formation of cGMP but not to reverse the inhibition of collagen or AA-induced aggregation, implying SNC might function by cGMP-independent mechanisms.

Platelet/Leukocyte Interactions

[A.13] Platelet/leukocyte attachment and significance

Several *in vivo* studies have demonstrated that leukocytes and platelets co-localize at sites of hemorrhage and within atherosclerotic lesions (Marcus, 1994; Ross, 1999). This interaction is said to occur via platelet P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes under static and shear conditions (Rinder *et al.*, 1991a; Rinder *et al.*, 1991b). This initial tethering is then followed by firm adhesion that is dependent on the leukocyte integrin, Mac-1 (Diacovo *et al.*, 1996; Evangelista *et al.*, 1996). Utilizing an *in vitro* flow model, Sheikh and Nash demonstrated that unstimulated neutrophils rolled steadily over a surface that was coated with platelets. Upon neutrophil stimulation with fMLP, neutrophils underwent a concentration dependent transition from rolling to stationary attachment that was itself blocked if the setup was perfused with antibody against CD18/CD11b (Mac-1) (Sheikh and Nash, 1996).

Simon *et al* (2000), in a novel series of experiments identified a direct interaction between the leukocyte integrin Mac-1 and platelet GPIbα. Neutrophil adhesion to platelets was inhibited by monoclonal antibodies to Mac-1 and GPIba, along with demonstrating decreased basal and agonist-stimulated leukocyte-platelet aggregates in blood samples from patients with Bernard-Soulier syndrome compared with normal control subjects.

The physiologic importance of a platelet-leukocyte interaction has received considerable attention because of the pro-inflammatory consequences of such an interaction. The binding of platelets to leukocytes influences a number of key cellular responses, such as neutrophil activation, upregulation of cell adhesion molecules, integrin activation, chemokine activation and a respiratory burst that is responsible for production of various ROS (Ott *et al.*, 1996; Weyrich *et al.*, 1996; Weyrich *et al.*, 1995). However, the impact of leukocytes on platelet-mediated functions remains controversial with a number of *in vitro* studies demonstrating a potentiation (Del Maschio *et al.*, 1990; Faraday *et al.*, 2001; Pratico *et al.*, 1993; Selak *et al.*, 1988) or an inhibition of platelet activity/activation (Nagata *et al.*, 1993; Salvemini *et al.*, 1989a; Schattner *et al.*, 1994; Valles *et al.*, 1993).

Platelet/Endothelium Interactions

[A.14] Platelet/endothelium attachment and significance

As described in section A.5, one of the first events following blood vessel injury is adhesion of platelets to the areas denuded of endothelium via attachment of platelet surface membrane receptor complexes with a number of proteins that include vWf, fibronectin, fibrinogen and thrombospondin. Apart from their critical role in haemostasis and thrombus development, platelets through their direct interaction with the endothelium are involved in various inflammatory mechanisms said to result in atherogenesis development.

Gawaz *et al* (2000), examining the platelet endothelium interaction demonstrated that thrombin activated platelets induce the secretion of monocyte chemotatic protein-1 (MCP-1) and increase the expression of ICAM-1 in cultured endothelial cells via an IL-1 dependent mechanism. Only IL-1 and not various platelet-derived products such as PF4, PGFβ and PDGF were demonstrated to have significant effects on MCP-1 or ICAM-1 generation in endothelial cells. These observation were also reconfirmed even with a transient interaction of thrombin activated platelets with endothelial cells (10-20 minutes) (Dickfeld *et al.*, 2001a),

further emphasizing the importance of limiting the interaction of platelets with the endothelium in an atherogenesis setting.

Section B:

Assessment of Platelet and Endothelial Function

[B.1] Models for evaluation of platelet function

[B.1.1] Platelet activation/aggregation techniques

Platelets play an integral role in both haemostasis and thrombus and as such, methods that accurately measure platelet function have been developed for both research and clinical use.

[B.1.1.1] Optical (turbidometric) platelet aggregometry

Born, in 1962, whilst investigating the induction of platelet aggregation by adenosine, described a method of assessment of platelet function that involved examining the changes in light transmittance through a platelet-rich plasma sample (Born, 1962). Briefly, platelet rich plasma (PRP) samples are stirred in a cuvette at 37°C between a light source and a photocell within an aggregometer. Upon the addition of a platelet agonist, platelets aggregate and the transmission of light increases. From its inception, this method of assessment of platelet function has been utilized extensively. However, there is now evidence to suggest that this optical turbidometric method of analysis of platelet function is insensitive to pre-existing micro-aggregates or insensitive to the development of micro-aggregates post agonist administration (reviewed by Harrison, 2000). A new aggregometer has been developed that utilizes a combination of laser light scattering and aggregometry to effectively monitor the formation of platelet micro-aggregates (Ozaki *et al.*, 1994).

[B.1.1.2] Impedance platelet aggregometry

Given that the traditional method of assessment of platelet function via the optical turbidometric method involves utilizing PRP or a washed platelet preparation, contributions from other blood constituents that may affect platelet function are absent. Therefore Cardinal and Flower, developed an aggregometer that quantifies platelet function within a whole blood

sample via changes in electrical impedance (Cardinal and Flower, 1980). Briefly, whole blood is stirred at 37°C between two platinum wire electrodes set at a fixed distance (Mackie *et al.*, 1984). Platelets when exposed to an agonist aggregate around these electrodes increasing the electrical impedance. In a comparative study Mascelli *et al* (1997), demonstrated that during and after 12-hrs of GPIIb/IIIa receptor antagonist treatment, the extent of inhibition of aggregation utilizing both the impedance and turbidimetric platelet aggregometry were correlated with the amount of GPIIb/IIIa receptor blockade, a relationship however that was lost for the turbidimetric platelet aggregometry at 36-hrs post GPIIb/IIIa receptor antagonist administration. Like the turbidimetric method of assessment of platelet function, impedance platelet aggregometry is also suspected to be insensitive to pre-existing micro-aggregates or insensitive to the development of micro-aggregates post agonist administration (Harrison, 2000). Despite these limitations whole blood impedance aggregometry still remains a popular and reliable method for assessment of platelet function (Elwood *et al.*, 1990; Chirkov *et al.*, 1993; Diodati *et al.*, 1995/1998) and as such serves as the method of assessment of platelet function used throughout the experimental sections of this thesis.

[B.1.1.3] The rapid platelet function assay (RPFA)

The rapid platelet function assay (RPFA) provides a simple and rapid means of monitoring the efficacy of GPIIb/IIIa receptor antagonist pharmacotherapy and is based on the principle that fibrinogen-coated beads agglutinate in whole blood in proportion to the number of GPIIb/IIIa receptors (Frelinger and Hillman, 1998; Harrison, 2000). A citrated whole blood sample is added to a cartridge that contains fibrinogen-coated beads and a platelet agonist. Platelet activation/aggregation commences resulting in fibrinogen binding to exposed GPIIb/IIIa receptors not already blocked by the receptor antagonist being examined.

[B.1.1.4] The cone and plate(let) analyzer (CPA)

As the classical methods of assessment of platelet aggregation (turbidimetric and impedance aggregometry) do not mimic totally all the physiological processes that occur *in vivo* (i.e. platelet adhesion) a number of other techniques that evaluate platelet function have been developed. The cone and plate(let) analyzer is a system that examines the level of platelet adhesion to an extracellular matrix under shear induced flow conditions and is dependent upon the presence of GPIb, vWf and GPIIb/IIIa (Kenet *et al.*, 1998; Varon *et al.*, 1997; Varon

et al., 1998). The analyzer consists of a cone and plate device in which a whole blood sample (150-250µl) is subjected to arterial flow conditions for a period of 2 minutes. An image analyzer monitors platelet adhesion/aggregation upon the extracellular matrix. In an examination comparing the effectiveness of monitoring GPIIb/IIIa receptor blockade, Osende *et al* (2001), demonstrated that the CPA was better correlated with the percentage of free GPIIb/IIIa receptors than was RPFA or platelet aggregometry.

[B.1.1.5] Other methods

A number of other devices that assess platelet function by either shear induced platelet activation such as the PFA-100 or the high shear filterometer or instruments that examine the global haemostasis (the clot signature analyzer (CSA) and the thrombotic status analyzer), have also been developed over recent years (see Harrison *et al.*, 2000 for a summary). Of the aforementioned devices, the PFA-100 has been extensively used. It functions by exposing whole blood samples to high shear within a capillary. It then monitors the drop in flow rate as the platelets within the whole blood sample form a haemostatic plug within an aperture of a membrane that is coated with collagen and either ADP or adrenaline. The test has been particularly useful in evaluating platelet function in patients with von Willebrand disease (Favaloro *et al.*, 2001; Harrison, 2000), and may also examine the contribution of systemic inflammation on platelet function (Homoncik *et al.*, 2000). The test has also been used to examine the effectiveness of GPII/IIIa pharmacotherapy during PTCA (Madan *et al.*, 2001), despite several limitations being raised when using it to assess platelet function in subjects with congestive heart failure (Serebruany *et al.*, 2001).

[B.1.2] Measures of platelet activation

A number of biochemical tests have now been developed to measure the degree of platelet activation *in vivo*.

[B.1.2.1] Activation markers

One method of assessing *in vivo* platelet activation is to measure platelet specific release products with examples including PAF-4 (Jafri *et al.*, 1993) and β-thromboglobulin (Hughes *et al.*, 1982; Kjeldsen *et al.*, 1987; Rossi *et al.*, 1998), that are released from platelet α-granules upon platelet activation/aggregation (Blockmans *et al.*, 1995). Other platelet activation assays have quantified the extent of the stable metabolites of thromboxane B_2 in

either plasma or urine (de Boer *et al.*, 1982; FitzGerald *et al.*, 1983; Nidorf *et al.*, 1989; Zahavi *et al.*, 1989). More recently the use of soluble P-selectin as a marker of platelet activation has also been performed by a number of investigators. P-selectin is an α-granule membrane protein that is translocated to the platelet membrane surface upon platelet activation and are cleaved by specific enzymes to produce a soluble platelet activation marker that can be quantified (Blockmans *et al.*, 1995; Michelson *et al.*, 1996a).

Utilizing flow cytometric analysis, a number of platelet specific, cell surface activation markers have also been utilized to determine a level of *in vivo* platelet activation. Such activation markers include P-selectin (Holmes *et al.*, 2001; Ritchie *et al.*, 2000), CD63 (Chakhtoura *et al.*, 2000; Hagberg and Lyberg, 2000), and CD40L (Hermann *et al.*, 2001; Lee *et al.*, 1999). It is also now possible to assess activation dependent changes in the conformation of the GPIIb/IIIa receptor complex using PAC-1 (Moshfegh *et al.*, 2000; Rossi *et al.*, 2001) and LIBS (ligand-induced binding sites) (Dickfeld *et al.*, 2001b). Other methods also include binding of secreted proteins such as thrombospondin (Griesshammer *et al.*, 1999) and exposure of phosphatidyl serine (factor Va and VIIIa binding, annexin V) (Furman *et al.*, 2000; Nomura *et al.*, 2000; Poley and Mempel, 2001).

[B.2] Assessment of endothelial function

Endothelial dysfunction as described in section C.2 is associated with a number of disease states that include atherosclerosis, hypertension and diabetes. The assessment of endothelial function has developed into an important diagnostic and research tool. A number of invasive and non-invasive techniques for the assessment of endothelial function have evolved over the past fifteen years, some of which are described below.

[B.2.1] Intra-coronary studies

Infusion of acetylcholine into the coronary circulation as pioneered by Ludmer *et al* (1986), has served as the "gold standard" for examination of coronary endothelial function. Traditionally the coronary artery diameter is measured using quantitative angiography before and after intra-coronary administration of acetylcholine. In arteries with preserved endothelial function, acetylcholine infusion stimulates the release of nitric oxide resulting in vasodilatation. However, in subjects with atherosclerosis, as described in section C.2, a paradoxical vasoconstrictor effect results, the phenomenon of which is now said to be an

early manifestation of endothelial dysfunction (Zeiher *et al.*, 1991). This technique has now provided insight into potential risk factors for endothelial dysfunction in coronary arteries (Vita *et al.*, 1990), and in predicting long-term atherosclerotic disease progression and cardiovascular event rates (Schachinger *et al.*, 2000; Suwaidi *et al.*, 2000).

[B.2.2] Impedance plethysmography

Venous occlusion plethysmography is a widely used technique for the semi-invasive assessment of limb blood flow and hence serves as a tool for the assessment of endothelial function. This technique uses infusion of acetylcholine or methacholine (Bruning *et al.*, 1996) to assess endothelium-dependent endothelial function, usually in the brachial artery and determines the increase in blood flow in the forearm following short-term occlusion. A mercury in-silastic strain gauge is placed at the upper third of the forearm. Venous occlusion is then achieved by a blood pressure cuff applied proximal to the elbow (Lind *et al.*, 2000).

Decreased changes in blood flow post venous occlusion have been shown to be related to the extent of coronary atherosclerosis (Anderson *et al.*, 1995b) along with the occurrence of various cardiovascular risk factors such as hypertension, hypercholesterolaemia, aging, male sex, diabetes mellitus and smoking (Celermajer *et al.*, 1994; Celermajer *et al.*, 1993; Clarkson *et al.*, 1996; Gilligan *et al.*, 1994; Taddei *et al.*, 1993; Zeiher *et al.*, 1995). In a more recent study, a direct comparison between the increase in blood flow induced by infusion of methacholine and the degree of flow-mediated dilatation as measured by brachial ultrasound (below) was performed. A significant correlation was observed between both methods regarding the degree of endothelium-independent vasodilatation evoked by the nitric oxide donor SNP. However, no correlation was found between the two methods regarding the evaluation of endothelium-independent vasodilatation (Lind *et al.*, 2000), casting doubt as to what both techniques were actually measuring.

[B.2.3] Brachial ultrasound

Celermajer *et al* (1992), whilst investigating potential determinants of endothelial dysfunction was one of the first to described a non-invasive way of assessing the extent of flow mediated dilatation within the brachial or femoral artery. The technique is based on the principle that increases in blood flow, and thereby shear stress, induces an increase in nitric oxide release causing vasodilatation. Upper-arm occlusion for 5 minutes results in a reactive

hyperemia after the pressure cuff is released with a resultant increase in shear stress that causes flow mediated dilatation (Anderson, 1999). Anderson *et al* (1995b), described a correlation between brachial artery flow mediated dilatation and the coronary artery response to acetylcholine administration. Numerous research groups are now using this technique for assessment of endothelial function in a variety of disease states and pharmacological interventions (Clarkson *et al.*, 2001; Dogra *et al.*, 2001; Gokce *et al.*, 1999; Hamabe *et al.*, 2001), with the potential for it to be used as a screening test for CAD (Schroeder *et al.*, 1999).

[B.2.4] Pulse wave analysis

Pulse wave analysis employs applanation tonometry to record peripheral pressure waveforms (brachial/carotid), from which the augmentation index AI(x), central pressure waveform in systole, ejection duration, the timing of wave reflection and heart rate can all measured. The AI(x) is the ratio of the augmentation pressure (difference in pressure between the early and late systolic shoulders) and the pulse pressure expressed as a percentage and is a measure of the systemic arterial stiffness and wave reflection (Wilkinson *et al.*, 1998a; Wilkinson *et al.*, 2000). Arterial stiffness is known to increase with age (O'Rourke *et al.*, 2001), hypertension and is also enhanced in subjects with diabetes mellitus (Lehmann *et al.*, 1992), atherosclerosis (Wada *et al.*, 1994), and end stage renal disease (London *et al.*, 1990), and as such is becoming more widely accepted as a means of quantifying endothelial function. Further to this, Wilkinson *et al* (2002), recently demonstrated a significant linear relationship between the extent of vascular responsiveness to albuterol and the change in forearm blood flow during acetylcholine infusion as measured by venous occlusion plethysmography of the brachial artery.

Section C:

Disturbances of Endothelial/Platelet Function; Relationship to Ischaemic Heart Disease

[C.1] Normal endothelial function

The vascular endothelium is a one cell thick lining of the vessel wall that plays an integral role in vascular homeostasis through the release of a number of growth factors, vasoactive, thromboregulatory and signal transduction molecules. In addition to its vasodilatory properties, a healthy endothelium is anti-atherogenic due to its anti-platelet, and anti leukocyte properties. This localized vascular control depends on a balance between vasodilators (nitric oxide and prostacyclin), and vasoconstrictors such as angiotensin II and endothelin (Anderson, 1999).

Acetylcholine, bradykinin and substance P, along with increases in blood flow, elicit the endothelium dependent dilation of both large and small vessels (Crossman *et al.*, 1989; Drexler *et al.*, 1989; Groves *et al.*, 1995). Vallance *et al* (1989), utilizing *L*-N^G-monomethyl-*L*-arginine (*L*-NMMA) in human subjects, was the first to present evidence demonstrating a continuous release of endothelium derived nitric oxide that influences the degree of blood flow implying that the vasculature remains in a partial dilated state. As described in section A.11, nitric oxide is anti-thrombotic through its potent anti-aggregatory and anti-adhesive properties. It also has potent anti-inflammatory properties (Libby, 1995; Ross, 1999). Disturbances in the endothelial mediated production of nitric oxide is thought to be the initiating event in atherosclerosis development and is important in the pathophysiological manifestations of the disease process as well.

[C.2] Endothelial dysfunction

Endothelial damage/dysfunction resulting from a mechanical or biochemical stimulus, is now thought to result from an imbalance between vasodilator/vasoconstrictor factors, between anti-and pro-coagulant mediators, and/or growth promoting and inhibiting factors (Raitakari and Celermajer, 2000). Endothelial dysfunction, a term often used to denote an impairment of endothelium-dependent vasodilatation, is now thought to be an important component of the

pathogenesis of atherosclerosis, myocardial ischaemia and heart failure (Anderson, 1999; Ross, 1993). Ludmer *et al* (1986) observed that while acetylcholine infusion readily produces vasodilatation in normal coronary artery segments, it induced a paradoxical vasoconstrictive response in segments with varying degrees of atherosclerosis. This observation of a paradoxical vasoconstriction (or attenuation of vasodilator response) has been observed in a multitude of other conditions that include diabetes mellitus, hypercholesterolaemia, smoking, hypertension, heart failure and others (Kato *et al.*, 1997; Quyyumi *et al.*, 1992a; Zeiher *et al.*, 1991).

[C.2.1] Association with coronary risk

Endothelial dysfunction may precede the clinical evidence of atherogenesis. Confirming results obtained by Zeiher *et al* (1991), Reddy *et al* (1994), observed an impaired endothelial-dependent vasodilatation in patients with hypercholesterolaemia but without intra-vascular ultrasound defined evidence of atherosclerosis. Healthy young adults with a family history of premature CAD were found to have an impaired endothelium-dependent dilatation in the brachial circulation, even in the absence of other cardiovascular risk factors (Celermajer *et al.*, 1992; Clarkson *et al.*, 1997). By multivariate analysis, serum cholesterol, male gender, a positive family history, age and total number of coronary risk factors were found to be independent predictors of a reduced coronary acetylcholine responsiveness in subjects with angiographically defined normal coronary arteries (Vita *et al.*, 1990). In a more recent study examining the brachial artery endothelial function of patients undergoing bypass surgery, Gokce *et al* (2002), demonstrated that age, renal insufficiency and a lower brachial artery flow-mediated dilation were independent predictors of a major event within 30 days post surgery.

[C.3] Mechanism/s of endothelial dysfunction

Potential mechanisms responsible for endothelial dysfunction can be simplistically viewed as those that involve an increased vasoconstrictor function and/or those that relate to a reduced vasodilator influence (Cooke, 2000), with much of the recent research behind endothelial dysfunction being focused on the latter. Of the causes of a reduced vasodilator influence, a dysfunction within the NOS pathway has been the most extensively studied.

[C.3.1] Reduced sensitivity to nitric oxide

The idea of a reduced sensitivity towards nitric oxide as an explanation behind the phenomenon of endothelial dysfunction can also be viewed as attenuation (resistance) of its effectiveness. Evidence demonstrating a resistance towards nitric oxide at the vascular and platelet level will be discussed in detail later in this chapter (section E).

[C.3.2] Reduced synthesis of nitric oxide

Evidence exists for and against a reduced synthesis of nitric oxide serving as the candidate for the mechanism behind endothelial dysfunction. Whether this reduction results from a lack of sub-units required for successful production by NOS (see section C.3.2.1) or results from a direct inhibition of the enzyme system, many studies have been performed addressing this potential mechanism.

[C.3.2.1] Attenuated L-arginine transportation or reduced NOS sub-unit availability

The lipid component of ox-LDL lysophosphatidylcholine has been demonstrated to inhibit *L*-arginine transport in bovine endothelial cells (Kikuta *et al.*, 1998). These results added weight to the findings of Jay *et al* (1997), who demonstrated a marked attenuation of an agonist-stimulated release of nitric oxide following exposure of human endothelial cells or vascular smooth muscle cells to ox-LDL. Further evidence of a possible impairment in *L*-arginine transportation, serving as the mechanism behind endothelial dysfunction, comes from an investigation by Kaye *et al* (2000), in which subjects with congestive heart failure were shown to have a significantly reduced clearance of [^{3}H] *L*-arginine compared to control subjects. This attenuated clearance of [^{3}H] *L*-arginine in the heart failure subjects was shown to be associated with a significant reduction in the expression of the cationic amino acid transporter CAT-1, partly responsible for *L*-arginine transportation.

[C.3.2.2] Inhibition of NOS by asymmetrical dimethyl-L-arginine (ADMA)

Asymmetric dimethyl-arginine (ADMA) and *N*-monomethyl-arginine (NMA), synthesized in many cells including vascular endothelial cells, are endogenous competitive inhibitors of NOS, with the former being the predominant plasma species (Cooke, 2000). Vallance *et al* (1992a/b) and subsequently others demonstrated that endogenous ADMA antagonized endothelium dependent vasodilatation (Calver *et al.*, 1993). Plasma levels of ADMA were

found to be elevated in patients with chronic renal failure, a condition associated with accelerated atherogenesis (Vallance *et al.*, 1992a).

Following on from these observations, plasma levels of ADMA were found to be elevated in subjects with pre-eclampsia (Fickling *et al.*, 1993), congestive heart failure (Feng *et al.*, 1998; Usui *et al.*, 1998), hypertension (Matsuoka *et al.*, 1997; Surdacki *et al.*, 1999) and hypercholesterolaemia (Boger *et al.*, 1998), all risk factors or disease states previously demonstrated to be associated with endothelial dysfunction. Plasma levels of ADMA have been demonstrated to be acutely elevated following ingestion of a high fat meal in patients with NIDDM. This increase in ADMA was demonstrated to occur in association with an acute reduction in the vasodilator response of the brachial artery (Fard *et al.*, 2000). As ADMA is a competitive inhibitor of *L*-arginine induced activation of NOS, a more appropriate approach may be ADMA:arginine ratios.

Given ADMA is elevated in many disorders associated with atherosclerosis, it has been speculated to represent the pathway by which endothelial dysfunction occurs. The exact mechanisms by which this may result, remain unclear. Pre-incubation of endothelial cells with ADMA has been demonstrated to increase the adhesiveness of the endothelial cells to a monocytoid cell line (Boger *et al.*, 2000), the binding of which is required for atherosclerosis initiation. Moreover, in haemodialysis dependent end-stage renal failure patients, plasma ADMA was a significant factor in predicting overall mortality and cardiovascular events (Zoccali *et al.*, 2001).

[C.3.2.3] Reduced half life of nitric oxide

Acute and chronic oxidative stress to the vascular endothelium is said to be a serious causative factor of vascular endothelial dysfunction by accelerated inactivation by ROS (Harrison, 1997). The reaction of superoxide and nitric oxide occurs at a rate of $6.7x10^{9}mol/L^{-1}\cdot s^{-1}$ and is faster than the reaction between superoxide and superoxide dismutase (Thomson *et al.*, 1995). Given that atherosclerosis, cigarette smoking, hypertension, hypercholesterolaemia, diabetes mellitus and heart failure are associated with both elevated levels of ROS and a depressed endothelial function, several research groups have examined the above hypothesis (Bauersachs *et al.*, 1999; Bouloumie *et al.*, 1997; Indik *et al.*, 2001; Zalba *et al.*, 2000).

Potential sources of ROS that have received particular attention in recent years have been the NAD(P)H oxidase, xanthine oxidase and NOS enzyme systems. A number of investigators have determined that the predominate substrate driving superoxide production within endothelial cells and VSMC's is NAD(P)H oxidase (Cai and Harrison, 2000; Zalba *et al.*, 2000). Each enzyme system's role will be discussed separately (see section C.4 for NAD(P)H oxidase, C.5.1 for xanthine oxidase and C.5.2 for NOS).

[C.3.2.4] Evidence against reduced synthesis of nitric oxide

Evidence against reduced synthesis of nitric oxide in the phenomenon of endothelial dysfunction is limited but does exist. In a study performed by Bauersachs *et al* (1999), rats with heart failure resulting from MI and demonstrating endothelial dysfunction showed a marked up-regulation of eNOS and soluble guanylate cyclase expression in their aortas. Habib *et al* (1994), investigating the extent of basal nitric oxide produced in heart failure subjects, demonstrated an increase in production of nitric oxide, as measured by the responsiveness to *L*-NMMA, and suggested that it may serve as a defense mechanism in developing heart failure. Furthermore, Winlaw *et al* (1994), also reported an increase in endogenous nitric oxide production in subjects with heart failure that was later shown to correlate with New York Heart Association functional class (Winlaw *et al.*, 1995).

The results obtained from a study by Bouloumie *et al* (1997) go some way to explain the discrepancies between studies that show high levels of NOS activity and endothelial dysfunction to those that subscribe to the reduced levels of nitric oxide hypothesis. Two weeks after aortic banding of Sprague-Dawley rats, neither endothelial dysfunction nor changes in vascular endothelial cell eNOS expression/activity were detected. However, vascular superoxide levels were significantly elevated. At six weeks post banding a reduced endothelial vasodilator responsiveness was observed with a corresponding increase in eNOS expression/activity and superoxide production that together resulted in the enhanced appearance of nitrotyrosine residues (reflecting incremental peroxynitrite formation). This would suggest that the development of endothelial dysfunction is associated with an overproduction of both nitric oxide and superoxide that may lead to the formation of peroxynitrite (Bouloumie *et al.*, 1997).

[C.3.3] Angiotensin II

The renin-angiotensin-aldosterone system via induction of various vasoconstrictive agents, such as angiotensin II (AngII) and endothelin, is also known to participate in the phenomenon of endothelial dysfunction. Given the potentency of Ang II as a vasoconstrictive agent, stimulation and release of Ang II functionally antagonizes endothelium-dependent vasodilatation (Schmidt-Ott *et al.*, 2000). Moreover, because of the suppression of bradykinin production, Ang II further limits the generation of nitric oxide by the endothelium (Sato *et al.*, 1999). A further discussion on the therapeutic benefits of limiting Ang II effectiveness via ACE inhibition or angiotensin receptor antagonism in respect to restoring endothelial function is found in section C.6.3.

Apart from counteracting the vasodilatory properties of nitric oxide, Ang II induces the production of superoxide via an NAD(P)H oxidase dependent mechanism, independent of its haemodynamic effects (Rajagopalan *et al.*, 1996), thereby generating superoxide that scavenge nitric oxide and therefore limiting its effectiveness. A separate discussion on the role of Ang II induced superoxide production via NAD(P)H oxidase is in section C.4.3.1.

[C.4] Pathophysiology of NAD(P)H oxidase

[C.4.1] Endothelium

Several studies have identified superoxide as a potential second messenger for a variety of cellular processes within the vasculature, with much attention being focused lately on its role in the initiation, development and complications associated with endothelial dysfunction and atherosclerosis (Meyer and Schmitt, 2000).

[C.4.2] Genetic polymorphisms

In terms of NAD(P)H oxidase per se and its involvement in the development of atherosclerosis, recent interest has focused on an association of the polymorphism in the *CYBA* gene that encodes $p22^{phox}$, with the progression of atherosclerosis. The *CYBA* C242T polymorphism results in a substitution of tyrosine for histidine at residue 72 of $p22^{phox}$, leading to modulation of the enzyme.

In subjects from the prospective lipoprotein and coronary artery study (LCAS) a significant association between the CYBA 242T polymorphism and accelerated progression of coronary

atherosclerosis was observed on post hoc analysis (Cahilly *et al.*, 2000). On the other hand, in a study by Guzik *et al* (2000a), investigating the functional significance of this polymorphism on vascular superoxide production, it was demonstrated that the 242T allele was associated with a significantly reduced vascular NAD(P)H oxidase activity and superoxide level. Schachinger *et al* (2001) examined the vasodilator function of the epicardial arteries and its relationship to the presence of the C242T $p22^{phox}$ polymorphism, and revealed that the CC genotype is independently associated with a reduced endothelium-dependent vasodilator response, compared with patients with the C242T polymorphism. Results of that study also showed a trend towards an impaired responsiveness to nitroglycerine (NTG) in subjects carrying the CC genotype of the C242T $p22^{phox}$ gene. Further investigations into the significance of these polymorphism are required in-order to clarify these and other discrepancies (Cai *et al.*, 1999; Li *et al.*, 1999a; Stanger *et al.*, 2001).

Adding further uncertainty to the role of NAD(P)H oxidase and hence superoxide in the initiation or progression of atherosclerosis, an investigation performed by (Kirk *et al.*, 2000) demonstrated no difference in the extent and rate of atherosclerotic lesion development in mice deficient in $gp91^{phox}$ compared to wild type controls when fed a high fat diet.

[C.4.3] Stimuli for NAD(P)H oxidase

[C.4.3.1] Angiotensin II

A number of groups have now demonstrated that Ang II stimulates the activity of membrane-bound NAD(P)H oxidase in both endothelial cells and VSMC's with the resultant production of superoxide (Griendling *et al.*, 1994; Pagano *et al.*, 1997; Rajagopalan *et al.*, 1996). Warholtz *et al* (1999), utilizing an animal model of hypercholesterolaemia, demonstrated that hypercholesterolaemia was associated with an increase in AT_1 receptor expression, a reduced endothelial function and increased NAD(P)H oxidase dependent superoxide production. Treatment of cholesterol fed rabbits with an AT_1 receptor antagonist reduced the degree of plaque formation, decreased NAD(P)H oxidase activity and improved endothelial function, implying an essential role for Ang II mediated (NAD(P)H oxidase dependent) endothelial dysfunction (Warnholtz *et al.*, 1999). A number of investigators have demonstrated the importance of NAD(P)H oxidase subunits, especially $gp91^{phox}$, $p67^{phox}$, nox-1 (mox-1), nox-4, in Ang II stimulated superoxide generation (Bendall *et al.*, 2002; Cifuentes *et al.*, 2000; Lassegue *et al.*, 2001; Mollnau *et al.*, 2002; Wang *et al.*, 2001; Wingler *et al.*, 2001).

[C.4.3.2] Others

Thrombin, platelet derived growth factor (PDGF), TNF-α and laminar sheer stress have also been demonstrated to stimulate NAD(P)H oxidase dependent superoxide generation in vascular smooth muscle cells (De Keulenaer *et al.*, 1998; Holland *et al.*, 1998; Li *et al.*, 2002; Marumo *et al.*, 1997).

[C.4.4] Association of NAD(P)H oxidase with endothelial dysfunction

In a landmark investigation by Ohara *et al* (1993), excess of endothelial derived superoxide was detected in aortic segments obtained from rabbits fed a high fat diet, and was also associated with a reduced acetylcholine-induced relaxation. Chronic treatment of these rabbits with either superoxide dismutase (Mugge *et al.*, 1991) or probucol (Keaney *et al.*, 1995) was shown to restore or preserve endothelial function, suggesting that superoxide released from the endothelium of hypercholesterolaemic vessels attenuates endothelial nitric oxide release and/or vascular responsiveness to nitric oxide. (Nguyen-Khoa *et al.*, 1999; Warnholtz *et al.*, 1999). More specifically, a number of investigators have also shown an association between the reduced vascular responsiveness to acetylcholine and an upregulation of NAD(P)H oxidase, with a resultant increase in superoxide (Zalba *et al.*, 2000).

[C.5] Other sources of reactive oxygen species

In mammalian cells, enzymatic sources of ROS include the mitochondrial respiration chain, lipoxygenase, cyclooxygenase, peroxidases, haemoproteins and endothelium derived hyperpolarizing factor synthase (Cai and Harrison, 2000; Fleming *et al.*, 2001). Although many of the aforementioned sources could potentially produce ROS that inactivate nitric oxide; two other enzyme systems will be discussed below.

[C.5.1] Xanthine oxidase

The xanthine oxidoreductase is a molybdoenzyme capable of catalyzing the oxidation of hypoxanthine and xanthine in the process of purine metabolism (Moriwaki *et al.*, 1999). Xanthine oxidoreductase has the ability to exist in two separate but interconvertable forms. Either as xanthine dehydrogenase or xanthine oxidase, of which both forms can generate superoxide (Moriwaki *et al.*, 1999).

One of the first investigators to postulate that superoxide derived from xanthine oxidase may modulate the bioavailability of nitric oxide came from Ohara *et al* (1993). As discussed before, rabbits fed a high cholesterol diet were found to have high superoxide levels, amounts of which were subsequently normalized following pre-incubation of the segments with oxypurinol. More recently, allopurinol pharmacotherapy has been shown to improve endothelial function in subjects with diabetes as assessed by venous occlusion plethysmography (Butler *et al.*, 2000). This result contrasts that obtained by O'Driscoll, in which endothelium dependent responses to acetylcholine remained unaffected in hypercholesterolaemic subjects chronically treated by allopurinol, implying that an alternative source of superoxide may be responsible for the postulated oxidative stress induced endothelial dysfunction (O'Driscoll *et al.*, 1999). In humans with hypercholesterolaemia, administration of oxypurinol was demonstrated to improve an impaired endothelium dependent vasodilatation that was not observed in subjects with hypertension alone (Cardillo *et al.*, 1997).

A potential explanation for some of the inconsistencies observed in some of the above investigations is the demonstration that xanthine oxidase may exist in a molybdenum-deficient form in many tissues. The modified enzyme is unable to utilize xanthine as a substrate and as such is not inhibited by oxypurinol or allopurinol, but rather utilizes NADH as a substrate to produce superoxide (Sanders *et al.*, 1997).

[C.5.2] Endothelial derived nitric oxide synthase

Another alternative source of vascular ROS that has received recent attention is eNOS. As described in section A.2.2.1, in the absence or low concentrations of the substrate *L*-arginine or co-factors such as BH_4, NOS is capable of catalyzing the uncoupled reduction of molecular oxygen to superoxide (Vasquez-Vivar *et al.*, 1998). Utilizing spontaneously hypertensive rats that have high levels of vascular superoxide, Kerr *et al* (1999), demonstrated a reduction in the amount of aortic superoxide generation following treatment with *L*-NAME or removal of the endothelium. More recently, using transgenic BH_4 deficient mice, Cosentino *et al* (2001), demonstrated that endothelium-dependent relaxation to acetylcholine was inhibited by catalase and enhanced by superoxide dismutase, suggesting that BH_4 deficiency leads to eNOS dysfunction with the subsequent formation of ROS,

influencing endothelium-dependent relaxation; similar observations were made by Shinozaki *et al* (1999).

Pharmacotherapy for Endothelial Dysfunction

Experimental evidence now exists that endothelial dysfunction in a variety of disease states can be attenuated via a number of pharmacotherapeutic interventions.

[C.6.1] Interventions targeting nitric oxide, NOS production/regulation

[C.6.1.1] L-arginine/tetrahydrobiopterin supplementation

Attempts to limit endothelial dysfunction through diet supplementation with *L*-arginine have been inconclusive. Diet supplementation with *L*-arginine has been shown to improve agonist mediated endothelium dependent vasodilatation in subjects with CHF (Hambrecht *et al.*, 2000), in hypercholesterolaemic patients (Creager *et al.*, 1992) but not in subjects with CAD alone (Blum *et al.*, 2000), or young subjects with type I diabetes mellitus (Mullen *et al.*, 1998b). These inconsistencies may be explained by the results obtained by Thorne *et al* (1998), who demonstrated a significant improvement in flow mediated dilatation of hypercholesterolaemic subjects and current smokers, but not in control or diabetic patients following *L*-arginine infusion, implying that endothelial dysfunction in diabetic patients does not result from an impaired utilization of *L*-arginine.

Despite the lack of conclusiveness in the results regarding the effectiveness of *L*-arginine supplementation for the amelioration of endothelial dysfunction, *L*-arginine dietary supplementation has been shown to attenuate some processes involved in atherosclerosis initiation. Monocyte adhesiveness to human or bovine aortic endothelial cells is diminished in subjects with hypercholesterolaemia (Chan *et al.*, 2000; Theilmeier *et al.*, 1997) or proven CAD (Adams *et al.*, 1997) following dietary *L*-arginine supplementation.

Combinations of other nitric oxide synthase co-factors required for successful nitric oxide generation such as tetrahydrobiopterin either alone (Heitzer *et al.*, 2000; Maier *et al.*, 1998; Verma *et al.*, 2000) or in combination with *L*-arginine (Jiang *et al.*, 2000) have also been demonstrated to improve the extent of endothelial function in human saphenous vein preparations and in animal models of advanced atherosclerosis. In insulin-resistant rats, oral supplementation with BH_4 (10mg/kg/day) for 8 weeks reversed the impaired endothelium

dependent arterial relaxation. Furthermore, BH_4 pharmacotherapy was associated with a two-fold increase in eNOS activity and a 70% reduction in endothelial superoxide production compared to control rats (Shinozaki *et al.*, 2000).

[C.6.1.2] Cholesterol lowering

A number of studies to date (but not all) have demonstrated that endothelial dysfunction is improved following cholesterol lowering, especially with the use of statins. Reduction of fat intake in hypercholesterolaemic monkeys resulted in improved endothelial function (Harrison *et al.*, 1987). This initial study was then reconfirmed in human subjects with hypercholesterolaemia but normal coronary arteries (Leung *et al.*, 1993), and in subjects with a moderately elevated total cholesterol, > 6mmol/L (O'Driscoll *et al.*, 1997b). Additional improvement in vasomotor responsiveness to acetylcholine was observed with a combination of a statin and anti-oxidant compared to statin treatment alone (Anderson *et al.*, 1995a). Cholesterol reduction was demonstrated to reduce the number of ischaemic episodes in subjects with CAD (Andrews *et al.*, 1997), via a mechanism that involves improvement in endothelial function (Dupuis *et al.*, 1999; Huggins *et al.*, 1998). Lundman *et al* (1997), in seven healthy males without risk factors for CAD, demonstrated that transient elevations of triglyceride were associated with decreased flow mediated dilatation of the brachial artery. However, transient moderate hypertriglyceridaemia through consumption of a high fat meal did not impair endothelial function of the forearm resistance vessels (Gudmundsson *et al.*, 2000). Contrary to the above positive studies, a randomized placebo controlled trail demonstrated no significant effect of six months of cholesterol-lowering therapy with simvastatin on coronary endothelial vasomotor function in patients with CAD and mildly elevated cholesterol levels (Vita *et al.*, 2000).

Despite the uncertainty arising from the use of cholesterol lowering pharmacotherapies at improving endothelial function, several groups have examined a potential mechanism that may involve a nitric oxide dependent pathway. Laufs *et al* (1998) demonstrated that treatment of human saphenous vein preparations with HMG-CoA reductase inhibitors resulted in an up-regulation of eNOS expression. An up-regulation of eNOS expression and/or activity was then reconfirmed by Kaesemeyer *et al* (1999) and Hernandez-Perera *et al* (1998) who also demonstrated that addition of atorvastatin or simvastatin to bovine aortic endothelial cells results in an inhibition of pre-pro ET-1 mRNA and ET-1 protein expression via prevention of

LDL oxidation. This improvement in endothelial function following HMG-CoA reductase inhibition was also shown to be independent of fibrinogen, soluble L-selectin, P-selectin and ICAM-1 concentrations (Rauch *et al.*, 2000), but dependent on caveolin abundance (Feron *et al.*, 2001).

Sumi *et al* (2001), utilizing rabbits fed a high cholesterol diet, demonstrated an up-regulation of eNOS mRNA expression (determined by competitive RT-PCR) and a decrease of superoxide production in vascular endothelial cells (quantified using an analogue of lucigenin, 2-methyl-3, 7-dihydroimidazol [1,2-α]pyrazine-3-one) in rabbits which were randomized to 12-weeks of fluvastatin. Fluvastatin treatment was also shown to improve endothelium dependent responses to acetylcholine and to increase the tissue cGMP concentrations of the aortas without facilitating a significant reduction in serum cholesterol (Sumi *et al.*, 2001). This result parallels the findings of Wassmann *et al* (2001), in which statin pharmacotherapy improved endothelial dysfunction, and profoundly reduced Ang II-induced vasoconstriction in spontaneously hypertensive rats. Angiotensin type 1 (AT_1) receptor, eNOS, and p22phox mRNA expression were also significantly inhibited by atorvastatin (Wassmann *et al.*, 2001).

[C.6.2] Interventions that reduce nitric oxide clearance

Clearance of nitric oxide by superoxide has been a mechanism postulated to explain the phenomenon of endothelial dysfunction and as such a number of pharmacotherapeutic interventions have been examined to limit this clearance.

[C.6.2.1] Antioxidant pharmacotherapy

[C.6.2.1.1] Vitamins

Numerous investigations have suggested that acute supplementation with water/lipid-soluble anti-oxidant vitamins, either vitamin C or E, improves endothelial function in subjects with cardiovascular risk factors that include hypercholesterolaemia (Ting *et al.*, 1997), smoking (Raitakari *et al.*, 2000), hypertension (Natali *et al.*, 2000; Solzbach *et al.*, 1997), and diabetes (Cinar *et al.*, 2001; Skyrme-Jones *et al.*, 2000; Ting *et al.*, 1996). Both vitamin C and E supplementation have also been shown to ameliorate endothelial dysfunction in subjects with vasospastic angina (Hirashima *et al.*, 2000; Motoyama *et al.*, 1998).

Levine *et al* (1996), demonstrated an acute improvement in the brachial artery flow-mediated dilatation of patients with CAD following a single oral dose of ascorbic acid with similar improvements being observed by others (Gokce *et al.*, 1999; Ting *et al.*, 1996). However, adding confusion to the hypothesis are the results obtained by a series of separate investigations in which no obvious benefit in stroke/MI prevention from vitamin C administration was observed (Ascherio *et al.*, 1999; Klipstein-Grobusch *et al.*, 1999).

Keaney *et al* (1993), reported that when rabbits were fed α-tocopherol or β-carotene in conjunction with a high cholesterol diet, the development of endothelial dysfunction was prevented; these results were confirmed by others (Andersson *et al.*, 1994; Keegan *et al.*, 1995; Klemsdal *et al.*, 1994). However, contrary to the beneficial results observed with vitamin C, there was no improvement in endothelial function following long term vitamin E supplementation in male subjects post MI (Elliott *et al.*, 1995), and no observed restoration of normal endothelial function in male smokers (Neunteufl *et al.*, 2000).

Despite the above negative studies, the beneficial effects of anti-oxidant pharmacotherapy with vitamin C and E have been thought to arise from an enhanced bioavailability of vascular nitric oxide. By scavenging superoxide, vitamin C may theoretically decrease the extent of nitric oxide consumption. However, it has been reported that only high, supra-physiological concentrations of vitamin C (>1mM) are capable of scavenging superoxide (Jackson *et al.*, 1998). Supplementation of human umbilical vein endothelial cell culture with vitamin C increased ionomycin-stimulated eNOS activity, via a mechanism that may enhance the affinity of eNOS for tetrahydrobiopterin (Heller *et al.*, 1999a). Other mechanisms vitamin C effects may include a direct reduction of nitrite to nitric oxide (May, 2000) and release of nitric oxide from both low-molecular weight *S*-nitrosothiols and *S*-nitroso-albumin (Scorza *et al.*, 1997).

[C.6.2.1.2] Other anti-oxidants

Alternative anti-oxidant interventions demonstrated to improve endothelial function include the acute administration of glutathione (GSH). GSH was demonstrated to improve vascular responsiveness to acetylcholine in patients with angiographically defined atherosclerosis or a number of coronary risk factors, and depressed endothelial function (Prasad *et al.*, 1999). These results reconfirm the observations made by Kugiyama *et al* (1998) in which GSH was

also demonstrated to improve acetylcholine responsiveness of epicardial coronary arteries of patients with a number of risk factors for atherosclerosis.

[C.6.3] Angiotensin-converting enzyme (ACE) inhibition/angiotensin receptor antagonists

Angiotensin-converting enzyme inhibitors were proven effective in the treatment of hypertension and congestive heart failure and showed a benefit in preventing adverse cardiovascular events in subjects with CAD without elevated blood pressure or heart failure (Lonn *et al.*, 1994; Weir and Dzau, 1999; Yusuf *et al.*, 2000). A number of studies to date have also demonstrated an improvement in endothelial dependent vasodilatation following treatment with ACE inhibitor (Antony *et al.*, 1996; Finta *et al.*, 1993; O'Driscoll *et al.*, 1997a), despite no benefit being demonstrated by Mullen *et al* (1998a) in type I diabetic patients.

ACE inhibitors exert a myriad of effects on the cardiovascular system through interruption of the renin-angiotensin-aldosterone system. Ang II is a powerful vasoconstrictor that not only counteracts nitric oxide, but also stimulates mitogenesis, resulting in smooth muscle cell hyperplasia and collagen deposition that increases arterial wall mass and a reduction in left ventricle compliance (Weir and Dzau, 1999). Ang II, as described in section C.4.3.1, stimulates the generation of ROS in a NAD(P)H oxidase dependent fashion, but also stimulates the release of noradrenaline, endothelin-1 and promotes the breakdown of bradykinin (O'Keefe *et al.*, 2001).

In the TREND (Trial on Reversing Endothelial Dysfunction) study, chronic therapy with quinapril in patients with CAD was demonstrated to improve coronary vasodilatory response to acetylcholine (Mancini *et al.*, 1996), results of which have been confirmed by others using ATRBs (Prasad *et al.*, 2000; Schiffrin *et al.*, 2000). Anderson *et al* (2000), utilizing high-resolution ultrasound to assess endothelium dependent brachial artery flow-mediated dilatation, demonstrated a significant improvement in endothelial function following quinapril treatment. No significant improvement was observed with losartan, enalapril or amlodipine and was only observed in subjects with an ID and II ACE genotype (Anderson *et al.*, 2000), suggesting that there are differences between these drugs in their ability to improve endothelial function.

For a series of specific reviews discussing the actions of Ang II and the benefits associated with ACE inhibitor/ATRB pharmacotherapy regarding restoration of endothelial function and overall clinical benefit see the following reviews by Mancini (2000), O'Keefe *et al* (2001), Raij (2001) and Tabibiazar *et al* (2001).

[C.6.4] Summary

As described above, the phenomenon of endothelial dysfunction can be explained mechanistically in a number of ways, and as such a number of pharmacotherapeutic interventions are available that limit its progression. A schematic diagram (Figure 1.5) summarizes some of the potential mechanisms and pharmacotherapeutic interventions.

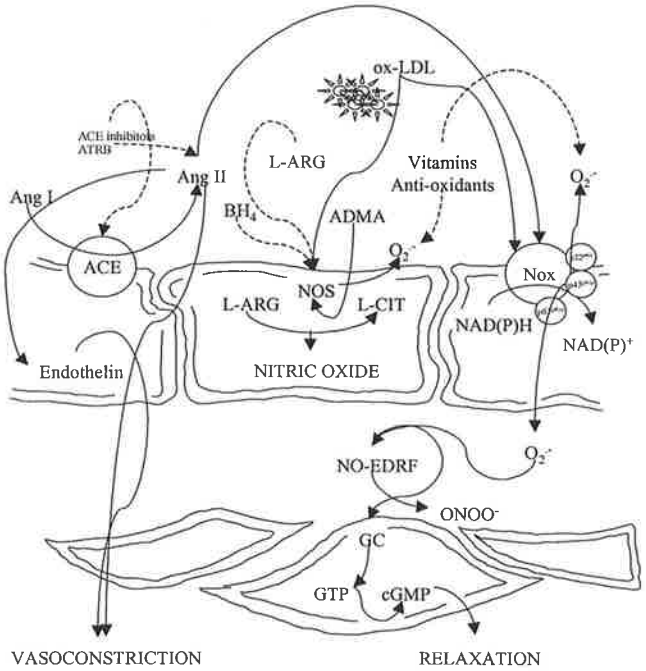


Figure 1.5 Endothelial dysfunction:

Mechanisms and pharmacotherapeutic interventions

Superoxide, produced by NAD(P)H oxidase, limits the effectiveness of nitric oxide, formed during the conversion of L-arginine to L-citrulline by NOS. Angiotensin II upregulates NAD(P)H oxidase, stimulates the release of endothelin, counteracting the vasodilatory properties of nitric oxide. ACE inhibitors, angiotensin receptor blockers, NOS subunits, vitamins and anti-oxidants limit endothelial dysfunction. Ang = angiotensin, ACE = angiotensin converting enzyme, ATRB = angiotensin receptor blocker, ADMA = asymmetric dimethyl-arginine, BH_4 = tetrahydrobiopterin, L-ARG = L-arginine, L-CIT = L-citrulline, oxLDL = oxidized low density lipoprotein, NAD(P)H = nicotinamide adenine dinucleotide phosphate, $ONOO^-$ = peroxynitrite, $O_2^{\cdot-}$ = superoxide, NOS = nitric oxide synthase, GTP = guanosine triphosphate, cGMP = cyclic guanosine monophosphate, EDRF = endothelial derived relaxing factor, GC = guanylate cyclase.

Pathological Platelet Aggregation:

[C.7] Evidence for a role of platelets in cardiovascular disease states

To aid in the discussion about the role of platelets in the pathogenesis of CAD some of the pathophysiological aspects of atherosclerosis will firstly be described.

[C.7.1] Lesion initiation/development

Atherosclerosis is a progressive disease that is characterized by the accumulation of low-density lipoprotein (LDL) within the subendothelial matrix and secondary inflammatory changes (Ross, 1999). Early lesions consist of cholesterol-engorged macrophages called "foam cells" that can be found in the early decades of life especially in the aortas (PDAY, 1993; Napoli *et al.*, 1997). Fatty streaks rich in foam cells serve as the precursors of more advanced lesions that contain lipid rich necrotic debris, smooth muscle cells that have migrated from the medial layer and a number of immune effector cells such as T-lymphocytes and others. For a recent discussion on the mechanisms and implications of atherosclerotic lesion development see the following reviews by Berliner *et al* (1995), Lusis (2000), Navab *et al* (1996) and Ross (1999).

[C.7.2] Advanced atherosclerotic lesion and plaque rupture

There are two mechanisms by which thrombus formation at or near the sites of an atherosclerotic lesion can occur. One involves endothelial erosion, in which removal of the endothelium exposes areas of the sub-endothelium connective tissue of the plaque to circulating platelets (Arbustini *et al.*, 1999). Alternatively thrombus formation at the site of an atherosclerotic lesion can also occur by plaque disruption (fissuring), where the thin fibrous caps of the lesion tears exposing the highly thrombogenic lipid core to circulating platelets (Davies, 2000). By histological examination of advanced atherosclerotic lesions that had either nearly ruptured or ruptured, Davies *et al* (1993), identified a higher number of monocyte/macrophages, rather than vascular smooth muscles within the advanced lesions. These immune effector cells were then identified to reside at or near the sites of plaque rupture or erosion (shoulder regions) (van der Wal *et al.*, 1994). Therefore, via either mechanism, thrombus formation is a reflection of an enhanced inflammatory activity within the atherosclerotic plaque (Ross, 1999).

The CD40-CD40 ligand-signaling pathway, which mediates a host of inflammatory responses (Laman *et al.*, 1997), has been suggested to function as a critical mediator of plaque instability. Originally thought to be expressed only on activated $CD4^+$ helper T lymphocytes, CD40-CD40L has now been localized on activated endothelial cells (Karmann *et al.*, 1995), vascular smooth muscle cells and monocyte derived macrophages (Mach *et al.*, 1997b). Hakkinen *et al* (2000), using a double immuno-staining technique, demonstrated that the normal human intima was free of CD40 and CD40L immunoreactivity. In samples obtained from fatty streaks and advanced atherosclerotic lesions, co-localization of CD40 and CD40L was observed in $CD3^+$ T-lymphocytes, $CD68^+$ monocyte derived macrophages and smooth muscle cells. This observation was reconfirmed by others (Bruemmer *et al.*, 2001).

Macrophages isolated from rabbit aortic lesions were initially demonstrated to contain interstitial collagenase (MMP-1) and stromelysin (MMP-3), whereas alveolar macrophages were not. Release of collagenase, stromelysin and gelatinase occurred with or without stimulation by phorbol ester or bacterial lipopolysaccharide (LPS), suggesting constitutive production of these MMP's (Galis *et al.*, 1995). Rather than concluding that MMP's were expressed constitutively in macrophages isolated from atherosclerotic lesions, Schonbeck *et al* (1997) demonstrated that stimulation of CD40 in isolated human VSMC's by either membranes of activated T-lymphocytes or recombinant CD40L was responsible for the regulation of the expression of the matrix degrading enzymes interstitial MMP-1, MMP-3, gelatinase-3 (MMP-9) and activated gelatinase A (MMP-2). Reconfirming this work, monocytes and macrophages were also demonstrated to stimulate the release of MMP-1, MMP-3 and tissue factor following exposure to CD40L (Mach *et al.*, 1997a).

Disruption of the CD40-CD40L signaling utilizing a monoclonal antibody directed against the CD40L has resulted in a reduction in the size of aortic atherosclerotic lesions in mice deficient in a LDL receptor (Mach *et al.*, 1998), and decreased expression of MMP-3 within the atherosclerotic plaques (Schonbeck *et al.*, 1999). Recently treatment of transgenic mice with a monoclonal antibody directed against CD40L has also been demonstrated to reduce further evolution of an established atherosclerotic lesion (Schonbeck *et al.*, 2000).

[C.7.3] Other factors contributing to plaque instability and rupture

Apart from factors that derive from the direct interaction of the CD40-CD40L such as the generation of matrix metalloproteinases as outlined above, interactions between monocyte derived macrophages and vascular smooth muscle cells have also been demonstrated to enhance plaque instability, potentially leading to rupture. Through the actions of monocyte derived prostaglandins, monocytes were shown to inhibit VSMC secretion of pro-collagen (Fitzsimmons *et al.*, 1999). Following direct cell-to-cell contact between monocytes and VSMC, an enhanced MMP-1 production resulted (Zhu *et al.*, 2000). Various cytokines including IL-1β, TNF-α and INF-γ released from various inflammatory mediators within atherosclerotic lesions have also been demonstrated to be cytotoxic to VSMC's, inducing apoptosis (Weissberg, 2000) and thus, potentially contributing to plaque instability.

Mechanical forces such as those generated from blood flow across a vulnerable plaque may play a key role in determining the extent of lesion stability (Gutstein and Fuster, 1999). Utilizing computer modeling, Richardson *et al* (1989) documented an increased level of stress occurs at the shoulder regions of the fibrous cap. However, in those subjects with a reduced lipid pool, the point of maximum stress was found to be over the center of the plaque.

Thus, due to generation of factors that include matrix metalloproteinases, cytokines and chemokines, an inhibition of VSMC proliferation and the inducement of apoptosis, a general weakening of the fibrous cap results, leaving the atherosclerotic plaque prone to rupture or erosion. For a schematic representation of the events that contribute to plaque instability and rupture see Figure 1.6.

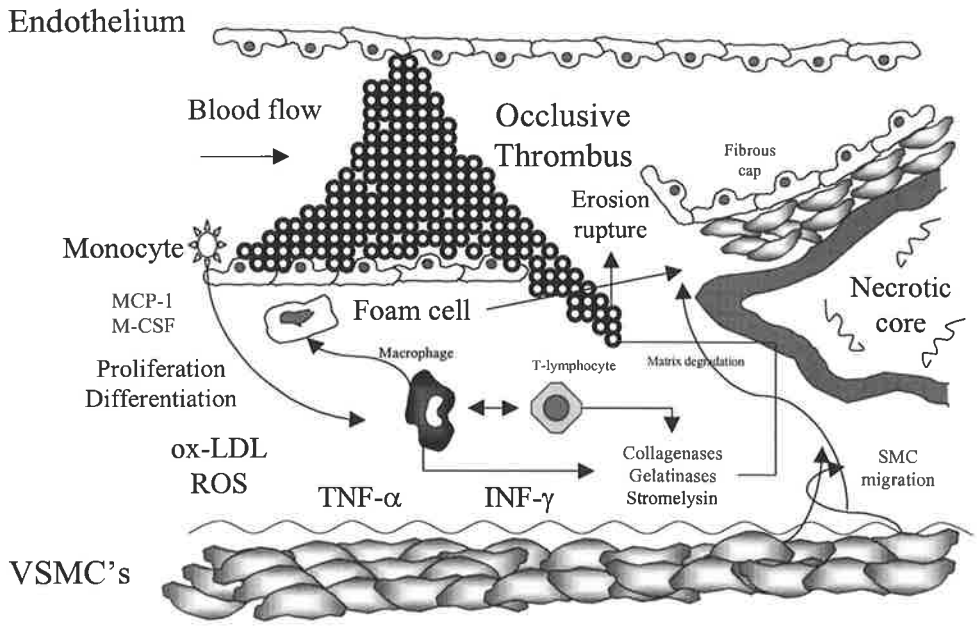


Figure 1.6 Plaque erosion/rupture leading to thrombus formation

An increased number of immune-effector cells including monocyte derived macrophages and T-lymphocytes, produce proteases (collagenases, gelatinases, stromelysin and cathepsins) that degrade the extracellular matrix proteins of the fibrous plaque. Erosion and rupture of the plaque usually occurs at the lesion's edge. Circulating blood platelets are then exposed to the contents of the lipid core of the lesion, resulting in thrombus formation. Adapted from (Lusis, 2000).

[C.7.4] Implications of plaque rupture

Upon plaque disruption, circulating blood platelets are exposed to the contents of the highly thrombotic lipid core. The coagulation cascade ensues leading to the formation of a thrombus, initially within the ruptured plaque itself (Falk, 1991). The thrombus may then expand into the arterial lumen thereby restricting distal blood flow. Thrombi that project into, but do not occlude the lumen, are said to be responsible for unstable angina (Theroux and Fuster, 1998). Thrombi that continue to develop, leading to total occlusion of the lumen, in the absence of sufficient collateral blood flow, serve as the basis of acute MI (Fuster *et al.*, 1992). Moreover, the flow of blood continues over an exposed thrombus dislodging activated platelet aggregates that are swept down stream from the initial thrombus, potentially resulting in distal micro-emboli (Falk, 1991).

Despite the potential for the development of a totally occlusive thrombus, the majority of episodes of plaque disruption do not cause major events (Davies, 2000). Large proportions of plaque rupturing episodes are not noticed, as the developing thrombus does not sufficiently occlude the lumen to cause ischaemia. Davies (1995), determined that approximately 70% of

haemodynamically significant stenosis of large coronary arteries (> 50% diameter determined angiographically), have had an episode of plaque rupture and lesion healing.

The dynamic process of plaque rupture, platelet aggregation, thrombus formation and lesion healing emphasizes the importance of therapeutic interventions that limit inflammation and strengthen plaque caps. It also highlights the importance of limiting platelet function in patients prone to plaque rupture. Various agents that limit the extent of platelet function/activation and the various mechanisms by which these anti-platelet agents function, are discussed in the following sections. Particular reference is given to the anti-platelet properties of organic nitrates.

[C.8] Evidence of platelet hyper-aggregability

Much of the evidence for platelet hyper-aggregability/activity associated with atherosclerotic states comes from studies performed on subjects with SAP in which enhanced platelet hyper-aggregability was observed post exercise. Evidence also comes from studies in which platelet hyper-aggregability was observed in subjects with risk factors for atherosclerosis. Additional evidence of platelet hyper-aggregability has stemmed from investigations into long-term effects of oral GPIIb/IIIa receptor blockade. Some of this evidence is examined below along with possible mechanisms behind the phenomenon.

[C.8.1] Stable angina pectoris

Moderate and strenuous exercise is known to enhance platelet aggregability in both healthy subjects (Andrews *et al.*, 1999a; Kestin *et al.*, 1993; Kishi *et al.*, 1992; Li *et al.*, 1999c; Piret *et al.*, 1990) and patients with SAP (Tokuue *et al.*, 1996; Wallen *et al.*, 1997; Winther and Rein, 1990). However, with SAP patients, there is also debate as to what extent exercise-induced ischaemia and coronary atherosclerosis contribute to the enhanced platelet aggregability. Some investigations have reported increases in platelet aggregability markers (such as PAF-4 or β-thromboglobulin) post exercise (Green *et al.*, 1980; Mehta and Mehta, 1982), but results are not uniform in this regard (Mathis *et al.*, 1981; Wennmalm *et al.*, 1990). Diodati *et al* (1994) demonstrated that platelets became activated across an atherosclerotic bed following rapid atrial pacing and a consequent increase in coronary blood flow. However, his increase in platelet reactivity was only demonstrated in those subjects that had a haemodynamically significant narrowing of a major coronary artery, suggesting that the

mechanism behind platelet hyper-aggregability following exercise may be partially shear stress related (Diodati *et al.*, 1994).

In a comprehensive study performed by Furman *et al* (1998a), unstimulated peripheral blood samples from patients with SAP were found to contain increased numbers of surface P-selectin positive platelets compared with platelets obtained from a series of normal volunteers (NVs). Moreover, in SAP patients compared to control subjects an increased platelet surface expression of P-selectin in response to stimulation with various agonists was observed. These results indicate an increased presence of circulating degranulated platelets in patients with SAP. Furthermore, monocyte-platelet aggregates were more likely to form in samples from patients with SAP than in control subjects, in response to *in vitro* stimulation with either ADP, adrenaline, or thrombin receptor agonist peptide (Furman *et al.*, 1998a).

[C.8.2] Unstable angina pectoris and myocardial infarction

Evidence of enhanced platelet activation in subjects with UAP or an acute MI comes from a series of studies in which indices of platelet activation such as thromboxane B_2, PAF-4 and β-thromboglobulin were found to be elevated in these subjects (de Boer *et al.*, 1982; Fitzgerald *et al.*, 1986; Hamm *et al.*, 1987; Hirsh *et al.*, 1981; Hughes *et al.*, 1982). Significantly higher levels of these platelet activation markers were observed in subjects with a recent onset of UAP compared to those patients whose anginal pain occurred more than 96-hrs previously (Hirsh *et al.*, 1981).

UAP patients and patients with progressive angina without rest pain were both shown to be hyper-aggregable in response to arachidonic acid compared to healthy control subjects (Grande *et al.*, 1990). Utilizing impedance aggregometry, the response to ADP, PAF-4 and collagen for subjects with UAP or MI was similar to that of subjects with no CAD (Nidorf *et al.*, 1989). However, in agreement with results obtained from the studies outlined above, aggregation in response to collagen resulted in a greater release of TXB_2 in subjects diagnosed with an MI compared to UAP and control subjects, an abnormality that persisted for 2-4 months (Nidorf *et al.*, 1989).

Further evidence demonstrating platelet activation during and after an ACS comes from results obtained in the Thrombolysis in Myocardial Infarction-12 (TIMI-12) trial, that was

designed to evaluate the effects of the oral GPIIb/IIIa receptor antagonist sibrafiban in patients post ACS (Ault *et al.*, 1999). In a subset of patients enrolled in the initial study (n=90/329), a significant degree of spontaneous platelet activation was observed in those subjects at enrolment compared to two cohorts of controls, one being healthy NVs and another consisting of patients admitted to an emergency unit for various acute medical problems not involving cardiovascular disease (Ault *et al.*, 1999). The extent of platelet activation following exposure of platelets to ADP (1 or 5μM) for 5 minutes was not different from those of the control group either at baseline, or 28 days post study enrolment. However, when compared to either control group, the extent of spontaneous platelet activation and the response to ADP remained significantly higher within the study cohort at day 28, implying that elevated platelet activation persists for a considerable period post an ACS despite the presence of a GPII/IIIa receptor antagonist.

[C.9] Evidence that coronary artery disease is associated with platelet hyper-aggregability

As discussed in the sections C.13-C.15, several large scales clinical trials with various anti-platelet agents that include aspirin (Baigent *et al.*, 1998), GPIIb/IIIa antagonists (Chan and Moliterno, 2001; Chew and Moliterno, 2001; Coller, 2001; McKay and Boden, 2001) and ADP receptor antagonists (Jarvis and Simpson, 2000) have demonstrated an improved clinical outcome in patients with an ACS. These results provide inferentially supportive evidence for a significant role for enhanced platelet activation in the pathogenesis of UAP/acute MI. However, evidence of an association between CAD and platelet reactivity in the general population is limited or inconclusive.

[C.9.1] Epidemiology

In one of the first large scale studies performed by Meade *et al* (1985), (958 subjects) there was a trend towards a significantly higher degree of platelet aggregability in male subjects who had a history of IHD and/or documented electrocardiographic (ECG) abnormalities compared to those without. In the Caerphilly collaborative heart disease study (Elwood *et al.*, 1990; Elwood *et al.*, 1991), the extent of platelet aggregation induced by collagen, thrombin and ADP determined in both PRP and in whole blood preparations were correlated against the prevalence of angina, history of MI and ECG evidence of IHD. Significant associations

between the degree of platelet responsive to ADP, a history of MI and to a lesser degree evidence of myocardial ischaemia by electrocardiography, was observed.

However, in the first re-examination of the Caerphilly cohort (phase II) five years after the first examination, no measures of platelet aggregation were found to be significantly predictive of a future clinical event (Elwood *et al.*, 1998). In a post hoc examination of the data, a trend towards a significant association between the secondary response to ADP and an early IHD event was noted when events were grouped according to their time post aggregation assessment (Elwood *et al.*, 1998). Reasons for the discrepancies between the studies performed in the early 1990's (Elwood *et al.*, 1990; Elwood *et al.*, 1991) compared to the late 1990's (Elwood *et al.*, 1998) on the same Caerphilly cohort remain undefined as exclusion criteria and experimental conditions were similar. Interestingly, it was more recently observed that neither primary nor secondary platelet aggregation was predictive of a subsequent MI in 2000 males of the Caerphilly Cohort study of Heart Disease, Stroke and Cognitive Decline (Elwood *et al.*, 2001).

In a retrospective examination of a smaller cohort of healthy male subjects (n = 150), Thaulow *et al* (1991), found no significant association between the extent of platelet aggregation induced by ADP, adrenaline or collagen and the incidence of future coronary heart disease mortality. However, this study did reveal that subjects with an extent of ADP-induced platelet aggregation above the median level, had a significantly higher IHD mortality than those below the median level, implying that enhanced platelet reactivity may increase the risk of cardiovascular death. These results were in agreement with the observations made by Trip *et al* (1990b), who also confirmed that platelet activity as determined by the incidence of spontaneous platelet aggregation, in subjects that had survived a recent MI, was a predictor of future coronary events and mortality.

In an investigation that examined subjects with IHD only, platelet responsiveness to thrombin was shown to be predictive of CAD progression determined by repeat quantitative angiography (Lam *et al.*, 1994). Moreover, these subjects were also shown to have a significantly worse prognosis than those with no disease progression do. Platelet hyper-activity was concluded to be not a consequence of CAD per se, but rather a determinant of disease progression (Lam *et al.*, 1994).

[C.10] Risk factors for atherosclerosis and platelet hyper-aggregability

[C.10.1] Diabetes

In patients with diabetes, CAD tends to be more extensive and severe, with cardiovascular related deaths being three times more common in diabetic patients than in non-diabetic patients (Stamler *et al.*, 1993). Furthermore, diabetic patients without a documented history of a previous MI have the same degree of risk of MI as non-diabetic patients with a previous history of infarction (Haffner *et al.*, 1998). The mechanisms accounting for this incremental risk remain poorly understood. However, there is some evidence to suggest that a hyper-aggregable state may at least in part contribute to the increased risk.

Platelets from patients with type II diabetes have increased platelet activation compared to non-diabetic subjects as demonstrated by an increased adhesion to an extra-cellular matrix (Knobler *et al.*, 1998). These results are in agreement with the observations made by Iida *et al* (1993) and others (Davi *et al.*, 1990; Mandal *et al.*, 1993; Tomaselli *et al.*, 1990), in which platelets from diabetic rats or humans were found to be hyper-aggregable or have an increased degree of activation. Along with platelet hyper-aggregability a significant degree of spontaneous platelet aggregation has been observed in platelet samples obtained from type II diabetics compared to age matched non-diabetic subjects (Iwase *et al.*, 1998). In an interesting investigation Oskarsson and Hofmeyer (1996), demonstrated an impaired ability of platelets from patients with diabetes to mediate vasodilatation of pre-constricted normal rabbit carotid artery segments compared to platelets from non-diabetic control subjects. Moreover, normal platelets incubated with a high D-glucose solution also lost their ability to mediate dilation in a concentration-dependent and time-dependent manner, suggesting that the impaired vasodilating capabilities of diabetic platelets may be mediated by elevated glucose concentrations.

Perhaps the most compelling evidence for a significant role of platelets in the pathogenesis of diabetes mellitus comes from the observed benefit with use of GPIIb/IIIa receptor antagonists during acute ischaemic episodes for subjects with diabetes (Bhatt *et al.*, 2000; Lincoff, 2000; Marso *et al.*, 1999).

[C.10.2] Hypercholesterolaemia

Initial studies demonstrated platelet hyper-aggregability to a range of agonists, including ADP, adrenaline and collagen, in samples from subjects with familial hypercholesterolaemia compared to samples obtained from healthy controls (Carvalho *et al.*, 1974). Furthermore, patients with hypercholesterolaemia were demonstrated to have elevated levels of β-thromboglobulin and other markers of platelet activation compared to age matched control subjects. Aoki *et al* (1997b), demonstrated that platelet-dependent thrombin generation was increased in patients with hypercholesterolaemia, a phenomenon that was also observed in patients with hypercholesterolaemia plus hypertriglyceridaemia compared to patients with hypertriglyceridaemia alone and to control subjects.

[C.10.2.1] ox-LDL serving as a pro-aggregant

Initial observations of a platelet dysfunction in subjects with hypercholesterolaemia led to a series of experiments that demonstrated that ox-LDL serves as a pro-aggregant (Aviram, 1989; Aviram *et al.*, 1989). Platelets exposed to ox-LDL alone, undergo immediate platelet shape change and pseudopodia formation (Zhao *et al.*, 1995), a transformation that enhances adhesion of platelets to endothelial cells under flow condition (Dardik *et al.*, 2000).

In a study assessing the effect of various forms of low-density lipoprotein, ox-LDL was demonstrated to increase the degree of platelet activation compared to native LDL (Katzman *et al.*, 1994). This observation was reaffirmed by Weidtmann *et al* (1995), who demonstrated irreversible platelet aggregation following treatment of platelets with mildly oxidized LDL rather than highly oxidized LDL. Tornvall *et al* (1999), demonstrated that pre-incubation of platelets with native and ox-LDL did not modify the degree of platelet aggregability in platelet-rich plasma or washed platelets but increased aggregation markedly in whole blood.

In a series of studies addressing the mechanism/s by which ox-LDL serves as a pro-aggregant, Siess and colleagues hypothesized that the lysophosphatidic acid (LDA) formed during oxidation of LDL may serve as the active moiety of both mildly and minimally modified LDL responsible for platelet activation (Siess *et al.*, 1999). Antagonists of LDA were demonstrated to prevent both mildly and minimally modified LDL induced platelet activation. Further to this, lovastatin has been demonstrated to prevent ox-LDL activation of platelets via a mechanism that involves a LDA dependent pathway (Essler *et al.*, 2000).

[C.10.3] Hypertension

Hypertension, another known risk factor for CAD, is also associated with a platelet hyper-aggregable state. In an investigation performed by Kjeldsen *et al* (1987), plasma concentrations of β-thromboglobulin in untreated males with essential hypertension were found to be significantly higher than in healthy normotensive men. Plasma β-thromboglobulin in the hypertensive group correlated significantly with the total serum cholesterol and LDL suggesting that subjects with untreated essential hypertension have an increased blood platelet release reaction that is related at least in part to the concentration of atherogenic blood lipids. In agreement with these observations, a number of other investigations have observed an increased platelet activity in subjects with essential hypertension (Lande *et al.*, 1988; Lande *et al.*, 1987; Lechi *et al.*, 1989; Thomas *et al.*, 1992; Winther *et al.*, 1991).

Dockrell *et al* (1999), utilizing platelets from the offspring of parents known to have hypertension, demonstrated that the presence of high blood pressure was associated with a potentiation of the primary phase of platelet aggregation by endothelin-1. These results suggested that enhanced platelet sensitivity to the potent vasoconstrictor appears to exist in the familial predisposition to hypertension. In subjects with essential hypertension, platelet aggregability towards ADP and serotonin, and plasma β-thromboglobulin concentration were increased compared to subjects with less severe hypertension (World Health Organization Class I) and normotensive subjects (Tomoda *et al.*, 1999).

A number of studies have indicated that platelet dysfunction in subjects with essential hypertension can be normalized following anti-hypertensive pharmacotherapy. Islim *et al* (1992), determined that the degree of platelet activation in subjects with essential hypertension, treated with either β-adrenergic antagonists or diuretics, was not significantly different from the activation observed in untreated hypertensive subjects. However, treatment with ACE inhibitors was associated with a significantly lower plasma β-thromboglobulin compared to control subjects. Moser *et al* (1997), whilst comparing the anti-platelet properties of captopril, enalapril and fosinopril observed no significant anti-aggregatory effect, but did observe a significantly decreased level of TXB_2 generated from platelets in hypertensive subjects that had been treated with fosinopril. Furthermore, the utilization of Ca^{2+} antagonists for the management of hypertension has also demonstrated some anti-

platelet properties (Osmialowska *et al.*, 1990) despite evidence to the contrary (Birkebaek *et al.*, 1988).

[C.10.4] Smoking

Smoking is strongly associated with CAD and has also been shown by some, but not all, to increase the extent of platelet aggregation (Inoue *et al.*, 2001; Taylor *et al.*, 1987). Smokers have also been demonstrated to have more small spontaneous platelet aggregates and more medium and large aggregates induced by adrenaline (1-5μM) than non-smokers (Fusegawa and Handa, 2000) reconfirming results from a previous investigation where similar observations were made (de Padua Mansur *et al.*, 1997).

[C.11] "Sick platelet behavior"

Sick platelet behavior whether manifesting itself as platelet hyper-aggregability or hyper-reactivity to platelet agonists, can be viewed simplistically as resulting from an imbalance between the pro-aggregatory and the anti-aggregatory mediators that govern platelet function. The formation and function of particular ROS and their possible role in the phenomenon of platelet hyper-aggregability are discussed below.

[C.12] Mechanisms

[C.12.1] Reactive oxygen species serving as pro-aggregants

Numerous groups over many years have investigated the hypothesis that ROS including superoxide, hydrogen peroxide and others, serve as pro-aggregants that are responsible for platelet hyper-aggregability.

[C.12.1.1] Superoxide

As described earlier superoxide can be generated in a number of ways. One electron reduction of molecular oxygen occurs to produce superoxide in many enzymatic processes. Alternatively superoxide can be generated through the specific enzyme system of NADPH oxidase located within neutrophils or other cellular sources (see section A.3.2).

$$O_2 + e^- \rightarrow O_2^{\cdot -}$$

$$2O_2 + NADPH \rightarrow 2O_2^{\cdot -} + NADP + H^-$$

As it was demonstrated that superoxide limits the effectiveness of nitric oxide/EDRF (Gryglewski *et al.*, 1986; Rubanyi and Vanhoutte, 1986) and that NO/EDRF serves as a principal inhibitor of platelet function (Radomski *et al.*, 1987b; Radomski *et al.*, 1987c), several studies have examined the effects of superoxide on platelet function. The addition of pyrogallol (a superoxide generator) to a platelet suspension was demonstrated to enhance thrombin stimulated platelet aggregation and adhesion to a gelatin coated plastic (Salvemini *et al.*, 1989b). Moreover, the addition of the superoxide scavenger superoxide dismutase (SOD) significantly inhibited thrombin-stimulated platelet aggregation and platelet adhesion. However, the addition of catalase serving as a scavenger of hydrogen peroxide (H_2O_2) failed to affect any of the experimental parameters, indicating superoxide rather than hydrogen peroxide, enhances the extent of platelet aggregability and adhesiveness.

Following on from these initial observations the flavoprotein inhibitor diphenyleneiodonium (DPI) was shown to inhibit thrombin-induced platelet aggregation in a concentration dependent fashion, presumably through its inhibition of NAD(P)H oxidase and therefore superoxide generation (Salvemini *et al.*, 1991). Interpretation of this result is complicated by the non-specific nature of DPI as it was subsequently shown to inhibit all flavoprotein containing enzymes including NOS (Stuehr *et al.*, 1991) and xanthine oxidase (Doussiere and Vignais, 1992).

Superoxide apart from having direct effects on platelets also influences platelet aggregability through its ability to form other ROS. The various other ROS generated from superoxide and their ability to mediate platelet hyper-aggregability is discussed below.

[C.12.1.2] Hydrogen peroxide

In much the same way as superoxide, the interaction of H_2O_2 and platelets *in vitro* has been extensively examined, as it is formed readily from the spontaneous dismutation of the superoxide anion, a reaction that is catalyzed by superoxide dismutase:

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Throughout the literature the contribution hydrogen peroxide plays in platelet homeostasis remains uncertain with several investigations reporting that it readily serves as a powerful anti-aggregatory agent (Clark and Klebanoff, 1980; Ohyashiki *et al.*, 1991; Rodvein *et al.*, 1976) whilst others have suggested that it has a pro-aggregant function (Del Principe *et al.*, 1985).

Platelets primed with sub-threshold concentrations of AA or collagen underwent platelet aggregation, TXA_2 production and serotonin release following the addition of SOD, the effect of which was abolished following addition of catalase, implying a role for hydrogen peroxide in the triggering of platelet activation (Iuliano *et al.*, 1991). These results were then reconfirmed when it was demonstrated that exposure to hydrogen peroxide triggers platelet activation via a cycloxygenase dependent pathway (Pratico *et al.*, 1991; Pratico *et al.*, 1992).

As platelets and polymorphonuclear leukocytes, specifically neutrophils, interact very closely, especially in subjects with CAD (Nagata *et al.*, 1993; Ott *et al.*, 1996), Pratico *et al* (1993) demonstrated that activation of human neutrophils by fMLP induces platelet aggregation in platelets that had been primed with sub-threshold concentrations of AA or collagen, via a H_2O_2 dependent pathway. Furthermore, this effect was shown to be enhanced by the addition of SOD and blocked by catalase, indicating that hydrogen peroxide already known to be generated and released from human neutrophils (Test and Weiss, 1984; Wymann *et al.*, 1987), activates primed platelets.

In a comprehensive investigation that summarizes the effects of hydrogen peroxide on platelet function Ambrosio *et al* (1994), demonstrated that low concentrations of hydrogen peroxide promoted platelet activation/aggregation in a cyclooxygenase dependent pathway. However, the addition of hydrogen peroxide in higher concentrations inhibits platelet aggregation, via a cGMP dependent mechanism.

[C.12.1.3] Hydroxyl radical

Evidence throughout the literature also exists suggesting that the hydroxyl radical that is formed from hydrogen peroxide via either the Haber-Weiss reaction or Fenton reaction (Kehrer, 2000), can also induce platelet hyper-aggregability (see chemical reactions below).

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^{-} \text{ [Haber-Weiss reaction]}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \text{ [Fenton reaction]}$$

Following removal of hydroxyl radicals, platelet aggregation was significantly inhibited in response to a range of platelet agonists (Violi *et al.*, 1988). The pro-aggregant effect observed with hydrogen peroxide for platelets primed with sub-threshold concentrations of agonists has also been demonstrated to be mediated via the hydroxyl radical generated from hydrogen peroxide in an extra-cellular Fenton-like reaction (Iuliano *et al.*, 1994). Furthermore, human platelets exposed to 15 or 30 minutes of anoxia and then reoxygenation were shown to undergo spontaneous platelet aggregation, an effect that was blocked following the addition of the hydroxyl radical scavenger mannitol (Leo *et al.*, 1997).

These results not only illustrate the potential of the hydroxyl radical to function as a pro-aggregant, but also reinforce the importance of hydrogen peroxide and superoxide as mediators of platelet aggregation.

[C.12.1.4] Peroxynitrite

As attention has been focused on the regulation of nitric oxide by superoxide and its association with CAD (Ronson *et al.*, 1999), several investigators have examined the actions of peroxynitrite on platelets as it is readily formed from the interaction of NO and $O_2^{\cdot -}$. As has been demonstrated for superoxide, hydrogen peroxide and hydroxyl radical, peroxynitrite also acts as a pro and anti-aggregant (Brown *et al.*, 1998; Moro *et al.*, 1994). Several reports, but not all, have demonstrated that peroxynitrite inhibits platelet aggregation (Mondoro *et al.*, 1997; Moro *et al.*, 1994; Yin *et al.*, 1995) whilst others have demonstrated that it potentiates platelet aggregation in washed platelet preparations (Moro *et al.*, 1994). Platelets contain or generate appreciable amounts of nitric oxide (Freedman and Keaney, 1999; Freedman *et al.*, 1997; Radomski *et al.*, 1990; Sase and Michel, 1995), superoxide (Caccese *et al.*, 2000;

Freedman and Keaney, 1999; Leoncini *et al.*, 1991) and SOD (Marcus *et al.*, 1977), implying the formation of peroxynitrite may occur in both the resting and stimulated state.

In an investigation by Boulos *et al* (2000), a theoretical rate of platelet derived peroxynitrite formation has been determined (~$2x10^4s^{-1}$), which is faster than the rate of reaction between superoxide and SOD (~$1.2x10^4s^{-1}$). These results imply that in activated platelets, significant amounts of superoxide will react with nitric oxide to form peroxynitrite rather than hydrogen peroxide through its reaction with SOD. Adding evidence to the notion that platelets produce peroxynitrite Tannous *et al* (1999), demonstrated the presence of peroxynitrite in platelets obtained from IDDM subjects, speculating that it may relate to the platelet dysfunction associated with diabetes.

[C.12.2] Products of reactive oxygen species

[C.12.2.1] Clinical implications

Evidence for a significant role of ROS mediating platelet activation *in vivo* comes from a series of investigations that determined a significant reduction of cyclic flow variations following administration of superoxide dismutase alone (Yao *et al.*, 1993) or in combination with catalase (Ikeda *et al.*, 1994). Infusion of xanthine plus xanthine oxidase (Ikeda *et al.*, 1994) or hydrogen peroxide (Yao *et al.*, 1993) was also shown to induce the cyclic flow variations indicative of thrombus formation. Products generated through of the actions of various ROS such as isoprostanes have also been shown to induce platelet hyper-aggregability (Pratico *et al.*, 1996).

Indirect evidence that ROS are involved in activation of platelet *in vivo* comes from a series of observations made in NIDDM and IDDM patients. In both types of diabetic subjects, urinary excretion of 8-epi-$PGF_{2\alpha}$ was found to be twice that of healthy age matched control subjects (Davi *et al.*, 1999b). 8-epi-$PGF_{2\alpha}$ a member of the F_2-isoprostane family, is formed following the attack of cell membrane phospholipids or circulating LDL by ROS, and have been found to correlate with the amount of TxB_2 (Davi *et al.*, 1999b; Patrono and FitzGerald, 1997; Pratico, 1999; Roberts and Morrow, 1997). Moreover, platelet aggregation is induced by 8-epi-$PGF_{2\alpha}$ if prior to isoprostane exposure the platelets had been exposed to sub-threshold concentrations of various platelet agonists (Pratico *et al.*, 1996).

Reduction of 8-epi-$PGF_{2\alpha}$ formation through improved metabolic control or vitamin E supplementation has also been demonstrated to reduce the extent of platelet activation (Davi *et al.*, 1999b). These results may go some way to explaining the results obtained by Szuwart *et al* (2000), who demonstrated a reduction in platelet adhesion to endothelial cells following vitamin E administration. This was despite recent doubts being raised over the effectiveness of vitamin E utilization in the reduction in the extent of oxidative stress (Patrignani *et al.*, 2000). More recently Iuliano *et al* (2001), have demonstrated a transient increase in coronary sinus F_2-isoprostane formation post PTCA suggesting localized oxidative stress that may serve as a basis for vessel re-stenosis post PTCA or stent implantation.

Pharmacomodulation of Platelet Function

Inhibition of Platelet Aggregation: Clinical Strategies

[C.13] Aspirin

[C.13.1] History

It has long been known that platelets exposed to aspirin have a diminished degree of aggregation in response to a range of platelet agonists (O'Brien, 1968; Zucker and Peterson, 1968). Roth *et al* (1975) and Roth and Majerus (1975), demonstrated that acetylation of prostaglandin synthase was responsible for the observed anti-aggregatory effects of aspirin.

[C.13.2] Prostanoid Structure and Function

Cyclic prostanoids such as prostaglandins and thromboxane are the products of the metabolism of arachidonate by prostaglandin H synthase, also known as cyclo-oxygenase (COX-1/2) (Bjorkman, 1998; Smith and DeWitt, 1995; Williams and DuBois, 1996). Cyclo-oxygenase converts arachidonate to prostaglandin G_2, then further catalyzes a peroxidation reaction to form prostaglandin H_2 (PGH_2). After the biosynthesis of PGH_2, the endoperoxide is converted to one of a series of potential prostanoid products, depending on its location. Within platelets and neutrophils PGH_2 may be converted into the potent platelet agonist and vasoconstrictor thromboxane-A_2 (TxA_2) by TxA synthase. Within the vascular endothelium PGH_2 is converted into the potent anti-aggregatory and vasodilating agent prostacyclin (PGI_2), by prostacyclin synthase. TxA_2 is now thought to be primarily synthesized by platelet

COX-1, whereas PGI_2 biosynthesis is largely meditated by the COX-2 isoform (Catella-Lawson and Crofford, 2001). For a schematic representation of prostanoid formation and the action of aspirin, see Figure 1.7.

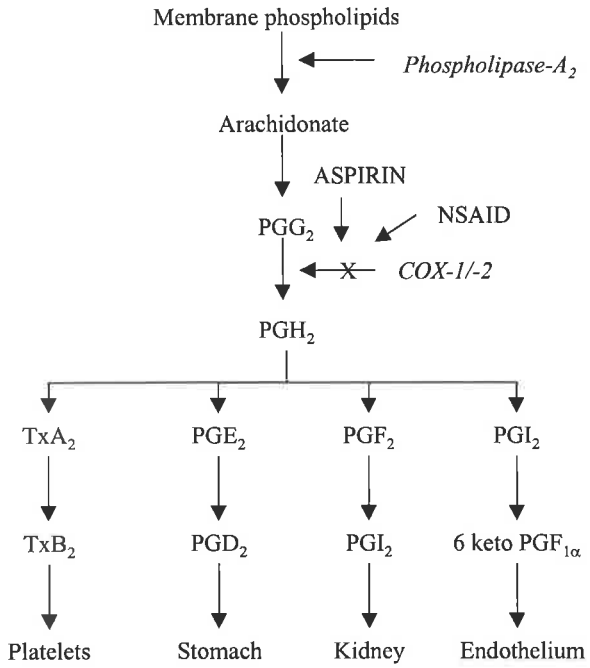


Figure 1.7 Prostanoid biosynthesis

Prostanoids are synthesized from arachidonate by the actions of cyclo-oxygenase. Cyclo-oxygenase then converts arachidonate to prostaglandin G_2 and then to prostaglandin H_2. Prostaglandin H_2 is then converted to prostaglandin E_2, D_2, $F_2\alpha$, I_2 or thromboxane A_2. (adapted from (Bjorkman, 1998). Not all prostanoids shown.

[C.13.3] Mechanism of Action

The two cyclo-oxygenase enzymes (COX-1, COX-2), also referred to as prostaglandin G/H synthase isozymes (PGH synathase 1 & 2), both exhibit cyclo-oxygenase and peroxidase activities. Both COX-1 and COX-2 enzymes are inhibited by aspirin through irreversibly acetylating a hydroxyl group of a single serine residue at position 529 within COX-1 and position 516 for COX-2 (Vane and Botting, 1995; Williams and DuBois, 1996). When blocked by the actions of aspirin, both COX-1/-2 are incapable of producing PGH_2, the necessary precursor of a number of prostaglandins and thromboxane. COX-2 on the other hand is capable of converting arachidonate to 15-*R*-hydroxyeicosatetraenoic acid (15-R-HETE) (Bjorkman, 1998).

Platelets are extremely sensitive to aspirin. Patrono *et al* (1980) illustrated a time and dose dependent inhibition of platelet production of TxB_2 following administration of aspirin

(100mg) in 45 healthy subjects. These results were later confirmed by others with even lower doses of aspirin producing a similar effect (Kallmann *et al.*, 1987; Patrignani *et al.*, 1982). Aspirin (75mg/day) was demonstrated to completely block TxB_2 production in ~80% of IHD patients who had recently undergone coronary artery bypass graft surgery (Ratnatunga *et al.*, 1992).

[C.13.4] Secondary Prevention of Ischaemic Events

In a prospective, randomized, double blind placebo controlled trial the RISC study group examined the effects of low dose aspirin administration in 796 males with either UAP or a non-Q-wave MI, and showed a reduction in the risk of both an MI and death after 5 days of therapy (The RISC Group, 1990). These benefits of aspirin utilization were then reconfirmed for the medium (3 months) (The RISC Group, 1990b) and long term (1 year) (Wallentin, 1991). Similar reductions in event rates for UAP and non-Q-wave MI patients were observed in other trials utilizing higher doses of aspirin (Cairns *et al.*, 1985; Lewis *et al.*, 1983). The Second International Study of Infarct Survival (ISIS-2) examining the efficacy of aspirin (160mg/day) utilization post MI in ~17 000 MI patients, demonstrated that aspirin utilization during the first 24-hrs of an infarct and for 1 month subsequently, significantly reduced the incidence of five week cardiovascular mortality, nonfatal re-infarction and non-fatal stroke (ISIS-2, 1988). This early benefit was maintained in the long term with neither a loss nor gain in benefit of aspirin randomization following 10 years follow-up (Baigent *et al.*, 1998). Moreover, the combination of aspirin with streptokinase was shown to be significantly better than either agent alone.

Aspirin utilization has also demonstrated benefit in preventing further vascular events in patients who have had a transient ischaemic attack or ischaemic stroke. In an overview analysis of 31 randomized trails, daily doses of aspirin ranging from 300-1500mg/day, reduced the risk of subsequent stroke, MI, or vascular death by ~22% (Antiplatelet Trialists' collaboration, 1988). These benefits were also observed with a lower dosing regimen (The SALT Collaborative Group, 1991).

[C.13.5] Aspirin Utilization in Primary Prevention of Ischaemic Events

As aspirin was shown to demonstrate a significant protective effect in the secondary prevention of an ischaemic event, the possible benefit of its use in primary prevention has

also been examined in a series of randomized trials. In the Physicians' Health Study (Steering Committee of the Physicians' Health Study Research Group, 1988), a randomized double blind placebo controlled trial of aspirin use on alternate days (325mg) was examined in ~22,000 men. The subjects randomized to receive aspirin had a 44% reduction in the risk of MI. This included significant benefits for aspirin on both nonfatal and fatal events (Steering Committee of the Physicians' Health Study Research Group, 1988). However, the observed benefit in aspirin use was apparent only amongst those subjects 50 years and older (Steering Committee of the Physicians' Health Study Research Group, 1989) with the protective effect becoming apparent soon after initiation of therapy (Ridker *et al.*, 1991a). Despite the observed benefit, no significant reduction in mortality from all cardiovascular causes was associated with aspirin utilization. Interpretation of this study must be made with caution, as the subject population was not truly representative. Only 88 cardiac deaths out of ~22,000 subjects studied was recorded representing a cardiovascular mortality 88% less than expected. Furthermore, this selective study group may have made lifestyle changes that lowered the likelihood of them having serious CAD.

The fact that the physicians were examined within the Physicians Health study may also serve as a potential reason why the British Doctors Trial failed to show a significant difference between the treatment groups for the combined endpoint (MI, stroke and total cardiovascular mortality) (Peto *et al.*, 1988). Despite the limitations of both investigations, an overview of these two trials of primary prevention demonstrated an overall reduction in non-fatal MI by 33% a result that was highly significant (Hennekens *et al.*, 1989; Hennekens *et al.*, 1988).

In the Swedish Angina Pectoris Aspirin Trial (SAPAT), ~2000 SAP patients were randomized to receive aspirin (75mg) or placebo on a background of sotalol. Following a mean follow up of 4 years, aspirin randomization was associated with a 34% reduction in the primary outcome event of MI and/or sudden death. A significant reduction in the incidence of first MI was associated with aspirin randomization (Juul-Moller *et al.*, 1992). These results confirmed the observations made by (Ridker *et al.*, 1991b) in a small cohort of chronic SAP patients from the Physicians Health study.

Some patients with other cardiovascular conditions may also benefit from the anti-aggregatory effect of aspirin as suggested in a comprehensive overview (Antiplatelet

Trialists' Collaboration, 1994). In an overview of 46 randomized trials, they demonstrated that anti-platelet therapy (chiefly aspirin alone or aspirin plus dipyridamole) greatly reduced the risk of vascular occlusion in a wide range of patients.

[C.14] Glycoprotein IIb/IIIa receptor antagonists

As described in section A.9, the final step in the process of platelet activation is the Ca^{2+} dependent transformation of the GPIIb/IIIa receptor complex to reveal previously cryptic binding sites for fibrinogen, fibronectin and vitronectin. Over the last fifteen years there has been a large emphasis on the development of antagonists that interfere with the binding of fibrinogen to the GPIIb/IIIa receptor, with three therapeutic classes of GPIIb/IIIa receptor antagonists being developed. Monoclonal antibodies, synthetic peptide and non-peptide receptor antagonists and disintegrins have undergone various levels of basic experimental and clinical investigation, with the monoclonal antibody Abciximab being the most extensively studied.

[C.14.1] Abciximab (c7E3 Fab)

Coller *et al* (1983), were the first to successfully develop a monoclonal antibody that was capable of preventing the binding of fibrinogen to platelets. In order to limit any immunogencity that may develop from administration of the 7E3 antibody, the F_c (Fragment crystallizable) region was removed by pepsin to produce an $F(ab')_2$ fragment (Faulds and Sorkin, 1994). Initial *in vitro* and *ex vivo* studies on platelets obtained from humans and dogs demonstrated that 7E3 was capable of completely inhibiting platelet aggregation induced by ADP (2-5μM) (Coller, 1985; Coller and Scudder, 1985). In a collaboration with Folts, Coller *et al* (1986), using the cyclic blood flow variation model (CFV) of thrombus formation, demonstrated that infusion of 7E3-$F(ab')_2$ resulted in complete abolition of the cyclic flow variations and a restoration of blood flow within 10 minutes, in all the dogs studied. Unlike administration of aspirin with the CFV model, epinephrine infusion (0.5 or 1.0mg/kg/min) or a series of other provocative challenges, failed to restore the CFV following 7E3-$F(ab')_2$ infusion, implying long-term protection from thrombus formation. Mickelson *et al* (1989), using the CFV model of thrombus formation confirmed the results obtained by Coller *et al* (1986), but also demonstrated that 7E3-$F(ab')_2$ not only inhibited *ex vivo* platelet aggregation but also minimized the amount of platelet deposition on damaged vascular endothelium.

[C.14.1.1] Ischaemic heart disease

Utilizing chimeric 7E3Fab, Anderson *et al* (1992/1994a) were one of the first investigators to show that inhibition of GPIIb/IIIa with c7E3Fab was a valid treatment for prevention of abrupt closure of a coronary artery during percutaneous transluminal coronary angioplasty (PTCA). Tcheng *et al* (1994), in patients with a moderate to high risk of sustaining an ischaemic complication, demonstrated a dose dependent inhibition of platelet aggregation and GPIIb/IIIa receptor blockade. A bolus dose of 0.25mg/kg was found to result in blocking > 80% of the GPIIb/IIIa receptors and a reduction to < 20% aggregation compared to samples prior to treatment. In a randomized placebo controlled study of 60 patients with an ACS, Simoons *et al* (1994), demonstrated that a 0.25mg/kg bolus followed by 10mg/min for 18 to 24-hrs of c7E3Fab, reduced the recurrence of further ischaemic episodes.

Following on from these initial investigations, a multitude of large clinical trials have been performed demonstrating the effectiveness of c7E3Fab in different clinical settings. The evaluation of c7E3 for the Prevention of Ischaemic Complications (EPIC) trial was the first to demonstrate the importance of GPIIb/IIIa blockade in high-risk patients with UAP or evolving MI, with pronounced and sustained benefit being demonstrated (The EPIC Investigators, 1994; Lincoff *et al.*, 1997; Topol *et al.*, 1994; Topol *et al.*, 1997). Subsequent large-scale trials such as EPILOG and EPISTENT extended the findings of EPIC to lower risk patients undergoing PTCA/stent implantation (The EPILOG Investigators, 1997; Lincoff *et al.*, 1999a; Lincoff *et al.*, 1999b; Topol *et al.*, 1999).

[C.14.2] Tirofiban

Tirofiban, a potent anti-platelet agent, inhibits the binding of fibrinogen to the activated GPIIb/IIIa receptor complex. *In vitro* administration of Tirofiban dose-dependently inhibited platelet aggregation induced by ADP, collagen, arachidonic acid, thrombin and the thromboxane analogue U46619 (Peerlinck *et al.*, 1993). These *in vitro* anti-aggregatory effects were then reconfirmed *ex vivo* in both dogs (Lynch *et al.*, 1995), healthy volunteers (Barrett *et al.*, 1994; Peerlinck *et al.*, 1993) and in high-risk IHD patients undergoing coronary angioplasty (Kereiakes *et al.*, 1996). On the basis of these initial investigations a series of multi-center trials such as PRISM, PRISM-PLUS and RESTORE, were performed in patients undergoing coronary intervention, as well as in subjects with UAP/non–Q wave

MI (The RESTORE investigators, 1997; PRISM investigators, 1998a; PRISM-PLUS study investigators, 1998).

[C.14.3] Other GPIIb/IIIa receptor antagonists

The synthetic peptide Eptifibatide, developed from a 73 amino acid peptide Barbourin, found in the venom of the southeastern pigmy rattlesnake *Sistrurus barbouri,* is another GPIIb/IIIa receptor antagonist and is the only disintegrin derivative to undergo extensive clinical investigation for its anti-platelet/thrombotic properties (Scarborough, 1999). Its anti-aggregatory properties have been extensively examined in healthy subjects (Charo *et al.*, 1992), patients undergoing elective PTCA (Harrington *et al.*, 1995; Tcheng *et al.*, 1995) and in patients with UAP or MI (Ohman *et al.*, 1997; Schulman *et al.*, 1996). Following on from these initial investigations, Eptifibatide has also undergone extensive clinical evaluation in such trials as IMPACT-II and PURSUIT (IMPACT-II, 1997; The PURSUIT Trial Investigators, 1998).

As described above, intravenous administration of GPIIb/IIIa antagonists at the point of care has demonstrated a significant clinical benefit. It was initially thought that an extension of the platelet inhibitory effect beyond the initial hospitalization should enhance the long-term clinical outcome of these patients. Therefore a series of potent orally active GPIIb/IIIa inhibitors have been developed including xemilofiban, orbofiban, roxifiban and sibrafiban. These agents have all undergone a series of major clinical trials with disappointing results (The SYMPHONY investigators, 2000; Ault *et al.*, 1999; Cannon *et al.*, 2000; Holmes *et al.*, 2000; O'Neill *et al.*, 2000): a 37% relative increase in mortality with the use of oral GPIIb/IIIa inhibitors (from 1.3% to 1.7%) (Chew *et al.*, 2001).

[C.15] ADP receptor antagonists

Ticlopidine and its structural analogue clopidogrel are thienopyridine derivatives that are a class of anti-platelet agents that function as ADP receptor antagonists (Quinn and Fitzgerald, 1999). Both ticlopidine and clopidogrel are inactive *in vitro* and require biotransformation with clopidogrel being metabolized to its active component by cytochrome P_{450} 1A subfamily (Savi *et al.*, 1994).

[C.15.1] Mechanism of Action

Di Minno *et al* (1985), in a comprehensive study involving healthy subjects, demonstrated that ticlopidine (500mg) inhibited *ex vivo* platelet aggregation to a variety of platelet agonists including ADP, epinephrine, A23187, collagen, arachidonic acid and thrombin via a mechanism independent of the GPIIb/IIIa complex. Addition of the ADP scavenger apyrase, added *in vitro* to a washed human platelet preparation obtained prior to ticlopidine or clopidogrel administration, demonstrated a similar anti-aggregatory effect of ticlopidine/clopidogrel treatment alone, suggesting that they both selectively inhibit platelet responses to ADP (Cattaneo *et al.*, 1991; Weber *et al.*, 1999). Ticlopidine has no effect on Ca^{2+} influx or intracellular mobilization (Hardisty *et al.*, 1990). Platelet shape change in response to ADP and other platelet agonists such as collagen and arachidonic acid was also demonstrated to be unaffected by ticlopidine (Di Minno *et al.*, 1985) or clopidogrel (Humbert *et al.*, 1996), adding further confusion to not only the mechanism of action for the thienopyridine derivatives, but also to the identification of the ADP receptor.

Using flow cytometry analysis of intracellular VASP (vasodilator-stimulated phosphoprotein) phosphorylation, Schwarz *et al* (1999), confirmed that the anti-aggregatory effect of ticlopidine and clopidogrel occurred via the $P2Y_{AC}$ ADP receptor. Phosphorylation of VASP, a cytoskeleton and integrin-associated platelet protein, correlates with inhibition of binding of soluble fibrinogen to the platelet GPIIb/IIIa receptor and inhibition of platelet aggregation (Horstrup *et al.*, 1994). Reconfirming the hypothesis that VASP phosphorylation was a useful marker for inhibition of platelet aggregation Schwarz *et al* (1999), demonstrated a time dependent increase in VASP phosphorylation when platelets were treated with SNP (100μM). Ticlopidine and clopidogrel pretreatment showed an attenuation of the inhibitory effect of ADP on prostaglandin E_1-stimulated, cAMP-mediated VASP serine 239 phosphorylation.

The clinical utility of ticlopidine and clopidogrel has been extensively examined, especially in the prevention of coronary stent thrombus (Bertrand *et al.*, 1998; Leon *et al.*, 1998; Urban *et al.*, 1998) with ticlopidine utilization being shown to be beneficial in the prevention of adverse outcomes in stroke patients (Hass *et al.*, 1989). However, several studies have also demonstrated that both ticlopidine and clopidogrel are capable of inducing thrombotic

thrombocytopenic purura, casting doubt on the safety of each agent (Bennett *et al.*, 2000; Bennett *et al.*, 1998; Ellie *et al.*, 1992; Kupfer and Tessler, 1997; Page *et al.*, 1991).

Effects from Other Cardiac Drugs on Platelet Function

[C.16] ACE Inhibitors

In a study by Moser *et al* (1997), the anti-platelet effects of equivalent anti-hypertensive doses of captopril, enalapril, and fosinopril were assessed in subjects with stage I-II essential hypertension. ADP, adrenaline or thrombin-stimulated platelet aggregation remained unchanged from baseline and between each ACE inhibitor. However, compared with baseline, fosinopril and captopril decreased the TxB_2 concentration, whereas enalapril consistently increased TxB_2 concentrations. Moser *et al* (1997) concluded that different ACE inhibitors have distinct effects on platelet TxB_2 formation, but no effect on platelet aggregation. In patients with an acute MI, captopril infusion resulted in a 30% reduction in the level of platelet surface GPIIb/IIIa receptor expression (Zurbano *et al.*, 1999), suggesting a reduction in the degree of platelet activation compared to patients not treated with ACE inhibitors.

Contrary to the above example and therefore casting some doubt on the direct anti-platelet properties of ACE inhibitors, Gow *et al* (1991), observed no significant anti-platelet effect following both acute and chronic administration of captopril in patients with congestive heart failure. These results are in agreement with the results obtained from Birkebaek *et al* (1988) and Gupta *et al* (1991), who also demonstrated that captopril or quinapril in therapeutic doses did not affect platelet activity in patients with mild to moderate essential hypertension.

Apart from the benefits observed regarding survival rates in subjects with heart failure (The CONSENSUS trial study group, 1987; The SOLVD Investigators, 1991; Kjekshus *et al.*, 1992), benefits from the use of ACE inhibitors post MI have also been demonstrated in a number of large-scale clinical investigations. Such studies include the Survival of Myocardial Infarction Long-term Evaluation (SMILE) trial were patients were randomized to zofenopril or placebo, in which the combined endpoint of death and the incidence of heart failure were significantly reduced in the zofenopril treated patients (Ambrosioni *et al.*, 1995). Other major trials to show a similar benefit include the SAVE, GISSI, ISIS-4 and HEART trials (GISSI-3, 1994; Pfeffer *et al.*, 1992; Pfeffer *et al.*, 1997; Rutherford *et al.*, 1994). In the Heart

Outcomes Prevention Evaluation (HOPE) study, treatment with ramipril (10mg/day) was demonstrated to prevent cardiovascular events or stroke in a broad range of subjects without baseline left ventricular dysfunction, or chronic heart failure, or CAD (Yusuf *et al.*, 2000).

The mechanism behind the benefits observed in the aforementioned clinical trails multifactorial with one or more postulated mechanism occurring at anyone time: blockade of angiotensin II production, inhibition of bradykinin breakdown, suppression of endothelin secretion and inhibition of superoxide production, just to name a few (Khalil *et al.*, 2001). However, given that a number of studies have demonstrated a direct effect of ACE inhibitors on platelet function, it is not unreasonable to assume the benefits observed through their use may stem from their anti-platelet properties.

[C.17] Statins

It is known that platelets from patients with hypercholesterolaemia are more sensitive to aggregating agents than platelets from normal subjects. For a specific review of the literature regarding the effects of ox-LDL and the presence of hypercholesterolaemia on platelets see section C.10.2.1, where it is implied that a reduction in ox-LDL through the actions of statins indirectly attenuates platelet hyper-responsiveness to various agonists. A number of large trials have demonstrated considerable benefit from statin utilization (Scandinavian Simvastatin Survival Study, 1994; Scandinavian Simvastatin Survival Study, 1995; Jukema *et al.*, 1995; Sacks *et al.*, 1996; WOSCOPS, 1998). However, these benefits suggest an improvement in the vasculature rather than a direct anti-platelet mechanism. An example of this comes from a study in which simvastatin pharmacotherapy was demonstrated to reduce the degree of platelet aggregation and TxB_2 production following four to twenty four weeks of treatment, whereas a lipid lowering effect can be observed in as little as two weeks of therapy (Davi *et al.*, 1989; Notarbartolo *et al.*, 1995).

[C.18] Calcium Antagonists

L-type Ca^{2+} channel antagonists are in widespread use for the management of CAD, with the main mechanism of action being an inhibition of Ca^{2+} fluxes across plasma membranes resulting in negative inotropy and coronary/peripheral vasodilatation (Abernethy and Schwartz, 1999). As described in section A.8, platelet function is governed by the transport of Ca^{2+}, and through inhibition of this action, several calcium antagonists have been

demonstrated to posses anti-platelet properties (Han *et al.*, 1983; Johnson *et al.*, 1986; Mehta *et al.*, 1983; Pumphrey *et al.*, 1983; Ware *et al.*, 1986; Winther *et al.*, 1990), despite evidence to the contrary (Rostagno *et al.*, 1990).

In a study performed by Lacoste *et al* (1994), the degree of platelet thrombus formation and the extent of platelet aggregation in response to thrombin were significantly decreased following oral verapamil pharmacotherapy in subjects with SAP. This was then confirmed by Wallen *et al* (1995). In contrast to the above results Beaughard *et al* (1995), was unable to demonstrate any benefit in utilizing diltiazem, nifedipine or verapamil in inhibiting cyclic flow variations induced in dogs.

The role of Ca^{2+} antagonists in the management of cardiovascular disease has been controversial, with some clinical studies showing little benefit and others demonstrating a significant reduction in the rate of events post MI in the absence of heart failure (Boden *et al.*, 1991; Furberg *et al.*, 1995; Gibson *et al.*, 1986; Goldbourt *et al.*, 1993). In a more recent investigation Boden *et al* (2000), demonstrated that diltiazem (300mg/day), on a background of aspirin, in patients with MI who had first received thrombolytic therapy, serves as an effective secondary prevention measure against reinfarction, perhaps functioning at least in some part via its anti-platelet properties. These results are in agreement with the observations made by the DAISY study investigators in which intravenous diltiazem in unstable angina reduced both short term and 1-year cardiac event rates (Gobel *et al.*, 1995; Gobel *et al.*, 1998). With regards to verapamil, the DAVIT II study demonstrated a significant reduction in the incidence of a major cardiac event post MI, a result that was reaffirmed in a meta-analysis of data obtained from a number of clinical trails (DAVIT II, 1990; Pepine *et al.*, 1998). Furthermore, the anti-platelet properties of Ca^{2+} antagonists may have also contributed to the beneficial effects of Ca^{2+} antagonist utilization post coronary angioplasty (Hoberg *et al.*, 1994).

Section D:

Pharmacotherapy with Organic Nitrates and Other Nitric Oxide Donors: Effects on Vasomotor and Platelet Function

[D.1] Nitrate pharmacotherapy

[D.1.1] Nitrovasodilators

Ever since the first descriptions of the therapeutic use of nitrates for the treatment of angina pectoris (Brunton, 1867; Murrel, 1879), organic nitrates have been in widespread use in the management of IHD and congestive heart failure (Abrams, 1996). Nitrovasodilators include not only the organic nitrate esters but also a wide variety of non-nitrate donors of nitric oxide, a list of which is shown in Table 1.2.

Table 1.2: Nitrovasodilators

Organic nitrates	*Non-nitrate nitric oxide donors*
Nitroglycerine [NTG]	*S*-nitrosothiols
Isosorbide dinitrate [ISDN]	Sodium nitroprusside [SNP]
Isosorbide 5-mononitrate [ISMN]	No(NO)ates
Erythritol tetranitrate	Mannitol hexanitrate
Pentaerythritol tetranitrate	Hydroxylamine
Nicorandil	Molsidomine

A list of various nitrovasodilators was adapted from Fung (1992), Abrams (1996) and Ignarro et al (2002).

[D.1.2] Organic nitrate preparations

Amongst the various organic nitrates that have been utilized in the management of IHD and congestive heart failure, three preparations stand out: NTG, ISDN and ISMN.

[D.1.2.1] Nitroglycerine (NTG)

Nitroglycerine (glyceryl trinitrate) has been utilized in the management of myocardial ischaemia for greater than one hundred years (Abrams, 1996). The American Heart Association and the American College of Cardiology guidelines designate intravenous NTG as a class I indication for use in anterior or complicated ST segment elevation MI (Abrams,

2000). A meta-analysis of reported data has suggested an improvement in the survival of patients receiving intravenous NTG in ACS (Yusuf *et al.*, 1988) despite data being obtained from a time when thrombolytics, aggressive anti-platelet agents and other beneficial interventions were not common practice. Following delivery of NTG via various routes including intravenous, transdermal and sublingual, NTG is rapidly converted to the metabolites 1,2 and 1,3-glyceryl dinitrate from which nitric oxide is liberated.

[D.1.2.2] Isosorbide di-nitrate (ISDN)

Another organic nitrate, ISDN is rapidly metabolized in the liver to the two metabolites, isosorbide 2-mononitrate and the commercially available isosorbide 5-mononitrate (ISMN) with approximately 60% of the di-nitrate being converted to the 5-form (Abrams, 1995).

[D.1.2.3] Isosorbide mono-nitrate (ISMN)

As outlined above, isosorbide 5-mononitrate is a metabolite of ISDN. Contrasting to the di-nitrate, ISMN does not undergo significant hepatic metabolism and is therefore extensively bio-available (Abrams, 1995). However, in the fourth international study of infarct survival (ISIS-4), oral ISMN (60mg/day) utilization in subjects with an acute MI was demonstrated to have no significant effect on mortality at 5 weeks (ISIS-4, 1995). This dose of ISMN is without effect in SAP, largely because of tolerance induction (Chrysant *et al.*, 1993). Furthermore, in ISIS-4, ISMN was demonstrated to reduce infarct mortality within 24-hrs supporting the safety of early nitrate use post MI: 1.77% for ISMN vs 2.16% for placebo ($p < 0.01$).

Despite some negative investigations NTG, ISDN and ISMN are in widespread use for the treatment/management of subjects with angina and congestive heart failure (Horowitz, 2000). The chemical structures of these three commonly utilized organic nitrate preparations along with their bioactive metabolites including ISMN are shown in Figure 1.8.

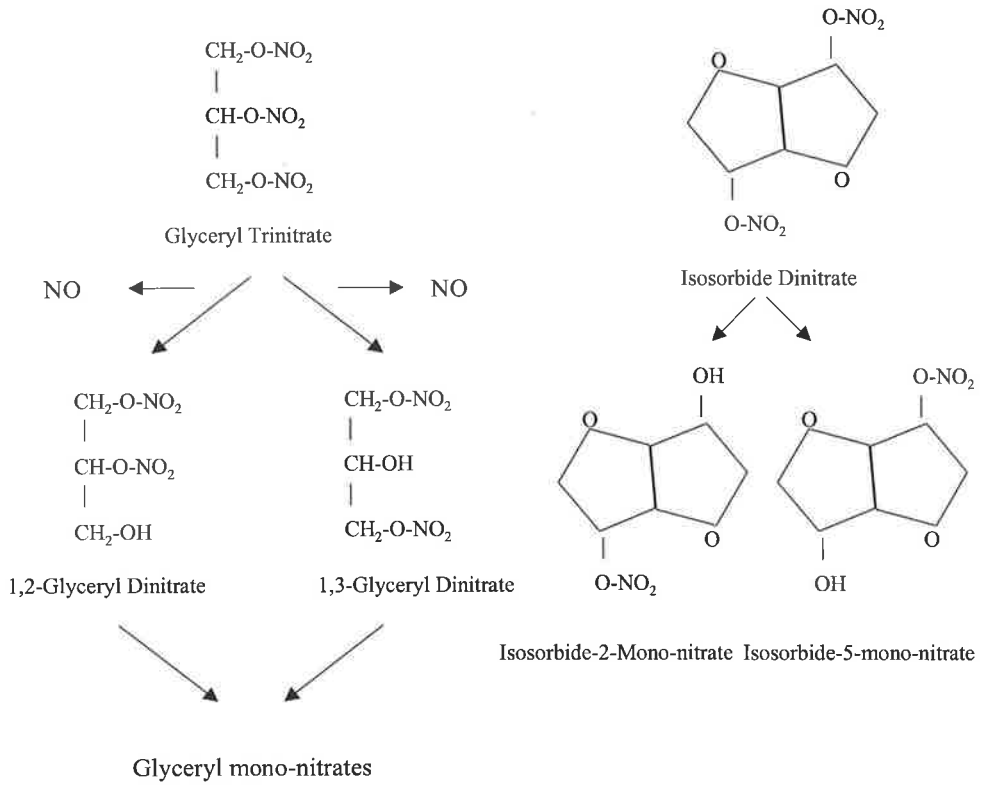


Figure 1.8 Chemical structures of the nitroglycerine (glyceryl tri-nitrate) and isosorbide dinitrate along with their respective metabolites

All organic nitrate preparations are pro-drugs that require enzymatic metabolism in order to generate the bioactive nitric oxide.

[D.1.3] Functional aspects of organic nitrates

[D.1.3.1] Vasculature

The haemodynamic and hence anti-anginal actions of organic nitrates are mediated largely through their capacity to dilate the capacitance veins and conductive arteries (Parker and Parker, 1998). Peripheral venodilation results in less blood returning to the right heart resulting in a reduction in preload that manifests itself as a decrease in ventricular filling pressures, wall tension and systemic blood pressure (Abrams, 1996).

Nitrates also induce arterial (rather than arteriolar) vasodilatation resulting in an improved vascular compliance, an effect that may be seen with very low concentrations of organic nitrates. The reduction in right and left ventricular preload and afterload caused by nitrate vasodilatation decreases cardiac work and lowers the myocardial oxygen requirements, preventing/alleviating myocardial ischaemia. For a generalized review of the role of nitrates in the vasculature see the following references (Abrams, 1995; Abrams, 1996).

NTG and the other organic nitrates have minimal vasodilator capacity in the smaller, distal vessels of the microcirculation. Reasons for this selectivity of function remain undefined. However, there is some suggestion that the enzyme/s responsible for nitrate bioconversion may be absent in these vessels (Harrison and Bates, 1993). Furthermore, both nitric oxide and *S*-nitrosothiols retain activity as dilators of arterioles (Harrison and Bates, 1993).

Organic nitrates are also coronary dilators, with significant effects on epicardial vessels as well as collaterals (Feldman *et al.*, 1982; Feldman *et al.*, 1979). These effects probably are of minor importance in the relief of exertional angina, but may be critically important in patients with vasospastic angina or threatened acute MI (Horowitz, 2000). Nitrates possess a number of extra-vascular effects including various anti-platelet properties, some of which are discussed below.

[D.1.3.2] Platelets

To date, numerous studies have demonstrated that nitrates possess considerable anti-platelet properties. A number of early studies demonstrated a potent anti-aggregatory effect of NTG *in vitro* (Schafer *et al.*, 1980) and *in vivo* (Lam *et al.*, 1988), although only at concentrations well above the therapeutic range. This was in contrast to other studies in which intravenous and sublingual NTG was shown to have no or limited *ex vivo* effect on platelet aggregation in PRP preparations (Fitzgerald *et al.*, 1984; Mehta and Mehta, 1980). Reasons for an absence of a discernable anti-platelet effect following nitrate treatment may include sub-optimal infusion duration (Stamler and Loscalzo, 1991), suggesting that only high concentrations of NTG elicit a significant anti-platelet effect. An example illustrating this comes from a study by Folts *et al* (1991), utilizing an open-chest dog model in which administration of 10-15 μg/kg/min NTG was demonstrated to reduce cyclic platelet thrombus formation, compared with 5μg/kg/min having no effect.

In UAP and acute MI patients, therapeutic concentrations of nitrates were demonstrated to significantly inhibit the extent of platelet aggregation assessed *ex vivo* (Diodati *et al.*, 1990). When platelet aggregometry experiments were performed on blood samples that had been preserved at room temperature for 30 minutes or had been obtained from subjects that had discontinued their NTG treatment 15 minutes prior to collection, the anti-platelet properties of NTG were absent, indicating a labile effect. Following on from earlier observations

(Diodati *et al.*, 1992; Diodati *et al.*, 1994), platelet hyper-aggregability induced during the passage of blood across the coronary circulation following rapid atrial pacing in patients with SAP was inhibited by therapeutic doses of NTG or SNP (Diodati *et al.*, 1995).

The above *ex vivo* observations lend further support to a multitude of other investigations where NTG and other nitrovasodilators such as SNP, ISDN, ISMN and *S*-nitroso-glutathione were demonstrated to possess potent anti-platelet properties (Aoki *et al.*, 1997a; Chen *et al.*, 1996; Chirkov *et al.*, 1992; Chirkov *et al.*, 1993; De Caterina *et al.*, 1984; De Caterina *et al.*, 1990; Hebert *et al.*, 1997; Hines and Barash, 1989; Karlberg *et al.*, 1992; Langford *et al.*, 1994; Langford *et al.*, 1996; Werns *et al.*, 1994; Wolfram *et al.*, 1996).

In a study performed by Salvemini *et al* (1993), the anti-aggregatory properties of NTG were absent when platelets were exposed to its metabolites, 1,2-GDN or 1,3-GDN. However, aggregation was inhibited when platelets were exposed to 1,2-GDN or 1,3-GDN in the presence of smooth muscle cells or endothelial cells, the effects of which were abrogated by co-incubation with oxyhaemoglobin indicating that the anti-aggregatory effects were due to NO release. Despite not addressing whether nitric oxide released from the co-incubated endothelial cells or smooth muscle cells was contributing to the anti-platelet effects, the study provided additional evidence that the metabolites of NTG require bioconversion to nitric oxide to elicit its anti-aggregatory affect.

[D.2] Nitrate Tolerance

The major limitation to widespread therapeutic nitrate utilization is the potential for the rapid development of tolerance to some or all responses to organic nitrates following sustained pharmacotherapy (Horowitz, 2000). Evidence demonstrating nitrate tolerance in both animals and humans is extensive. However, the exact mechanisms behind the phenomenon remain poorly defined.

[D.2.1] Evidence for tolerance induction: Ischaemic heart disease

Continuous administration of organic nitrates in all forms, have demonstrated an attenuation of efficacy within a short period of time. In a series of patients with SAP, Thadani *et al* (1982), demonstrated a marked attenuation in the effectiveness of ISDN pharmacotherapy following 1 week of 4 times-a-day treatment with a range of doses. These observations were

then reconfirmed by a number of other investigators, utilizing ISDN but also NTG patches, ISMN and intravenous NTG (Steering Committee, Transdermal Nitroglycerine Cooperative Study, 1991; Muiesan *et al.*, 1986; Parker and Fung, 1984; Sage *et al.*, 2000; Thadani *et al.*, 1987; Zimrin *et al.*, 1988).

[D.2.2] Congestive Heart Failure

In addition to tolerance in patients with chronic and acute IHD, progressive attenuation of the haemodynamic effects of nitrates have been demonstrated in patients with congestive heart failure using various nitrate preparations including continuous and intermittent intravenous NTG (Dakak *et al.*, 1990; Dupuis *et al.*, 1990; Gogia *et al.*, 1995; Makhoul *et al.*, 1990; Packer *et al.*, 1987), transdermal-NTG (TD-NTG) (Jordan *et al.*, 1986; Packer *et al.*, 1986), and ISDN (Elkayam *et al.*, 1991).

[D.2.3] Platelet aggregation

Demonstration of nitrate tolerance at the platelet level is limited, with only a number of studies addressing the phenomenon specifically. In a landmark investigation performed by Chirkov *et al* (1997), tolerance towards NTG at a platelet level was demonstrated following 24-hrs of a low infusion rate of NTG (5μg/min) and even after a single sublingual dose (300μg) in SAP patients. Tolerance toward NTG was also demonstrated not to be associated with cross tolerance to SNP, or related to a down-regulation of platelet guanylate cyclase. These results therefore suggest that bioconversion of NTG to nitric oxide is the most probable site of tolerance induction.

Other investigators have also described tolerance to the anti-platelet effects of nitrates. Continuous administration of NTG in dogs resulted in a loss of the coronary vasodilator responsiveness and a progressive increase in thrombin stimulated *ex vivo* platelet activity (Fink *et al.*, 1999). This enhancement of platelet activity was initially observed in 1997 in which continuous NTG infusion alone resulted in an increase in thrombin stimulated intra-platelet Ca^{2+} and increased thrombin-induced platelet aggregation (Fink and Bassenge, 1997). Somewhat contrasting to the results obtained by Chirkov *et al* (1997), impairment in both the unstimulated and SNP-stimulated platelet cGMP concentrations was also observed, implying a partial soluble guanylate cyclase inactivation (Fink *et al.*, 1999).

In a study performed by Herbert *et al* (1997), investigating the phenomenon of nitrate tolerance at both the vascular and platelet level, persistent inhibition of thrombin induced platelet aggregation and platelet deposition on porcine aortic media was observed, despite haemodynamically induced tolerance caused by NTG (0.8mg/h).

[D.3] Proposed mechanism/s of nitrate tolerance

Evidence now exists suggesting that there is no single cause for nitrate tolerance (potential mechanisms of which are examined below) with the phenomenon not necessarily reflecting a diminution in nitric oxide's effectiveness. Conceptually therefore, the phenomenon is now viewed as involving two separate components of one problem:

- "True tolerance": diminution in nitric oxide's efficacy and
- "Pseudotolerance": that involves activation of vasoconstrictor mechanisms that counteract and obscure the vasodilator effectiveness of nitric oxide.

[D.3.1] Historical perspective

It was initially suggested by Needleman and Johnson (1973) that tolerance towards NTG results from a reduction in the availability of vascular sulfhydryl groups necessary for nitrate biotransformation (or receptor interaction as concluded in 1973). Tolerance towards NTG in rabbit thoracic aortic segments was found to be time and dose dependent. Furthermore, an improvement in vascular responsiveness in tolerant vascular strips post administration of dithiothreitol was observed implying an interaction with sulfhydryl compounds, an observation that was also confirmed *ex vivo* (Needleman and Johnson, 1973).

Although initially postulated as the mechanism behind nitrate tolerance, a number of other mechanisms covering both "true tolerance and pseudotolerance", including the phenomenon of an impaired nitric oxide release, increased nitric oxide clearance, increased production of vasoconstrictors, plasma volume expansion and inactivation of soluble guanylate cyclase, have also been suggested.

[D.3.2] True tolerance

[D.3.2.1] Impaired nitric oxide release

[D.3.2.1.1] Attenuated bio-conversion

As described in section D.1.2.1, NTG is a prodrug that undergoes bioconversion to 1,2-glyceryl dinitrate (1,2-GDN) and 1,3-glyceryl dinitrate (1,3-GDN), an event that occurs prior to its pharmacological effects (Brien *et al.*, 1988). Fung and Poliszcuk (1986), demonstrated that tolerance towards NTG was accompanied by a reduced production of the 1,2-glyceryl dinitrate metabolite. These results confirmed the results obtained by Brien *et al* (1986), in which NTG-induced relaxation of rat aorta and tissue metabolite concentrations were significantly less for NTG-tolerant tissue compared with non-tolerant tissue. In cultured rat lung fibroblasts and porcine kidney epithelial cells re-exposed to NTG after prolonged exposure (0.1mM for 3-hrs), a marked attenuation of NTG biotransformation resulted. In nitrate tolerant preparations there was no formation of 1,2-GDN after exposure to NTG (Bennett *et al.*, 1989). Sage *et al* (2000) demonstrated *ex vivo* that SAP patients randomized to receive 24-hrs of NTG pharmacotherapy prior to elective CABG surgery had a reduced amount of 1,2-GDN in segments of saphenous vein compared to patients not treated with nitrates. This degree of 1,2-GDN loss paralleled attenuated NTG responsiveness, further indicating that a reduced bioconversion of NTG maybe responsible for nitrate tolerance induction. The observations of Sage *et al* (2000) have also been confirmed by Omura *et al* (2001).

The lack of cross-tolerance to other nitric oxide releasing vasodilators such nitroprusside, which readily donate nitric oxide without undergoing bioconversion (Ignarro *et al.*, 2002), also suggests that an impaired bioconversion of NTG is partly responsible for tolerance induction.

[D.3.2.1.2] Bioconversion enzyme

A decrease in the activity of the enzyme that mediates the formation of nitric oxide from NTG has been suggested to be responsible for nitrate tolerance induction. Multiple biotransformation mechanisms have been identified, with several candidate enzymes being suggested (Bennett *et al.*, 1994). An undefined microsomal protein isolated from bovine coronary arteries was demonstrated to mediate the formation of nitric oxide from NTG

(Chung *et al.*, 1992). Both vascular μ isotype of glutathione-S-transferases (Kenkare *et al.*, 1994; Nigam *et al.*, 1996; Tsuchida *et al.*, 1990) and the rat aortic cytochrome P_{450} (McDonald and Bennett, 1993; McGuire *et al.*, 1994) have also been demonstrated to mediate the denitration of NTG despite doubts raised by a number of other investigators (De la Lande *et al.*, 1996; Kurz *et al.*, 1993).

In an investigation addressing the role of NADPH-cytochrome P_{450} reductase (CPR) during *in vivo* nitrate tolerance induction, CPR expression as assessed by NADPH-dependent cytochrome *c* reductase activity of aortic microsomes, immunoblotting, and northern analysis, was demonstrated to remain unchanged in aorta from NTG-tolerant rats (Ratz *et al.*, 2000b). However, Minamiyama *et al* (2001) demonstrated that the appearance and disappearance of P_{450} parallels the conversion of NTG to nitric oxide in a rat model of nitrate tolerance.

Perhaps the most compelling evidence demonstrating a decrease in the activity of an enzyme that mediates the bioconversion of NTG serves as a mechanism responsible for nitrate tolerance induction, is the recent observations of Chen *et al* (2002), in which the mitochondrial aldehyde dehydrogenase (mtALDH) demonstrated to be responsible for the bioconversion of NTG to 1,2-glyceryl dinitrate, was significantly inhibited in tolerant tissue.

[D.3.2.1.3] Sulfhydryl hypothesis

Depletion of reduced sulfhydryl groups required for the biotransformation of NTG to nitric oxide is one of the most extensively studied hypotheses addressing the mechanism behind nitrate tolerance induction. Most of the evidence against a reduced sulfhydryl store hypothesis as an explanation for nitrate tolerance come from observations that thiol groups are not depleted in NTG tolerant tissue and that sulfhydryl containing drugs augment vascular responses to NTG in the presence and absence of nitrate tolerance (Boesgaard *et al.*, 1994; Fung *et al.*, 1988; Munzel *et al.*, 1989). These observations suggest that depletion of tissue sulfhydryl groups is not a major mechanism by which tolerance induction occurs.

Attenuation of tolerance induction via sulfhydryl addition has been postulated to occur via potentiation of nitrate effects. Nitrates interact with sulfhydryl donors extracellularly leading

to production of *S*-nitroso-thiols or nitric oxide that function as potent anti-platelet agents and vasodilators (Fung *et al.*, 1988).

[D.3.2.2] Increased nitric oxide clearance

Enhanced formation of the superoxide radical or alternative ROS stemming from the vascular endothelium with resultant increased clearance of nitric oxide, has also been postulated as a potential mechanism behind the development of nitrate tolerance.

This hypothesis was originally proposed following an investigation in which significantly higher concentrations of superoxide (as determined by LDCL) were found in rabbit aortas that had been made tolerant following TD-NTG treatment (Munzel *et al.*, 1995b). Pre-treatment of tolerant aorta with superoxide dismutase (SOD 600U/ml), significantly enhanced the maximal relaxation responses to NTG, SIN-1, and acetylcholine, whilst reducing the degree of chemiluminescence and hence superoxide.

In an attempt to identify the potential sources of superoxide, Dikalov *et al* (1998a), utilizing spin trapping techniques that are more specific to superoxide and other ROS than lucigenin, demonstrated a doubling in the degree of superoxide production from vascular smooth muscle cells, endothelial cells and washed platelets following exposure to NTG. Furthermore, the rate of ROS formation detected by spin traps in tolerant smooth muscle cells, treated for 24-hrs with 0.01mM NTG, was significantly higher than that of control (Dikalov *et al.*, 1998b). Membrane associated NAD(P)H oxidase and CuZn/SOD activity were significantly increased and decreased respectively, in homogenates of segments obtained from *in vivo* tolerant rabbits. In contrast, both NAD(P)H oxidase and CuZn/SOD activity were found to be unaltered in segments made tolerant *in vitro,* suggesting different levels of importance of superoxide in the mechanism of tolerance induction (Munzel *et al.*, 1999). Reconfirming the results observed earlier (Munzel *et al.*, 1995b), the addition of tiron functioning as a superoxide scavenger, effectively restored the vasorelaxant response to NTG in *in vivo* tolerant aortic rings, but not the reduced response to NTG in *in vitro* tolerant rings (Munzel *et al.*, 1999).

Studies demonstrating cross-tolerance to non-nitrate sources of nitric oxide both *in vitro* (Rapoport *et al.*, 1987) and *in vivo* (Laursen *et al.*, 1996) also support the hypothesis of superoxide scavenging of nitric oxide as a mechanism behind nitrate tolerance.

[D.3.2.2.1] Evidence against superoxide involvement

Evidence against superoxide involvement in the phenomenon of nitrate tolerance is about as extensive as the evidence supporting its involvement. Segments of rabbit aorta (Hussain *et al.*, 1996) or human internal mammary artery (Sage *et al.*, 2000) exposed to short term elevated levels of superoxide (levels approaching that was observed in tolerant segments), showed an unchanged NTG responsiveness. Despite the notion that a short term exposure to elevated levels of superoxide *in vitro* may not reflect the *in vivo* situation (i.e. prolonged exposure to potentially higher concentrations of superoxide), results demonstrating a lack of cross tolerance to non-nitrate sources of nitric oxide *in vitro* (Du *et al.*, 1991; Kowaluk and Fung, 1990) or *in vivo* (Bauer and Fung, 1991; De la Lande *et al.*, 1999) also add evidence against nitric oxide inactivation by superoxide during nitrate tolerance development.

Utilizing rat aorta made tolerant *in vitro*, Laight *et al* (1997) demonstrated no change in NTG responsiveness following pretreatment of segments with a variety of ROS scavengers. NTG induced *in vitro* tolerance was associated with a significant increase in 3-nitrotyrosine formation, indicative of peroxynitrite formation. Addition of free tyrosine to the segment incubation medium failed to alter tolerance development, but abolished 3-nitrotyrosine formation suggesting peroxynitrite formation is not involved in tolerance induction (Mihm *et al.*, 1999).

Plasma lipid peroxidation products and 8-iso-PGF_2 α, both serving as markers of oxidative stress, were also demonstrated to be unchanged following acute and sustained therapy with TD-NTG (Milone *et al.*, 1999b), suggesting further that nitrate therapy and tolerance induction is not associated with increased free radical production. Despite the utilization of DPI, an inhibitor of flavoprotein containing enzymes that includes NOS, Ratz *et al* (2000b), demonstrated that increased superoxide due to increased NAD(P)H oxidase activity also does not account for the development of *in vivo* tolerance to NTG in rats.

[D.3.2.2.2] Superoxide summary

As conflicting results have been obtained in studies examining the degree of cross-tolerance to non-nitrate nitric oxide sources, attenuation of tolerance induction following anti-oxidant pharmacotherapy (see below), and altered responsiveness to NTG following aortic segment exposure to elevated levels of superoxide, the exact role of superoxide within the phenomenon of nitrate tolerance, still remains undefined.

[D.3.2.3] Involvement of nitric oxide synthase

Munzel *et al* (2000a), investigating the role of eNOS in the phenomenon of nitrate tolerance demonstrated that Wistar rats treated with NTG (10μg/kg/min for 3 days) induced nitrate tolerance and marked up regulation in vascular eNOS expression that was associated with elevated levels of superoxide. The source of the elevated levels of vascular superoxide was hypothesized to result from an uncoupling of the NOS enzyme (Munzel *et al.*, 2000a) and may result from a depletion in intracellular L-arginine (Abou-Mohamed *et al.*, 2000; Kaesemeyer *et al.*, 2000).

However, the observations of Wang *et al* (2002) cast doubt on the involvement of eNOS in the phenomenon of nitrate tolerance, where the vascular effects of NTG (20mg/kg, 3 times a day for 3 days) was examined in eNOS knockout mice. In both the eNOS knockout and wild type mice, NTG induced tolerance was clearly evident. Furthermore, vascular tolerance did not lead to changes in eNOS protein expression in the wild type mice indicating the absence of any significant role for eNOS in the phenomenon of nitrate tolerance.

[D.3.3] Pseudotolerance

A decrease in blood pressure caused by NTG pharmacotherapy initiates a baroreflex stimulation that leads to the release of various vasoconstrictor agents that reduce the vasodilating effects of nitrates, an effect known as pseudotolerance. Administration of nitrates is associated with increases in plasma concentration of catecholamines, aldosterone, vasopressin and plasma renin activity (Munzel *et al.*, 1996a).

In a rabbit model of *in vivo* tolerance induction, Munzel *et al* (1995a), reported that tolerance towards NTG was associated with increased contractile sensitivity to a range of vasoconstrictive agents that included angiotensin II, serotonin, phenylephrine and phorbol

ester, a direct protein kinase C activator. Perhaps more interestingly, constrictions caused by endothelin-1 (ET-1) were paradoxically decreased in nitrate-tolerant vessels with ET-1 immuno-reactivity being observed in tolerant but not in control aortas. Enhanced vasoconstriction was mimicked in control vessels through the addition of sub-threshold concentrations of ET-1, suggesting ET-1 sensitizes vascular smooth muscle cells to a number of vasoconstrictors during tolerance induction.

Vasoconstriction induced by ET-1 occurs primarily through the ET_A receptor on vascular smooth muscle cells resulting in activation of protein kinase C (Giannessi *et al.*, 2001; Schiffrin, 2001). Chronic ET_A receptor blockade with ZD2574 had no effect on NTG induced relaxation, decrease in NTG induced cGMP accumulation or on the decrease in NTG biotransformation which occur in aortas from tolerant rats (Ratz *et al.*, 2000a). It was concluded that elevated levels of ET-1 observed post tolerance induction do not mediate tolerance, but rather occur as a consequence of vascular changes that are employed during chronic nitrate exposure. This study contrasts the observations made by Kurz *et al* (1999), in which co-administration of bosentan (a nonselective ET receptor antagonist) to tolerant rabbits resulted in a significantly increased sensitivity to NTG in aortic rings made tolerant to NTG. In this study NTG treatment caused a persistent increase in plasma renin activity. Following 72-hrs of NTG treatment ACE activity had returned to control values with AT_1 receptor mRNA expression being significantly above that of control, resulting in increased vasoconstriction (Kurz *et al.*, 1999).

[D.3.3.1] Plasma volume expansion

Dupuis *et al* (1990), whilst investigating the mechanisms of nitrate tolerance in subjects with congestive heart failure, demonstrated an extra-vascular to intra-vascular volume shift occurring within the first hour post commencement of a 1.5μg/kg/min NTG infusion. This study results confirmed results obtained by other investigators (Parker *et al.*, 1991; Parker *et al.*, 1992; Parker and Parker, 1993) and led to the development of a hypothesis for nitrate tolerance induction in which nitrate-induced expansion of plasma volume reverses the effects of nitrates on ventricular preload (Parker and Parker, 1998).

As with a number of possible hypotheses addressing the phenomenon of nitrate induced tolerance, the role plasma volume expansion remains unclear. Attempts to attenuate tolerance

development through diuretic pharmacotherapy have generated conflicting results. Treatment with hydrochlorothiazide had no effect on the development of tolerance to TD-NTG, but interestingly, diuretic therapy alone improved exercise capacity in patients with SAP (Parker *et al.*, 1996). Treatment of subjects with captopril has been demonstrated to limit (Stork *et al.*, 1997) and/or not affect nitrate tolerance (Dakak *et al.*, 1990). However, delineating the ACE inhibitor function from the diuretic properties of the drug is required to prove a diuretic effect on nitrate tolerance. Sussex *et al* (1994) and Mohanty *et al* (1995) also demonstrated a reduced degree of tolerance induction to nitrates with the co-administration of diuretics further confounding the role of plasma-volume expansion in the phenomenon of nitrate tolerance.

[D.3.4] Other hypotheses

Several other hypotheses addressing the mechanism of nitrate tolerance have also been suggested but not followed as thoroughly as the mechanisms outlined above. These include the idea of a desensitization of guanylate cyclase and an increased phosphodiesterase activity that correlate with a decrease in cGMP accumulation (Axelsson and Andersson, 1983; Bennett *et al.*, 1988; Schroder *et al.*, 1988; Waldman *et al.*, 1986). More recently Mulsch *et al* (2001), utilizing a rat and rabbit models of *in vivo* nitrate tolerance demonstrated that treatment of both rat or rabbit with NTG leads to a marked increase in expression of soluble guanylate cyclase, but also a down regulation of cGMP-dependent protein kinase, as detected by a reduction in the basal phosphorylation of the cGK-I substrate VASP following tolerance induction. Similarly Kim *et al* (2001), demonstrated a marked increase in cGMP phosphodiesterase expression in tolerant rat aortas. Moreover, administration of the phosphodiesterase-specific inhibitor vinpocetine partially restored the sensitivity of the tolerant vasculature to subsequent NTG exposure.

Regarding the phenomenon of nitrate tolerance at the level of platelets specifically, NTG (40-400 μmol/L) was shown to significantly attenuate platelet eNOS activity (Chen and Mehta, 1997). This effect was demonstrated by a reduction in the rate of conversion of *L*-arginine to *L*-citrulline, whilst having no effect on eNOS protein levels as detected by western analysis, prompting Chen and Mehta to suggest a negative feedback regulation of eNOS may also serve as a contributor to the phenomenon to nitrate tolerance, at least at the platelet level.

[D.4] Strategies that minimize true/pseudotolerance

[D.4.1] Regimen based

[D.4.1.1] Low dose and intermittent dosing regimens

There are now a number of studies demonstrating that the induction of nitrate tolerance can be minimized by the use of dosing regimens that give an extended nitrate-free interval (Chrysant *et al.*, 1993; DeMots and Glasser, 1989). A strategy that provides an interval of low nitrate exposure during a 24-hr period has also been demonstrated to be somewhat successful in limiting nitrate tolerance induction during chronic therapy (Cowan *et al.*, 1987; Ferratini *et al.*, 1989; Parker *et al.*, 1995). Despite the demonstration of a benefit in intermittent nitrate therapy over continuous therapy, withdrawal of nitrates may be associated with "rebound" myocardial ischaemia or the "zero hour" phenomenon that may result in increased episodes of ischaemia (DeMots and Glasser, 1989; Parker and Parker, 1998). Furthermore, nitrate-free intervals are unsuitable for monotherapy of ACS where the risk of ischaemia is not solely dependent on exercise-induced increases in heart rate (Horowitz, 2000).

[D.4.2] Co-therapy

[D.4.2.1] N-acetyl cysteine

Several studies have reported that thiol supplementation in the form of *N*-acetylcysteine (NAC) augments the haemodynamic effects of NTG in both tolerant and non-tolerant subjects (Horowitz *et al.*, 1983; May *et al.*, 1987; Packer *et al.*, 1987; Pizzulli *et al.*, 1997; Winniford *et al.*, 1986). Co-infusion of nitrates and NAC has been also demonstrated too partially prevent tolerance induction (Boesgaard *et al.*, 1992; Horowitz *et al.*, 1988; Shaffer *et al.*, 1992). Contrary to the above studies, other investigators have failed to show potentiation or reversal of NTG tolerance with sulfhydryl donors (Dupuis *et al.*, 1990; Munzel *et al.*, 1989; Parker *et al.*, 1987).

N-acetylcysteine's effects have traditionally been explained as the restoration of depleted thiol reserve. However, evidence now suggests that NAC restores the beneficial effects of nitrate administration via a thiol independent mechanism. Boesgaard *et al* (1993b), whilst investigating how intra/extracellular changes in sulfhydryl group concentration affect haemodynamic responsiveness to NTG *in vivo*, demonstrated that the hypotensive effect

following NTG chronic infusion remains unaffected in the presence of high intracellular concentrations of cysteine and glutathione.

The exact mechanism by which NAC reduces tolerance remains unclear. However, there is some evidence suggesting that it may function as an anti-oxidant (Aruoma *et al.*, 1989; Benrahmoune *et al.*, 2000) or have properties similar to that of an ACE inhibitor (Boesgaard *et al.*, 1993a). Recently a number of studies have demonstrated that NAC inhibits NFκB, previously demonstrated to play a functional role in oxidative stress responses and apoptosis (Fernandez *et al.*, 1999; Kim *et al.*, 2000). Despite these recent studies, a connection between a potentiation of true tolerance by NAC via a NFκB dependent mechanism has not been made to date.

[D.4.2.2] Anti-oxidants

In much the same way as anti-oxidant utilization is hypothesized to attenuate endothelial dysfunction (section C.6.2.1.1), anti-oxidant pharmacotherapy has also been demonstrated to reduce the degree and incidence of nitrate tolerance induction. Watanabe *et al*, on a number of occasions has demonstrated that vitamin utilization (either C or E) in NVs, patients with IHD or congestive heart failure attenuates nitrate tolerance induction (Watanabe *et al.*, 1997b; Watanabe *et al.*, 1998b; Watanabe *et al.*, 1998c). Partly reaffirming Watanabe's observations, vitamin-C combined with NTG was demonstrated to fully maintain the NTG-induced changes in the blood pressure, compared to NTG treatment alone, following prolonged exposure (Bassenge *et al.*, 1998).

Changes in vascular tolerance in NTG-treated subjects were paralleled by an upregulation of the activity of isolated platelets, which was also reversed by vitamin-C administration (Bassenge *et al.*, 1998). Vitamin-C co-administration with NTG reduced the degree of vascular superoxide formation compared to NTG treatment alone (Dikalov *et al.*, 1999). Co-administration of the carvedilol metabolite BM910228 or vitamin-C with NTG suppressed the degree of oxidative stress and reduced the increased platelet activity and impaired nitrate-induced vasorelaxation that results from continuous NTG utilization (Fink *et al.*, 1999). However, Milone *et al* (1999b) were unable to demonstrate any change in the haemodynamic or venous effects of NTG with vitamin C use.

[D.4.2.3] High dose ACE inhibitors

As the release of various vasoconstrictive agents to counteract the vasodilator effects of nitric oxide serves as one of the postulated mechanisms of nitrate tolerance (pseudotolerance), a number of groups have employed high dose ACE inhibitor pharmacotherapy in an attempt to limit its development.

Several studies have demonstrated an improved coronary vasodilator responsiveness following the combination of captopril and nitrates when compared to nitrate utilization alone (Meredith *et al.*, 1993; Pizzulli *et al.*, 1996), whilst other groups have demonstrated a failure of captopril to prevent tolerance induction, especially in subjects with congestive heart failure (Dakak *et al.*, 1990). Apart from its ACE inhibitor activity, captopril contains a sulfhydryl moiety. In a comparative investigation examining the efficacy of captopril and enalapril for the prevention of nitrate tolerance, captopril but not enalapril was demonstrated to attenuate nitrate tolerance induction *in vitro*, similar to that achievable by NAC. These results suggest that the sulfhydryl group contained within captopril or the diuretic properties, function to prevent nitrate tolerance rather than the ACE inhibitor activity (Lawson *et al.*, 1991).

Contrary to the above hypothesis, treatment with non-SH ACE inhibitors that include enalapril (Katz *et al.*, 1991; Munzel and Bassenge, 1996; Watanabe *et al.*, 1997a) and more recently ramipril (Berkenboom *et al.*, 1999) have also shown to attenuate nitrate tolerance.

The exact mechanisms by which ACE inhibition prevents/attenuates nitrate tolerance remains undefined. Attenuation of superoxide formation via direct/indirect inhibition of NAD(P)H oxidase sub-unit assembly has also been postulated as one mechanism by which ACE inhibitors limit nitrate tolerance. NAD(P)H oxidase enzyme activity is upregulated by angiotensin II as demonstrated in tissue culture (Griendling *et al.*, 1994) and *in vivo* (Rajagopalan *et al.*, 1996) (see also section C.4.3.1 and C.6.3). Kurz *et al* (1999), in a study investigating the effectiveness of losartan (AT_I receptor antagonist) in attenuating nitrate tolerance induction, demonstrated that *in vitro* pre-incubation of losartan did not alter vascular superoxide production. This was in contrast to the *ex vivo* results in which losartan inhibited the degree of vascular superoxide production in both tolerant and control vessel preparations. It was therefore concluded that losartan functions to limit tolerance induction by preventing AT_I receptor stimulation and hence release of angiotensin may stimulate

NAD(P)H oxidase rather than directly inhibiting the assembly of vascular NAD(P)H oxidase sub-units (Kurz *et al.*, 1999).

However, Milone *et al* (1999a) also examining the efficacy of losartan in attenuating nitrate tolerance, failed to demonstrate any prevention of haemodynamic or vascular tolerance to TD-NTG in humans partly agreeing with the observations of Cotter *et al* (1998) and adding further confusion to the role of ACE inhibition in nitrate tolerance development.

[D.4.2.4] Hydralizine

The co-administration of hydralizine with nitrates has showed some benefit in reducing nitrate tolerance induction despite a lack of recent research. In patients with chronic heart failure due to left ventricular systolic dysfunction, the concomitant use of oral hydralizine was demonstrated to prevent the early development of nitrate tolerance (Gogia *et al.*, 1995). These results were supported by the observations of Munzel *et al* (1996b) who demonstrated an absence of nitrate tolerance induction in rabbits treated with NTG and hydralizine compared to NTG alone. Within this investigation the mechanism for prevention of tolerance induction was suggested to be via a reduction in the extent of vascular superoxide generation. Alternative mechanisms include an enhancement of available sulfhydryl-containing compounds (Unger *et al.*, 1993) or via direct inhibition of $p22^{phox}$ mRNA expression a sub-unit required for production of vascular and polymorphonuclear derived superoxide (Fukui *et al.*, 1997).

[D.4.2.5] Other co-therapy

Addressing the hypothesis that continuous NTG utilization causes a reduction in the bio-availability of *L*-arginine required for nitric oxide production by NOS, Parker *et al* (2002), investigated the therapeutic benefit of co-administration of *L*-arginine in preventing/limiting tolerance to TD-NTG. In a cohort of SAP patients it was observed that *L*-arginine (700 mg) use was associated with a significant increase in the treadmill walking time prior to the onset of moderate angina during chronic but not acute administration of TD-NTG (0.4mg/h). Similarly it has also been suggested that the combined use of nitrates and folic acid may also prevent nitrate tolerance induction (Gori *et al.*, 2001).

[D.4.3] Tolerant resistant nitric oxide sources

In a study performed by Fink and Bassenge (1997), pentaerythryl-tetranitrate was demonstrated to display minimal tolerance development relative to NTG, an observation that has been recently confirmed (Jurt *et al.*, 2001; Mullenheim *et al.*, 2001). Nicorandil has also been suggested to have a decreased propensity to induced tolerance relative to NTG pharmacotherapy over 24-hrs in subjects with heart failure (Larsen *et al.*, 1997) and is suggested to function through its potassium channel opening function rather than its nitrate activity (Tsutamoto *et al.*, 1995). A number of other non-nitrate nitric oxide donors have also been recently developed as potential anti-ischaemic agents including molsidomine and linsidomine thought not to readily develop tolerance relative to NTG (Hinz and Schroder, 1999; Sutsch *et al.*, 1997).

[D.4.4] Summary

Attenuation of the clinical utility of organic nitrates is a major concern in the management of both subjects with IHD and congestive heart failure. Evidence exists both supporting and refuting a number of hypotheses regarding induction of nitrate tolerance, suggesting that it is a complex and multi-factorial phenomenon that involves a number of potential mechanisms.

Section E:

Combined Dysfunction of the Endothelium and Platelets. The Concept of Reduced Responsiveness to the Anti-Platelet and Vasodilator Properties of Nitric Oxide (Nitric Oxide Resistance)

[E.1] Phenomenon

Initially suggested in subjects with severe congestive heart failure (Armstrong *et al.*, 1980), reduced responsiveness to nitric oxide released from an organic nitrate and hence initially termed "nitrate resistance," has also been used to describe an apparently analogous

phenomenon demonstrated at the platelet level (see below). From these initial observations, reduced responsiveness to NTG has been utilized to characterize the phenomenon of reduced responsiveness both to exogenous sources other than NTG, and to endogenous sources of nitric oxide. The basis for the extension of the definition has been studies utilizing non-nitrate sources of nitric oxide such as SNP. The phenomenon therefore reflects nitric oxide resistance per se, rather than nitrate resistance. Evidence describing this phenomenon within the vasculature and at a platelet level along with a series of potential mechanisms is discussed below.

[E.1.1] Nitric oxide resistance at the vascular level

Many of the early studies delineating the phenomenon of "nitrate resistance" as it was called then, were performed on subjects with moderate to severe congestive heart failure (Armstrong *et al.*, 1980; Kulick *et al.*, 1988; Packer *et al.*, 1986). In an investigation examining the haemodynamic effects of ISDN in subjects with congestive heart failure, over half of the subjects examined remained unresponsive to 40mg ISDN (as measured by a < 20% decrease in the mean pulmonary artery wedge pressure after 1 hour). Approximately 25% of the initial non-responders to 40mg ISDN remained unresponsive following increases up to 120mg (Kulick *et al.*, 1988). These results clearly illustrated haemodynamic resistance to the effects of ISDN within these patients and supported a series of observations of resistance to NTG either given intravenously or sublingually in subjects with congestive heart failure (Armstrong *et al.*, 1980; Elkayam *et al.*, 1985; Magrini and Niarchos, 1980). It remains possible that a component of this "nitrate resistance" was imposed by elevated intrapericardial pressures, and thus is not entirely related to vasomotor effects of organic nitrates.

Resistance, to organic nitrates, demonstrated in subjects with congestive heart failure (Katz *et al.*, 1992; Katz *et al.*, 1993) and in animal models of heart failure (Wang *et al.*, 1994), has also been demonstrated in human atherosclerotic coronary segments (Forstermann *et al.*, 1988) and in patients with type II diabetes mellitus (Leung *et al.*, 1993).

[E.1.2] Further evidence of nitric oxide resistance and risk factors for coronary artery disease

The phenomenon of nitric oxide resistance closely resembles that of endothelial dysfunction, i.e. diminution or absence of vasodilator responsiveness. Analogously, subjects with classical risk factors for CAD such as hypercholesterolaemia and diabetes as well as animal models of these risk factors have also demonstrated vascular resistance to the effects of nitric oxide.

Forearm blood flow responses to SNP were observed to be less in hypercholesterolaemic patients compared to control subjects (Creager *et al.*, 1990; Adams *et al.*, 1998). Aortic vessels (Watanabe heritable hyperlipidaemic rabbits) with extensive atherosclerotic lesions, also exhibited attenuated responsiveness to NTG compared to segments devoid of atherosclerotic lesions (Kolodgie *et al.*, 1990) confirming results observed in human subjects (Berkenboom *et al.*, 1989; Lundman *et al.*, 1997)

Resistance to the vascular effects of nitric oxide has also been demonstrated in experimental models of diabetes by some investigators (Martinez-Nieves and Dunbar, 1999), whilst others observed no resistance (Huvers *et al.*, 1999; Koltai *et al.*, 1997; Van Buren *et al.*, 1998). The differences between the studies remain unresolved but may lie in the observation that normal endothelial function is still present at the early stages of diabetes (Brands and Fitzgerald, 1998). However, it should also be mentioned that some studies of endothelial dysfunction have failed to explore dose-response relationships for nitric oxide donors, preferring instead to examine only single large doses (Hamabe *et al.*, 2001; Komatsu *et al.*, 2002). Thus shifts in vascular responsiveness may have been missed.

Whilst investigating possible reasons for an increased prevalence of essential hypertension in African Americans, Cardillo *et al* (1999) demonstrated attenuated forearm vasodilator response to both SNP and acetylcholine in normotensive African Americans compared to Caucasian subjects. These results provide evidence of resistance to the effects of nitric oxide, with attenuated response to either exogenous or endogenous sources of nitric oxide contributing to the development of risk of vascular events closely associated with CAD.

[E.1.3] Nitric oxide resistance and its relationship to endothelial dysfunction

In an investigation utilizing quantitative angiography and intra-coronary ultrasound, vessel segments exhibiting a paradoxical vasoconstrictor response to acetylcholine suggestive of localized endothelial dysfunction, also demonstrated significantly reduced vasodilator capacity to NTG (Schachinger and Zeiher, 1995). An inverse correlation between NTG induced vasodilator capacity and local atherosclerotic plaque load was also noted. Moreover, coronary endothelial vasodilator dysfunction as measured by acetylcholine provocation testing and to a lesser degree NTG vasodilator capacity have recently been demonstrated to be an independent predictor of atherosclerotic disease progression and cardiovascular event rates (Schachinger *et al.*, 2000). As other investigators have shown an association between impairment of responses to nitric oxide donors and a paradoxical response to endothelium-dependent vasodilators (Adams *et al.*, 1998; Schachinger and Zeiher, 1995; Zeiher *et al.*, 1995), the abnormality in vasodilator function is not only confined to endothelial dependent mechanisms. It may also comprise of impairment in smooth muscle dilator function. Moreover, they must share some common mechanisms.

[E.1.4] Nitric oxide resistance at the platelet level

As described in section C.8, platelets from patients with angina pectoris, and from subjects with risk factors for CAD, are hyper-aggregable to a range of platelet agonists. Platelets from these subjects may also display a diminished degree of responsiveness to the anti-aggregatory effects of nitric oxide donors, a phenomenon apparently analogous to that of vascular nitrate resistance.

Examples of a reduced responsiveness to nitric oxide released from NTG or SNP initially came from a series of studies examining the anti-aggregatory properties of insulin. Insulin both *in vitro* and *in vivo* has been demonstrated to have potent anti-aggregatory properties mediated through a nitric oxide/cGMP dependent mechanism (Hiramatsu *et al.*, 1987; Trovati *et al.*, 1988; Trovati *et al.*, 1986; Trovati *et al.*, 1997; Trovati *et al.*, 1994; Trovati *et al.*, 1996). In obese subjects with and without NIDDM and in subjects with hypertension, insulin-induced anti-platelet effects were demonstrated to be significantly attenuated (Touyz and Schiffrin, 1994; Trovati *et al.*, 1995). Platelet responsiveness to NTG and nitroprusside (although the latter data were not reproduced in the article) was subsequently demonstrated to be significantly reduced in the insulin-resistant states of obesity alone and obesity with

NIDDM confirming the presence of a nitric oxide resistance phenomenon at the platelet level (Anfossi *et al.*, 1998).

To date potential restoration of platelet responsiveness to nitric oxide following administration of insulin has not been investigated. However, normalization of impaired platelet anti-aggregatory effect to prostaglandin E_1/I_2 in patients with an ACS was evident following bolus injections of insulin (0.1U/kg/7days). Subjects treated with saline only demonstrated no restoration in platelet responsiveness to prostaglandin (Kahn *et al.*, 1992).

Further evidence confirming the phenomenon of nitric oxide resistance at the platelet level comes from experiments performed by Woods *et al* (1993), in which platelets from patients with untreated essential hypertension also demonstrated a reduced anti-aggregatory effect of nitric oxide compared to control subjects. Moreover, platelets from patients with SAP exhibited reduced sensitivity to the anti-aggregatory effects of NTG *in vitro* (Chirkov *et al.*, 1993). Approximately 100-fold higher concentrations of NTG were required to achieve 50% reversal of aggregation compared to concentrations used on samples from control subjects. At the time of study enrollment no patient had received nitrates for a period of 24-hrs prior to sampling, eliminating the possibility that nitrate tolerance may serve as the explanation for the observed results (Chirkov *et al.*, 1993).

In a study reaffirming the above observations and utilizing a more direct donor of nitric oxide than NTG, Chirkov *et al* (1996) demonstrated an attenuated anti-aggregating and cGMP-stimulating effect of SNP in platelets from patients with SAP compared to a cohort of NVs.

[E.2] Mechanism/s

Is there a distinction between endothelial dysfunction and nitric oxide resistance? Many hypotheses addressing the mechanisms of both endothelial dysfunction and nitrate tolerance induction have also been used to explain the mechanisms underlying nitric oxide resistance. However, specific studies addressing the mechanisms of resistance are somewhat limited with most of the research addressing the phenomenon occurring at a vascular level. A number of mechanisms have been proposed and are outlined in an early review by Abrams (1991). These mechanisms include mechanical compression of the peripheral vasculature, salt and water infiltration of blood vessels rendering them unable to further dilate, a decreased bio-

availability of the nitric oxide, and excessive neurohormonal activation that results in a marked vasoconstriction (Abrams, 1991). However, this review failed to consider the critical haemodynamic phenomenon of diastolic ventricular interaction, and did not explore possible biochemical aberrations.

As a potential mechanism to explain the phenomenon of a significantly reduced haemodynamic response to ISDN in a cohort of heart failure subjects, Kulick *et al* (1988), observed a significantly higher right atrial pressure in those heart failure subjects that were unresponsive to low dose ISDN (40mg). Again this finding is consistent with diastolic ventricular interaction (Atherton *et al.*, 1997).

[E.2.1] The link generated from diabetes and insulin

As discussed above and in addition to its metabolic effects, *in vitro* and *ex vivo* administration of insulin restores platelet responsiveness to both nitric oxide and prostacyclin in subjects with insulin resistant conditions such as obesity and NIDDM (Anfossi *et al.*, 1998; Touyz and Schiffrin, 1994; Trovati *et al.*, 1995). Insulin has been demonstrated to increase blood flow in skeletal muscle by a nitric oxide dependent mechanism (Steinberg *et al.*, 1994). In normal volunteers, insulin at physiological concentrations was demonstrated to rapidly decrease wave reflection of the aorta (as determined by applanation tonometry), reflecting increased distensibility or vasodilatation of large arteries (Westerbacka *et al.*, 1999).

[E.2.2] Reactive oxygen species

Studies examining the role of superoxide or other ROS in the phenomenon of nitric oxide resistance are limited despite extensive evidence suggesting its involvement in the phenomena that it most closely resembles i.e. endothelial dysfunction and nitrate tolerance. Of the studies performed examining superoxides involvement in nitric oxide resistance, its function in the phenomenon at the platelet level has not been addressed previously and therefore serves as one of the major residual issues that this thesis will address.

Platelets obtained from two children with a history of arterial thrombosis were hyper-aggregable in response to ADP, but also hypo-responsive to the anti-platelet effects of nitric oxide. Plasma from both children was deficient in glutathione peroxidase (GSH-Px); addition of exogenous GSH-Px led to a restoration of the platelet responsiveness to nitric oxide

(Freedman *et al.*, 1996). These results suggest that impaired metabolism of ROS reduces the bio-availability of nitric oxide and provides a further impetus to examine the role of superoxide in the phenomenon of nitric oxide resistance at least at the platelet level. Further evidence of a significant contribution from ROS to the phenomenon of nitric oxide resistance comes from a study by Haramaki *et al* (2001), where the degree of platelet responsiveness to NTG was examined in chronic smokers and non-smokers. The extent of platelet responsiveness to NTG within the chronic smoker cohort was found to be significantly less than that of non-smokers. Moreover, the levels of intra-platelet reduced glutathione were significantly less in the chronic smokers compared to the non-smokers suggesting a heightened level of oxidative stress may play a role within the phenomenon of a reduced platelet responsiveness to NTG (Haramaki *et al.*, 2001).

Section F:

Major Residual Issues

Despite being two separate phenomena, the combination of platelet hyper-aggregability and decreased platelet responsiveness to nitric oxide in subjects with (or risk factors for) also implies reduced platelet sensitivity to endogenously available nitric oxide. Resultant failure of regulation of platelet aggregability may result and as such represents a potential contribution to the increased risk of ischaemic events observed in such subjects.

Within this context the thesis was designed to explore further the phenomena and potential mechanism/s behind platelet hyper-aggregability and nitric oxide resistance, and the interplay between these phenomena in context of angina management with nitrates.

Section G:

Scope of the Current Study

This thesis investigates further the phenomena of platelet hyper-aggregability, hypo-responsiveness to nitric oxide and the anti-platelet tolerance inducing effects of organic nitrate utilization.

Specific objectives of this work include: -

- To study further the phenomena of platelet hyper-aggregability and platelet hypo-responsiveness to nitric oxide in subjects with both acute and chronic ischaemic heart disease.

- To examine the role of superoxide in the phenomenon of platelet hypo-responsiveness to nitric oxide.

- To assess the clinical determinants of a reduced responsiveness to nitric oxide at the platelet level.

- To investigate the effects of both acute and chronic nitrate exposure on platelet function, along with examining any potential relationship to vascular reactivity in subjects with chronic SAP.

In order to achieve the above objectives this body of work is divided into three components: -

- Chapter 2 addresses the phenomena of platelet hyper-aggregability and hypo-responsiveness to nitric oxide. The chapter also assesses the role of superoxide within these phenomena.

- Chapter 3 examines the clinical determinants of reduced responsiveness to nitric oxide at the platelet level.

- Chapter 4 examines the inter-relationships between platelet hyper-aggregability, nitrate tolerance induction, platelet hypo-responsiveness to nitric oxide and vascular reactivity in the clinical setting of both acute and chronic exposure of nitrates in chronic SAP patients.

Chapter 2

Platelet hyper-aggregability and hypo-responsiveness to donors of nitric oxide; involvement of the superoxide anion

[2.1] Chapter Overview

This chapter describes the observations of platelet hyper-aggregability and hypo-responsiveness to donors of nitric oxide in whole blood samples obtained from patients with acute and chronic symptomatic ischaemic heart disease. It addresses the hypothesis that the superoxide anion by its reaction with nitric oxide, limiting the bioavailability of nitric oxide, is responsible for the phenomena of platelet hyper-aggregability and reduced responsiveness to nitric oxide.

The chapter also describes a method for quantifying superoxide within whole blood samples, prior to and post induction of platelet aggregation.

The chapter then addresses the relationship between both platelet hyper-aggregability and hypo-responsiveness to donors of nitric oxide, and elevated levels of superoxide in blood samples obtained from patients with SAP and ACS.

[2.1.1] Summary of the study examining the phenomena of platelet hyper-aggregability and hypo-responsiveness to donors of nitric oxide.

Objectives: To investigate the mechanisms underlying the phenomena of platelet hyper-aggregability in response to ADP and of reduced responsiveness of platelets to the anti-aggregatory effects of the nitric oxide donors SNP and NTG, in patients with ischaemic heart disease.

Methods: In blood samples obtained from a series of NV (n = 37), patients with either SAP (n = 43) or an ACS (n = 47), the anti-aggregatory effects of SNP (10μM) and NTG (100μM) on ADP (1μM)-induced platelet aggregation were examined. Via the addition of SOD/catalase (300U/mL), the putative role of superoxide in the phenomena of platelet hyper-aggregability and hypo-responsiveness to donors of nitric oxide was also examined.

Results: Platelets from patients with SAP and ACS were hyper-aggregable in response to ADP (1μM) compared to those obtained from NVs (ANOVA $F = 5.18$, $p < 0.01$). *In vitro* administration of SNP and NTG inhibited the extent of platelet aggregation in patients to a

lesser degree in comparison to responses obtained from NVs (SNP: ANOVA $F = 5.93$, $p < 0.01$; NTG: ANOVA $F = 6.1$, $p < 0.01$). Addition of SOD/catalase to blood samples obtained from patients inhibited the extent of ADP-induced platelet aggregation (paired analysis SAP $t = 4.4$, $p < 0.01$). However, the extent of platelet aggregation was not correlated with the degree of change in platelet aggregation post SOD/catalase administration, suggesting the presence of superoxide is independent of platelet hyper-aggregability (regression analysis; angina pectoris $r = -0.32$, $p = 0.15$, run test $p = 0.79$; NV $r = -0.11$, $p = 0.73$, run test $p = 0.87$). Administration of SOD/catalase did not overall significantly affect platelet responsiveness to SNP in samples from NV, SAP and ACS patients (ANOVA $F = 0.027$, $p = 0.87$). However, in those subjects with a history of angina (SAP/ACS patients), a significant inverse relationship existed between the initial platelet responsiveness to SNP and the extent of change in SNP responsiveness post administration of SOD/catalase (regression analysis: $r = -0.55$, $p = 0.0085$, run test $p = 0.74$). Moreover, this relationship was absent within the NV cohort (regression analysis: $r = 0.32$, $p = 0.28$, run test $p = 0.29$) indicating a heterogeneity of effect of superoxide between normals and patients regarding platelet responsiveness to SNP.

Conclusions: Platelets from patients with SAP or an ACS are hyper-aggregable in response to ADP and significantly less responsive to the anti-aggregating effects of both SNP and NTG. These phenomena may reflect, at least in part, an inactivation of the released nitric oxide by superoxide, thereby contributing to the observed platelet hyper-aggregability associated with angina pectoris.

[2.1.2] Summary of a study examining the development of a detection method for superoxide in whole blood samples.

Objectives: To examine levels of superoxide within whole blood samples prior to and post induction of platelet aggregation.

Methods: In blood samples obtained from a series of NVs and utilizing the luminescent component of a lumi-aggregometer, levels of superoxide prior to and post induction of whole blood ADP-induced platelet aggregation were examined utilizing lucigenin (bis-*N*-methylacridinium nitrate) as a probe.

Results: In whole blood samples, superoxide was detected prior to and post induction of platelet aggregation utilizing lucigenin (12.5μM). The extent and rate of superoxide generation post induction of platelet aggregation positively correlated with the extent and rate of platelet aggregation (extent of LDCL; Spearman rank = 0.67, $p < 0.01$; rate of aggregation-associated LDCL $r = 0.61$, $p < 0.01$). A lag period between the induction of platelet aggregation and the commencement of the aggregation-associated generation of superoxide was also observed and found to be a function of the concentration of ADP used (ANOVA $F = 5.4$, $p < 0.01$), the extent of aggregation detectable prior to the increase in LDCL ($r = 0.76$, $p < 0.01$) and the extent of aggregation-associated LDCL ($r = -0.29$, $p = 0.025$).

Conclusions: Superoxide was detected in an unstimulated whole blood sample. Induction of platelet aggregation initiated further generation of superoxide in a platelet aggregation-dependent fashion. These results emphasize the potential for release of superoxide at the sites of vascular injury where platelet aggregation plays an integral part in thrombus formation.

[2.1.3] Summary of a study examining the extent of LDCL in subjects with coronary artery disease; relationship to platelet aggregability and hypo-responsiveness to donors of nitric oxide

Objectives: To compare the levels of LDCL (pre- and post platelet aggregation) across cohorts of NVs and SAP, ACS patients. To examine the relationship between platelet aggregability, platelet hypo-responsiveness to a donor of nitric oxide and the superoxide content in a whole blood sample pre- and post induction of aggregation.

Methods: In samples obtained from a series of NVs (n = 21), patients with either SAP (n = 30) or ACS (n = 25), extent of platelet aggregation in response to ADP (1μM) and its inhibition by SNP (10μM) was compared with the extent of LDCL prior to and post platelet aggregation.

Results: Utilizing two separate concentrations of lucigenin, the extent of baseline and aggregation-associated LDCL was significantly greater in ACS patients than in the SAP and NV subject cohorts (lucigenin (125μM) baseline LDCL ANOVA $F = 7.1$, $p < 0.01$; aggregation-associated LDCL ANOVA $F = 3.26$, $p = 0.047$; lucigenin (12.5μM) baseline LDCL ANOVA $F = 6.2$, $p < 0.01$; aggregation-associated LDCL ANOVA $F = 11.1$, $p < 0.01$). With both concentrations of lucigenin, no significant relationship was observed for the SAP and ACS patient cohorts between (1) the extent of platelet aggregation and baseline/aggregation-associated LDCL or (2) responsiveness to SNP and baseline/aggregation-associated LDCL. However, when the extent of LDCL was expressed relative to aggregation or SNP responsiveness, ACS patients generated significantly greater amounts of superoxide per unit aggregation, compared to SAP patients.

Conclusions: ACS patients generate significantly greater amounts of superoxide than SAP/NV. As they also release significantly greater amounts of superoxide per Ohm/% inhibition of aggregation, an excess of superoxide at the site of thrombus formation may contribute to the pathological nature of ACS.

Table 2.1 Abbreviations used in this chapter

Abbreviation	Definition	Abbreviation	Definition
ACE	*Angiotensin converting enzyme*	MCLA	*2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol [1,2-a] pyrazin-3-one*
ACS	*Acute coronary syndrome*	NAD(P)H	*Nicotinamide adenine dinucleotide phosphate*
ADP	*Adenosine di-phosphate*	NQAMI	*Non Q-wave myocardial infarction*
ANCOVA	*Analysis of covariance*	NTG	*Nitroglycerine*
ANOVA	*Analysis of variance*	NV	*Normal volunteers*
ASA	*Acetyl salicylic acid; aspirin*	ODQ	*1H-[1,2,4] oxodiazolo [4,3,a] quinoxalin-1-one*
cGMP	*Cyclic guanosine monophosphate*	$O_2^{\cdot-}$	*Superoxide*
CLA	*2-methyl-6-phenyl-3,7-dihydroimidazol [1,2-α] pyrazin-3-one*	$OH^{\cdot}$	*Hydroxyl radical*
DEPMPO	*5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide*	$ONOO^-$	*Peroxynitrite*
DMPO	*5,5-dimethylpyrroline-N-oxide*	PPP	*Platelet-poor plasma*
EDRF	*Endothelial derived relaxing factor*	PRP	*Platelet-rich plasma*
ESR	*Electron spin resonance*	RBC	*Red blood cell*
fMLP	*N-formyl-methionyl-leucyl-phenylalanine*	ROS	*Reactive oxygen species*
GPIb	*Glycoprotein Ib*	SAP	*Stable angina pectoris*
GPIIb/IIIa	*Glycoprotein IIb/IIIa*	SD	*Standard deviation*
HOCl	*Hypochlorous acid*	SNP	*Sodium nitroprusside*
H_2O_2	*Hydrogen peroxide*	SOD	*Superoxide dismutase*
KS	*Kolmogorov-Smirnov*	UAP	*Unstable angina*
LDCL	*Lucigenin-derived chemiluminescence*		
L-NAME	*N^{ω}-monomethyl-l-arginine*		

[2.2] Introduction

[2.2.1] Platelets / activation / aggregation

Platelets are non-nucleated megakaryocyte derived disk shaped cells (George, 2000) that are responsible for the formation of the primary haemostatic plug at sites of vascular injury and the development of pathological thrombi at the sites of atherosclerotic plaque rupture (Davies, 1995). In a series of active processes (reviewed in sections A.4-A.9 of chapter 1), platelets adhere to particular proteins within the sub-endothelial matrix, become exposed to various platelet agonists, and undergo platelet activation and shape change to form platelet aggregates.

Limiting the activation and extent of platelet aggregation in particular conditions characterized by thrombus formation has been a subject of great interest following the discovery that platelet aggregation is a major component of the pathogenesis of UAP and acute MI (Davies, 1995; Stamler and Loscalzo, 1991).

[2.2.2] Effects of nitric oxide on platelet aggregation

Authentic nitric oxide / EDRF

Not long after the discovery that endothelial derived relaxing factor (EDRF) possessed a number of physiological properties that are related/identical to those of nitric oxide (Ignarro *et al.*, 1987a; Ignarro *et al.*, 1987b; Palmer *et al.*, 1987) Radomski *et al*, demonstrated that authentic nitric oxide and EDRF are potent inhibitors of platelet aggregation/adhesion (Radomski *et al.*, 1987a; Radomski *et al.*, 1987b; Radomski *et al.*, 1987c). For a further summary see section A.11 of chapter 1.

In vitro

The effects of nitric oxide donors on platelets have been studied extensively *in vitro* over the last thirty years. Despite developments in the understanding of how NTG and other nitric oxide donors affect platelets, a number of inconsistencies remain between various studies. It has long been recognized that the *in vitro* administration of NTG or other organic nitrates to a platelet suspension causes inhibition of platelet aggregation, but only at supra-pharmacological concentrations (Fitzgerald *et al.*, 1984; Loscalzo, 1985; Mehta and Mehta, 1980; Schafer *et al.*, 1980).

In a study performed by Chirkov *et al* (1993), the *in vitro* anti-platelet properties of NTG were investigated in blood samples from NV and patients with SAP. The *in vitro* anti-aggregatory effectiveness of NTG was found to be concentration-dependent with a $1.4 \pm 0.3 \times 10^{-6}$M concentration being associated with a 50% reversal of platelet aggregation. Moreover, this *in vitro* anti-platelet effect was associated with an elevation of cGMP, agreeing with results from other investigators (Mellion *et al.*, 1981). Furthermore, NTG added post induction of platelet aggregation caused marked disaggregation, an effect that was concentration dependent and consistent with the observations made using SNP and NTG in NVs (Chirkov *et al.*, 1991; Chirkov *et al.*, 1992; Chirkov *et al.*, 1993). For a further summary see section D.1.3.2 of chapter 1.

In vivo / ex vivo

Despite the inconsistencies in the concentration of nitric oxide donors used *in vitro*, a large number of studies have demonstrated that NTG inhibits platelet aggregation *in vivo* and *ex*

vivo. Lam *et al* (1988), utilizing ^{111}In-labeled platelets demonstrated that NTG significantly inhibited platelet deposition on porcine carotid artery preparations injured by balloon angioplasty, compared to control preparations.

Through the use of the "Folts" model of coronary thrombosis (Folts *et al.*, 1982), the *in vivo* effects of organic nitrates have been examined by a number of investigators (Folts *et al.*, 1991; Rovin *et al.*, 1993). The Folts model of coronary thrombosis permits to study periodic acute platelet thrombus formation (± associated coronary vasoconstriction) in stenosed canine coronary arteries that have undergone mechanical intimal damage (Folts, 1991a; Folts, 1991b). Platelet thrombus formation at the sites of intimal damage produces cyclical reductions in blood flow; inhibition of these cyclical flow reductions by various anti-platelet agents is related to their *in vivo* potency. However, the model is not one of "pure" platelet aggregation, given that cyclic flow reductions may also be modulated in part by associated changes in coronary vasomotor tone (Lam *et al.*, 1988).

Folts *et al* (1982), utilizing the canine model of coronary thrombosis were unable to demonstrate a change in the frequency of the cyclical reductions in blood flow when NTG was applied topically, proximal to the stenosis, despite causing a transient fall in the systemic blood pressure. Golino *et al* (1990), utilizing the same model, also failed to demonstrate any significant change in the cyclical reductions in blood flow when NTG was administered intravenously (5μg/kg/min). However, intra-coronary administration of NTG at high rates (21μg/kg/min) significantly inhibited cyclical flow reductions, suggesting higher concentrations of nitric oxide donors were required to produce an effect. Folts *et al* (1991), using NTG infused intravenously at 10-15μg/kg/min, demonstrated a significant reduction in cyclic flow reductions, an effect that was observed without any significant change in coronary blood flow in normal arteries. Furthermore, and akin to the *in vitro* results, pre-treatment of dogs with *N*-acetyl cysteine (100mg/kg) for 30 minutes prior to NTG administration potentiated NTG effects (Folts *et al.*, 1991).

Platelet hyper-aggregability/reactivity induced by rapid atrial pacing (section 2.2.4 of this chapter) in subjects with a significant narrowing within a coronary artery, was significantly inhibited following pretreatment of subjects with a 10-minute infusion of either NTG or SNP (titrated for each subject to achieve a 10% decrease in the mean arterial blood pressure)

(Diodati *et al.*, 1992). In a study examining the anti-aggregatory effects of intravenous NTG, Karlberg *et al* (1992) demonstrated that aggregation time increased with increasing concentrations of intra-venously administered NTG.

Therefore, investigations described above, along with those described in section D.1.3.2, provide evidence to support the view that nitric oxide, either endothelially derived or exogenously donated, has significant anti-platelet properties *in vivo*.

Mechanism of disparity between in vivo and in vitro effects of nitric oxide

In a study addressing the inconsistencies between the anti-aggregatory effects of NTG *in vitro* compared to *ex vivo*, Loscalzo demonstrated that the administration of *N*-acetylcysteine markedly potentiated the platelet inhibitory effects of NTG (Loscalzo, 1985). The idea that the anti-platelet properties of NTG were dependent on the availability of reduced thiols, effectively increasing the rate of bioconversion of NTG and hence nitric oxide liberation, was then reconfirmed in *ex vivo* experiments (Stamler *et al.*, 1988). Utilizing a multiple agonist approach to examine the anti-aggregatory effect of a number of compounds Willoughby *et al* (1996) demonstrated that the NTG concentration-response curve was shifted markedly to the left when multiple agonists were used to induce platelet aggregation, compared to ADP alone. Therefore two mechanisms are possibly contributing to a reduced potency of NO donors *in vitro*: (I) thiol depletion and (II) reduced concentration of some components of "multiple agonist"-induced aggregation in most *in vitro* models.

[2.2.3] Mechanism of inhibition

Nitric oxide

As described in section A.11 of chapter 1, nitric oxide derived from endothelial cells and platelets or from exogenous sources, suppresses platelet activation via a number of ways. It is now well established that nitric oxide (Moro *et al.*, 1996; Nguyen *et al.*, 1991) via the direct interaction with guanylate cyclase, stimulates cGMP production (Nguyen *et al.*, 1991) in human platelets. This leads to activation of cGMP-dependent protein kinase (Geiger *et al.*, 1992) and promotion of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) dependent refilling of Ca^{2+} stores, preventing platelet aggregation (Trepakova *et al.*, 1999).

As described in section A.8.1 of chapter 1, platelet activation is associated with the activation of the phosphoinositide 3-kinase pathway. Studies performed by Zhang *et al* (1996), suggested that the p85/PI3-kinase is integral to the activation of GPIIb/IIIa and hence platelet aggregation. Pigazzi *et al* (1999), utilizing *S*-nitroso-glutathione as a source of nitric oxide demonstrated that it prevents stimulation of PI3-kinase associated with tyrosine-phosphorylated proteins and the p85/PI3-kinase that is associated with LYN following platelet activation with thrombin receptor-activating peptide.

Furthermore, nitric oxide or peroxynitrite (formed via the interaction of nitric oxide with superoxide), is also capable of inhibiting platelet function through its direct actions on platelet 12-lipoxygenase and cyclooxygenase-1 (Boulos *et al.*, 2000; Fujimoto *et al.*, 1998). Inhibition of cyclooxygenase-1 effectively reduces the amount of TxA_2 production by inhibiting the conversion of arachidonic acid to the prostaglandins G_2/H_2.

[2.2.4] Platelet hyper-aggregability

Acute coronary syndrome

Increased platelet aggregability and the systemic secretion of platelet products (TxA_2) has been demonstrated by a number of investigators in subjects with an acute MI or UAP (Becker *et al.*, 1994; Fitzgerald *et al.*, 1986; Holmes *et al.*, 2000; Langford *et al.*, 1996). The apparent increase in platelet reactivity in subjects with ACS may account in part for the propensity of these subjects to have progressive or recurrent thrombosis (Ault *et al.*, 1999; Holmes *et al.*, 1999; Trip *et al.*, 1990a). See also section C.8.2 of chapter 1.

Stable angina pectoris

As described in section C.8.1 of chapter 1, the phenomenon of platelet hyper-aggregability/reactivity is not limited to subjects experiencing an ACS. Chirkov *et al* (1993), whilst investigating the anti-platelet properties of NTG demonstrated that platelets from patients with SAP who had not received prophylactic nitrates for at least 24-hrs prior to sampling, were significantly hyper-aggregable to ADP compared to blood samples from a cohort of NVs. The mechanisms responsible for increased platelet aggregability/activation remain uncertain but may be explained in part by the observations made by Diodati *et al* (1992). Marked platelet activation was demonstrated across the coronary vascular bed in response to rapid atrial pacing in patients with SAP and significant coronary stenosis, a result

that was absent either at rest or in subjects without significant disease. These observations were later confirmed by Tokuue *et al* (1996). However, it is questionable whether localized coronary venous evidence of platelet activation following tachycardia-induced ischaemia can account for finding of hyper-aggregability in peripheral venous samples, especially in the absence of recent symptomatic ischaemia. Interestingly, analogues data have been presented to suggest that this is indeed the case for neutrophil activation in patients with unstable coronary syndromes (Buffon *et al.*, 2002).

[2.2.5] Platelet hypo-responsiveness towards nitric oxide / nitro-vasodilators

Chirkov *et al* (1993), whilst investigating the *in vitro* and *ex vivo* anti-aggregatory effects of NTG, observed that platelets from patients with SAP exhibited significantly reduced sensitivity to the disaggregatory effects of NTG administered *in vitro*. Mean concentration of NTG associated with 50% reversal of platelet aggregation in PRP samples obtained from healthy volunteers was $1.4 \pm 0.2 \times 10^{-6}$M, contrasting to that in samples obtained from a series of SAP patients [$1.2 \pm 0.3 \times 10^{-4}$M] (Chirkov *et al.*, 1993). Moreover, the relative increase in intra-platelet cGMP post administration of NTG was significantly less in platelets obtained from SAP patients compared to that of samples from NVs (Chirkov *et al.*, 1993). See also section E.1.4 of chapter 1.

The aforementioned results by Chirkov *et al* (1996) were obtained with NTG as the primary nitric oxide donor. These results were then confirmed utilizing a more direct donor of nitric oxide, SNP. Concentrations of SNP required to induce 50% reversal in the extent of platelet aggregation were significantly greater in a cohort of SAP patients compared to that of a cohort of NVs. Moreover, SNP produced less pronounced elevation of the intra-platelet cGMP concentration within samples from subjects with SAP compared to controls. However, this decrease in cGMP production was not related to an impairment of the platelet guanylate cyclase (Chirkov *et al.*, 1996). The above findings are of particular importance as aggregation experiments were performed in PRP with presumably far lower concentrations of superoxide than those present in whole blood samples.

Investigations by others have also demonstrated reduced platelet responsiveness to the anti-aggregatory effects of nitric oxide donors in platelets from patients with hypertension (Woods

et al., 1993), diabetes/obesity (Anfossi *et al.*, 1998; Giugliano *et al.*, 1995) or post vigorous exercise in healthy NVs (Sakita *et al.*, 1997).

The frequent association of platelet hyper-aggregability (section 2.2.4) with the phenomenon of an attenuated platelet responsiveness to donors of nitric oxide (Chirkov *et al.*, 1993; 1996) and therefore endogenous sources, might contribute to an increased risk of platelet aggregation and intravascular thrombus formation observed in subjects with symptomatic and asymptomatic IHD.

Excessive production of ROS has been implicated in the pathogenesis of atherosclerosis and endothelial dysfunction by a number of investigators (Griendling *et al.*, 1994; Griendling *et al.*, 2000; Guzik *et al.*, 2000b; Guzik *et al.*, 2000c; Halliwell, 1989; Kunsch and Medford, 1999). Possible mechanisms for the observed platelet hyper-aggregability and also for platelet hypo-responsiveness to donors of nitric oxide might include the scavenging of nitric oxide by the ROS superoxide.

[2.2.6] Superoxide: Mechanisms of formation

The superoxide anion, the primary source of many ROS, is formed by the univalent reduction of the triplet-state molecular oxygen ($^3O_2$), in a process that is mediated by enzyme systems that include NAD(P)H oxidase (reviewed in section A.3.2.1 of chapter 1), nitric oxide synthase, xanthine oxidase, lipoxygenase, cyclooxygenase, peroxidases and other haemoproteins (Babior, 1999; Droge, 2002; Cai and Harrison, 2000). Superoxide can also be generated non-enzymatically by the semi-ubiquinone compound of the mitochondrial electron transport chain (Raha *et al.*, 2000; Raha and Robinson, 2000).

[2.2.7] Superoxide: Effects on platelet physiology

There is extensive evidence within the literature illustrating ROS, especially superoxide, have a potent pro-aggregant function (Caccese *et al.*, 2000; Iuliano *et al.*, 1991; Leo *et al.*, 1997; Pratico *et al.*, 1993) (see review, Chapter 1, sections C.12.1.1). Furthermore, evidence exists demonstrating that neutrophils are a major source of superoxide that may influence platelet behavior (Pratico *et al.*, 1993). In a feedback dependent mechanism, platelet activation triggers release of superoxide from neutrophils (Nagata *et al.*, 1993; Tsuji *et al.*, 1994), further potentiating the extent of platelet aggregation. Moreover, platelets have the ability to

produce superoxide themselves (Caccese *et al.*, 2000; Freedman and Keaney, 1999; Marcus *et al.*, 1977). Western blot analysis of proteins derived from platelets demonstrated the presence of $p22^{phox}$ and $p67^{phox}$ (Seno *et al.*, 2001), components of the NAD(P)H oxidase enzymatic system responsible for superoxide generation.

[2.2.8] Detection systems for superoxide

Numerous techniques to date have been developed to quantify ROS; for reviews see Nakano, Tarpey and Fridovich, and Togashi (Nakano, 1998; Tarpey and Fridovich, 2001; Togashi *et al.*, 2000). However, quantitation of superoxide is fraught with a number of important limitations. The survival of intra-cellularly formed superoxide is modulated by the actions of cytoplasmic and mitochondrial superoxide dismutases. Extracellularly, the balance between superoxides formation from plasma membrane-bound oxidases (NAD(P)H oxidase, xanthine oxidase; reviewed in section A.3.2 and C.4 of chapter 1) or release from anion channels and its degradation into other ROS, is also altered by extracellular-SOD and other anti-oxidants that include nitric oxide. The rate constant of superoxide reacting with SOD is $2.4 \times 10^{9} M^{-1}s^{-1}$ and $2 \times 10^{10} M^{-1}s^{-1}$ when reacting with nitric oxide to form peroxynitrite (Boulos *et al.*, 2000). Some superoxide detection systems are therefore required to compete with the aforementioned intracellular and extracellular superoxide scavengers' in-order to facilitate a correct estimation of the levels of superoxide.

Superoxide detection systems can be categorized into methods that either detect the actual presence of superoxide, or those that detect where it once was ("foot printing"). A description of some of the commonly utilized techniques is provided below.

[2.2.8.1] Cytochrome c reduction

Reduction of ferricytochrome *c* by superoxide to produce ferrocytochrome *c* and oxygen has been used to measure quantities and rates of superoxide formation in a multitude of cell systems and enzyme preparations (Leoncini *et al.*, 1991; Levine *et al.*, 1981; Rubanyi *et al.*, 1991). However, like most superoxide detection techniques, several problems exist. Apart from superoxide, various other compounds, including ascorbate, gluthathione and the byproducts of an interaction between xanthine oxidase and quinones have been demonstrated to reduce ferricytochrome *c* (Tarpey and Fridovich, 2001). Therefore, measurements of superoxide made by cytochrome *c* reduction are potentially subject to falsely elevated results.

Conversely, reduced cytochrome *c* can be re-oxidized by cytochrome oxidases, peroxidases and other ROS such as hydrogen peroxide and peroxynitrite, further complicating the analysis of superoxide generation as detected by cytochrome *c* reduction (Thomson *et al.*, 1995).

[2.2.8.2] Electron-spin resonance and spin trapping

An alternative approach to the quantification of superoxide and other ROS is the use of electron-spin resonance (ESR), which examines the magnetic properties of the unpaired electrons within the ROS molecule (Tarpey and Fridovich, 2001). Like many detection techniques, quantification of ROS by ESR is confounded by their low concentrations and short half-lives. However, these limitations are overcome by the addition of molecules known as spin traps, that readily react with the respective radical species to form more stable products that have characteristic ESR signatures (Tarpey and Fridovich, 2001).

5,5-dimethylpyrroline-*N*-oxide (DMPO), a superoxide spin trap, has been used by investigators examining the role of superoxide in the metabolism of NTG (Dikalov *et al.*, 1998a), superoxide from nitric oxide synthase (Xia *et al.*, 1998b), and assessed the anti-oxidant properties of particular compounds (Ide *et al.*, 1999; Ikeda *et al.*, 2001). However, much like many other reputed quantifiers of superoxide, a number of compounds are capable of interfering with DMPO to give false estimates of the levels of superoxide. In the presence of ferric ions, DMPO has the ability to detect hydroxyl radicals (Makino *et al.*, 1990). More recently a molecule that has a greater specificity for superoxide has been developed (5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO)) and used by a number of investigators (Jackson *et al.*, 2002; Sorescu *et al.*, 2001; Vasquez-Vivar *et al.*, 1997). However, Rouboud *et al* (1997) compared the efficiency of superoxide trapping by DEPMPO to that of the cytochrome *c* reduction method. When total superoxide production was > 20μM, cytochrome *c* detected approximately 100% of the superoxide produced, while DEPMPO trapped only 60 to 70%.

Thus, like a number of other techniques that quantify ROS, spin traps also have the potential for a broader reactivity with a number of compounds, effectively influencing the result. The considerable cost associated with ESR spectrometers is another important limitation to the

widespread use of ESR/spin trapping as a technique for the detection and quantification of ROS.

[2.2.8.3] Chemiluminescence reactions

Over the years several compounds have been developed and used for the chemiluminescence detection of superoxide. Such compounds include lucigenin (*bis*-*N*-methylacridinium nitrate), luminol, coelenterazine [2 - (4 - hydroxybenzyl) - 6 - (4 - hydroxyphenyl) - 8 - benzyl - 3, 7 dihydroimidazol [1,2-α] pyrazin-3-one] and its analogs CLA (2-methyl-6-phenyl-3, 7-dihydroimidazol [1,2-α] pyrazin-3-one) and MCLA [2-methyl-6- (4-methoxyphenyl)-3,7-dihydroimidazo [1,2-α] pyrazin-3-one].

Lucigenin

Lucigenin derived chemiluminescence relies upon the univalent reduction of lucigenin^{2+} to lucigenin^{+}, followed by the reaction of lucigenin^{+} with superoxide to yield an unstable dioxetane (Liochev and Fridovich, 1998). Decomposition of the unstable dioxetane to acridone then leads to a quantifiable light emission (Faulkner and Fridovich, 1993).

LDCL has been widely used to assay the formation of superoxide from a variety of cellular preparations that include neutrophils (Ellis *et al.*, 2000; Gyllenhammar, 1987), macrophages, platelets (Caccese *et al.*, 2000; Leo *et al.*, 1997) and vascular tissue (Bhunia *et al.*, 1997). Lucigenin has also been used to assess the involvement of superoxide in various disease conditions, nitrate induced tolerance (Munzel *et al.*, 1995b) atherosclerosis (Sumi *et al.*, 2001; Warnholtz *et al.*, 1999) and endothelial dysfunction (Bauersachs *et al.*, 1999), and also a measure of intracellular superoxide dismutase activity (Liochev and Fridovich, 1997).

Like many other systems that are designed to quantify ROS, LDCL has a number of limitations with the most notable being the theoretical ability of the reduced lucigenin to act as a redox-cycling compound capable of reducing oxygen to superoxide and hence overestimating the rate of superoxide production (Liochev and Fridovich, 1998; Tarpey and Fridovich, 2001; Vasquez-Vivar *et al.*, 1997).

Given the enormous interest in finding and utilizing an inexpensive probe for the detection and quantification of superoxide, a number of investigators have countered the

aforementioned claims by illustrating an absence (or minimization) of "redox cycling" of lucigenin when used at low concentrations (typically < 20μM) (Li *et al.*, 1999d), or none at all irrespective of the lucigenin concentration, due to its relative reduction potential (Afanas'ev, 2001). According to Afanas'ev *et al* (2001), lucigenin is unable to take part in "redox cycling" with molecular oxygen because of its positive one-electron reduction potential, directly counteracting the arguments put forward by Spasojevic *et al* (2000). Moreover, rather than overestimating the rate of superoxide production due to "redox cycling", as described by Liochev and Fridovich (1998), Afanas'ev *et al* (2001) demonstrated that the reduced form of lucigenin decreases superoxide production due to competition with one-electron reduction of dioxygen to superoxide ion.

Despite the legitimate caveats associated with the use of LDCL as a method for the quantification of superoxide, numerous investigators are still utilizing this methodology (Brar *et al.*, 2002; Hathaway *et al.*, 2002; Li *et al.*, 2002; Wassmann *et al.*, 2002). However, the majority of investigators tend to use lower concentrations of lucigenin than those associated with the initially described methodology, approximately 5-10μM (Skatchkov *et al.*, 1999).

Other compounds

In the quest to develop a luminescence agent that fails to undergo "redox cycling", coelenterazine, a lipophilic luminophore, originally isolated from the coelenterate *Aequorea*, has been introduced and claimed to be better than other superoxide detecting luminescent probes (Tarpey and Fridovich, 2001; Tarpey *et al.*, 1999; Teranishi and Shimomura, 1997). Superoxide-stimulated chemiluminescence occurs upon oxidation of the coelenterazine to the acetamidopyrazine anion. The use of coelenterazine as a probe for superoxide has been limited thus far (Hink *et al.*, 2001; Souza *et al.*, 2000), but like so many other superoxide detection systems, coelenterazine is not entirely specific for superoxide, as it has also been demonstrated to detect peroxynitrite (Tarpey *et al.*, 1999), the reaction product resulting from the interaction of superoxide and nitric oxide.

[2.2.8.4] Protection by superoxide dismutase

A role for enhanced generation of superoxide within particular disease states has been inferred by a number of investigators when the addition of the superoxide scavenger

superoxide dismutase or alternative scavengers of superoxide, alters the effect seen in the absence of the antioxidant (Tarpey and Fridovich, 2001).

Superoxide dismutase exists as two separate classes of enzymes, one consisting of SODs with Cu^{2+} and Zn^{2+} at the active site (EC-SOD), whereas the other comprises of SODs with either Mn^{2+} or Fe^{3+} at the catalytic center, with the former being the mammalian cytosolic SOD (Beyer *et al.*, 1991; Fridovich, 1995).

Examples throughout the literature in which extracellular SOD was added to a cellular preparation in order to assess the role of superoxide within that system include studies attempting to determine the mechanism of tolerance towards NTG (Munzel *et al.*, 1995b) and endothelial dysfunction (Bauersachs *et al.*, 1999; Mugge *et al.*, 1991). Similar experiments have been performed utilizing SOD-mimetics (Haj-Yehia *et al.*, 1999). For example, treatment of streptozotocin-induced diabetic rats with tempol reduced vascular concentrations of superoxide, malondialdehyde and 8-epi-prostaglandin $F_{2\alpha}$. It also was demonstrated to restore vascular responses to acetylcholine and NTG in diabetics rats to levels observed among control rats (Nassar *et al.*, 2002).

A number of problems are associated with using this method of assessing the role of superoxide. Results generated through the use of superoxide dismutase may be misleadingly negative. Many of the studies have utilized CuZn-SOD that is only capable of functioning as an extracellular scavenger of superoxide and is unable to gain access to intracellular sites of superoxide production such as mitochondria.

Overcoming some of these logistic problems, a number of investigators have deliberately used models of over-expression of SOD in order to assess its protective effects against superoxide, in conditions were superoxide is said to play a significant pathological role. In a rat model of hypertension, over-expression of EC-SOD but not Mn-SOD resulted in improvements in the levels of detectable nitric oxide (Fennell *et al.*, 2002). In isolated endothelial cells over-expression of EC-SOD was shown to prevent endothelial cell mediated oxidation of LDL (Takatsu *et al.*, 2001).

[2.2.8.5] Enhancement of effect by inhibition of superoxide dismutase

Similar to the method outlined above, enhanced production/concentration of superoxide has been inferred when diminished superoxide dismutase activity results in an alteration in the variable being examined (Tarpey and Fridovich, 2001). Examples in the literature include studies utilizing various metal chelators such as diethyldithiocarbamate. Addition of diethyldithiocarbamate to aortic rings of hypercholesterolaemic rabbits, greatly enhanced LDCL (Mugge *et al.*, 1994). The addition of agents that inhibit SOD has also been used to determine if inhibition of CuZn-SOD mimics the mechanism of nitrate tolerance, implying a functional role for superoxide within the phenomenon (Munzel *et al.*, 1999).

[2.2.8.6] Malondialdehyde and isoprostane ("foot printing")

ROS react with a number of biological molecules that include proteins, lipids, nucleic acids and carbohydrates (Meagher and FitzGerald, 2000). The interaction of ROS with lipids resulting in lipid peroxidation and the formation of a semi stable end product serves as an alternative method for the detection and quantification of oxidative stress.

Malondialdehyde

A widely used index of lipid peroxidation is the measurement of malondialdehyde (MDA) by the thiobarbituric acid-reacting substance (TBARS) assay (Halliwell and Chirico, 1993). Examples of its use include as a measure of oxidative stress post MI (Arstall *et al.*, 1995), in subjects with CAD (Buczynski *et al.*, 1993), the extent of lipid peroxidation in hypercholesterolaemic patients (Chirico *et al.*, 1993) and has been used to examine the effectiveness of ACE-inhibition (Napoli *et al.*, 1999). Despite its use as a measure of lipid peroxidation, a number of important limitations are associated with the TBARS assay (Gutteridge, 1986). Aldehydes other than malondialdehyde have the ability to form chromogens that have the same absorbance at 532nm, artificially elevating the level of MDA (Kosugi *et al.*, 1987). Furthermore, amino acids and bilirubin are also reactive towards TBA (Meagher and FitzGerald, 2000). Finally, malondialdehyde production is not just a reflection of the extent of lipid peroxidation, but it is also a byproduct of cycloxygenase activity in platelets (Hamberg *et al.*, 1975), questioning further the clinical utility of MDA as an index of lipid peroxidation.

Isoprostanes

In much the same way as malondialdehyde is a marker of lipid peroxidation and general level of oxidative stress, so too are a group prostaglandins isomers, the isoprostanes. The clinical implications of their formation are reviewed in section C.12.2.1 of chapter 1. Isoprostanes are formed via the interaction of ROS with esterified arachidonate, with the most extensively studied being the 8-epiPGF$_{2\alpha}$ or 8-isoPGF$_{2\alpha}$. Given the measurement of the F_2 sub-group isoprostanes has been accepted as a sensitive and specific index of the extent of lipid peroxidation (Pratico *et al.*, 2001), these isoprostanes have been used by a number of investigators to asses *in vivo* oxidant stress in several human disease states (Davi *et al.*, 1999a; Mehrabi *et al.*, 1999; Moore *et al.*, 1995; Patrono and FitzGerald, 1997; Roberts and Morrow, 1997), and even post coronary angioplasty (Iuliano *et al.*, 2001).

Moreover, isoprostanes are not simply markers of the extent of lipid peroxidation and hence a level of oxidative stress:- they have also been demonstrated to possess potent biological effects that include potentiation of platelet activation/adhesion (Minuz *et al.*, 1998). A dose-dependent increase in platelet adhesion to fibrinogen- and plasma-coated micro-wells by 8-epi-PGF$_{2\alpha}$ (1 to 1000 nmol/L) was observed when resting platelets and thrombin-stimulated human platelets were used. The expression of glycoprotein IIb/IIIa was also significantly increased post exposure to 8-epi-PGF$_{2\alpha}$ (Minuz *et al.*, 1998).

Other lipid peroxidation products

By no means are malondialdehyde and 8-isoprostane F$_{2\alpha}$ the only lipid peroxidation products that have been used to assess the overall level of oxidative stress. Measurements of a variety of other aldehydes and conjugated dienes have also been used to assess the role of lipid peroxidation and hence ROS in particular disease states. For comprehensive reviews see Meagher and FitzGerald (2000), Subbanagounder *et al* (2000).

[2.2.9] Unresolved issues

The experiments performed within this chapter where designed to address a number of unanswered questions. Are the phenomena of platelet hyper-aggregability and reduced platelet responsiveness to the anti-aggregatory effects of nitric oxide donors, initially observed within a cohort of SAP patients, also present in subjects with an ACS? What is the effect of removal of the extracellularly generated superoxide on platelet activity? Utilizing

LDCL as a method for the quantification of superoxide, what is the relationship between superoxide, platelet hyper-aggregability and hypo-responsiveness to SNP?

The experimental work described in this chapter consists of three sections:

Section I: *A description of platelet hyper-aggregability and nitric oxide hypo-responsiveness in whole blood samples obtained from patients with acute and chronic ischaemic heart disease. This section also examines the effects of superoxide scavengers on platelet aggregation and responsiveness to nitric oxide.*

Section II: *Development of a detection system that assesses whole blood levels of superoxide prior to and post induction of platelet aggregation.*

Section III: *Examination of the relationship between the extent of platelet aggregation, platelet responsiveness to SNP and superoxide content in whole blood samples obtained from normal volunteers and patients with SAP or ACSs.*

[2.3] Platelet hyper-aggregability and hypo-responsiveness to donors of nitric oxide; the role of the superoxide anion

[2.3.1] Introduction

Platelet hyper-aggregability

Platelets have a critical role in normal haemostasis and thrombotic disorders (George, 2000). Formation of a platelet plug at the site of a ruptured atherosclerotic lesion is a component of the physiological mechanism behind UAP and acute MI (Davies, 1994). Platelet hyper-aggregability has been documented in several cardiovascular disease states (Ault *et al.*, 1999; Chirkov *et al.*, 1996; Chirkov *et al.*, 1995; Diodati *et al.*, 1992; Eto *et al.*, 1998) and has been proposed as a factor predisposing subjects towards future thrombotic events (Grande *et al.*, 1990; Meade *et al.*, 1985). It remains uncertain whether platelet hyper-aggregability is

correlated with the inflammatory reaction, which seems to antedate the appearance of symptoms in patients with acute coronary syndromes.

Platelet hypo-responsiveness to nitric oxide

One of the major limiting factors for the clinical utility of organic nitrates has been the induction of nitrate tolerance through continuous nitrate pharmacotherapy (Glasser, 1999; Munzel *et al.*, 1996a; Parker, 1992), the phenomenon and potential mechanism/s of which are described in detail within section D.2-D.4 of chapter 1. The phenomenon of nitrate tolerance has been documented largely at the vascular level (Bassenge and Zanzinger, 1992; Munzel *et al.*, 1996a; Sage *et al.*, 2000) and to a lesser degree at a platelet level (Chirkov *et al.*, 1997). Interestingly, a poor haemodynamic responsiveness to nitrates may also occur on a *de novo* basis (independent of any prior nitrate pharmacotherapy), a phenomenon which was described originally in patients with heart failure (Armstrong *et al.*, 1980; Magrini and Niarchos, 1980) but now has also been documented in subjects with CAD (Forstermann *et al.*, 1988) and in those with risk factors for CAD (Leung *et al.*, 1993).

Whilst investigating the *in vitro* and *ex vivo* anti-aggregatory effects of NTG, a diminished disaggregatory effect of NTG (and then also SNP) was observed in PRP samples from SAP patients (Chirkov *et al.*, 1993; 1996). The extent and potential mechanism/s of this phenomenon, which is apparently analogous to that of nitrate resistance described within the vasculature of subjects with heart failure or CAD, has not been investigated to date.

As described within section A.11 and D.1.3.2 of chapter 1, the anti-aggregatory effects of NTG and other nitrovasodilators are mediated via the actions of nitric oxide, which through its interaction with platelet guanylate cyclase leads to the generation of cGMP; cGMP dependent protein kinases that are responsible for Ca^{2+} retention and platelet inhibition (Anderson *et al.*, 1994b). While the effects of nitric oxide liberated from NTG occur largely via enzymatic thiol-dependent bioconversion, SNP is considered to be a more direct donor of nitric oxide (Ignarro *et al.*, 2002). Given the studies performed by Chirkov *et al.*, (1993; 1996) demonstrating a significantly reduced sensitivity of platelets from patients with SAP to both SNP and NTG, the phenomenon of platelet hypo-responsiveness to NTG/SNP reflects a reduced sensitivity to nitric oxide rather than to organic nitrates. Moreover, it was demonstrated that a decrease in platelet sensitivity to the anti-aggregatory effects of NTG and

SNP was also associated with a decrease in intra-platelet cGMP accumulation in response to these nitric oxide donors (Chirkov *et al.*, 1996; Chirkov *et al.*, 1993), indicating a reduced supply or effectiveness of nitric oxide may serve as the mechanism behind the phenomenon.

Role of superoxide

Superoxide readily reacts with nitric oxide, diminishing the bioavailability of nitric oxide. This interaction has served as a basis for a number of hypotheses addressing the mechanism/s behind such phenomena as nitrate tolerance (Munzel *et al.*, 1995b) and endothelial dysfunction (Cai and Harrison, 2000; Guzik *et al.*, 2000c; Warnholtz *et al.*, 2001). It may be hypothesized that a decreased platelet responsiveness to SNP and NTG relates to an increased clearance of nitric oxide by superoxide, concentrations of which or byproducts thereof, are known to be elevated within various cardiovascular disease states (Cai and Harrison, 2000; Meyer and Schmitt, 2000; Miller *et al.*, 1998; Pratico, 1999; Warnholtz *et al.*, 1999). An additional component of the postulated role of superoxide as a modulator of nitric oxide effects is its production in association with the biological effects of some organic nitrates (Dikalov *et al.*, 1998), molsidomine (Munzel *et al.*, 1995) and dysfunctional NOS (Munzel *et al.*, 2000).

Experimental study

The following study was designed to investigate further the phenomena of platelet hyper-aggregability and hypo-responsiveness to donors of nitric oxide. In blood samples obtained from a cohort of NVs, patients with SAP or ACS (UAP/NQAMI), the whole blood anti-aggregatory effects of both SNP and NTG were examined. Possible interactions between superoxide and the extent of platelet hyper-aggregability and platelet responsiveness to the nitric oxide donor SNP were also determined by examination of the effects of a superoxide/hydrogen peroxide scavenger (superoxide dismutase/catalase).

[2.3.2] Current study hypothesis

This study was designed to test the following *null* hypotheses in a cohort of subjects that included healthy normal volunteers, patients with SAP or an ACS:-

Primary:

- *Platelets from patients with SAP or ACS are not hyper-aggregable in response to ADP or hypo-responsive to the anti-aggregatory effects of SNP or NTG compared to those from normal volunteers.*

Secondary:

- *Addition of the superoxide radical scavenger, superoxide dismutase in combination with catalase, does not inhibit platelet aggregation or normalize platelet responsiveness to SNP in blood samples obtained from patients with SAP or ACS.*

[2.3.3] Methods

[2.3.3.1] Subjects

Studies were performed on blood samples obtained from the following groups:

- Healthy NVs (n = 37; 16 males and 21 females) aged 22 to 76 years; 46 ± 12 years (mean ± S.D) the majority of whom (80%) were not taking medication that may influence platelet aggregation. The remaining 20% of NVs were receiving aspirin pharmacotherapy at the time of blood collection.

- Patients with SAP (n = 44; 27 males and 17 females aged 39 to 78 years; 65 ± 10 years (mean ± S.D)) undergoing elective cardiac catherization and coronary angiography. In all cases at least one haemodynamically significant (> 50%) stenosis was present in a major coronary artery.

- ACS patients (n = 47; 29 males and 18 females aged 39 to 83 years; 68 ± 10 years mean ± S.D)) who were admitted for treatment of prolonged chest pain occurring at rest and were studied during the first few hours after admission. Eventual diagnosis was UAP (n = 34) or non Q-wave acute myocardial infarction (NQAMI) (n = 13).

For all patients a background medication profile was recorded at the time of recruitment with the clinical characteristics of the study cohort being displayed in Table 2.2. No patient was receiving an ADP or glycoprotein IIb/IIIa receptor antagonist at the time of study enrolment. Numbers of subjects used in individual experiments are indicated below (Results: Section 2.3.4). The study was approved by the North Western Adelaide Health Service Ethics of Human Research Committee with informed consent being obtained prior to study entry.

[2.3.3.2] Blood Sampling

Blood samples from patients undergoing cardiac catheterization were drawn prior to the commencement of the procedure, via a femoral arterial sheath. Blood samples obtained from other patients and NVs was by venesection from an antecubital vein. It has been shown previously that there is no arteriovenous difference in platelet function (Chirkov *et al.*, 1993; Diodati *et al.*, 1995). Blood was collected in plastic tubes containing 1:10 volume acid citrate anticoagulant (2 parts of 0.1M citric acid to 3 parts of 0.1M tri-sodium citrate) in order to minimize deterioration of platelet function during experiments (Kinlough-Rathbone *et al.*, 1983).

[2.3.3.3] Platelet Aggregation Studies

All experiments commenced within 5-10 minutes following blood collection. Whole blood platelet aggregation studies were performed using a dual-channel impedance aggregometer (Model 560, Chrono-Log). Tests were performed at 37°C and a constant stirring speed of 900rpm. Samples of whole blood were diluted 2-fold with physiological saline (0.9% NaCl) to a final volume of 1mL and pre-warmed for 5 minutes at 37°C. Aggregation was induced with adenosine 5'-diphosphate (ADP) (final concentration of 1μM). Platelet aggregation was monitored continuously for 7 minutes with the responses being recorded for electrical impedance in Ohms (RO-3 Rikadenki chart recorder). These methods have previously been described (Chirkov *et al.*, 1999; Willoughby *et al.*, 1998). SNP (10μM) and NTG (100μM) were added to samples 1 minute prior to the addition of ADP. SOD and catalase (final concentration of 300U/mL for both enzymes) were added immediately before SNP or NTG and 1 minute prior to the addition of ADP. The duration of incubations was estimated as those optimal in preliminary experiments (data not shown). The anti-aggregatory effects of the agents studied were normalized to the extent of ADP-induced aggregation. In control tests

physiological saline was added in appropriate volumes. Each test was performed in at least triplicate, with mean values calculated.

[2.3.3.4] Chemicals

Adenosine 5'-diphosphate (ADP) sodium salt, sodium nitroprusside (SNP), superoxide dismutase (SOD) (from bovine erythrocytes), catalase (from bovine liver) were purchased from Sigma (St.Louis, MO, USA). Physiological saline (0.9% NaCl) was purchased from Baxter Healthcare (Old Toongabbie, NSW, Australia). NTG was purchased from Fisons (Thornleigh, NSW, Australia).

[2.3.3.5] Statistical Analysis

Gaussian distributions of data were determined by the Kolmogorov-Smirnov test. Evaluation of the differences in platelet responsive to ADP, SNP and NTG between NVs and the cohorts of studied patients, was made utilizing ANOVA followed by Bonferroni's post hoc multiple comparison test. Differences between SAP and ACS patients for the numbers of subjects with particular coronary risk factors and pharmacotherapy with common anti-anginal medications were performed using Fisher's exact test. Significance of correlation was determined by regression analysis with linearity determined by a run test. Differences between correlation curves were examined by analysis of co-variance (ANCOVA). Statistically significant differences were limited to $p < 0.05$ or $p < 0.01$ with the results being expressed as a mean ± S.E.M unless otherwise indicated. Statistical analysis was performed using a combination of Excel (Microsoft Office 98), Graph Pad Instat 3.0a, Graph Pad Prism 3.0a a Macintosh version by Software MacKiev and GB-Stat 6.5 for Windows 2000.

[2.3.4] Results

[2.3.4.1] Clinical characteristics

The clinical characteristics of the SAP (n = 44) and the ACS (n = 47) patients examined in this study are summarized in Table 2.2. Proportions of male subjects, subjects with diabetes, hypertension, hypercholesterolaemia and the numbers of current smokers were similar across the cohorts examined.

Medication profile

Almost all patients with SAP or ACS were on multiple anti-anginal pharmacotherapy prior to study enrollment. There were no significant differences in the numbers of subjects that were being treated with aspirin, ACE inhibitors, statins, Ca^{2+} antagonists and β-adrenoceptor antagonists for the SAP and ACS patient cohorts. A significantly greater proportion of ACS patients were receiving prophylactic nitrate pharmacotherapy compared to the SAP (100% vs. 61%; Fishers exact test $p < 0.01$). Perhexiline pharmacotherapy was significantly more frequent in the SAP patients than the ACS patients (41% vs. 6%; Fishers exact test $p < 0.01$). The results summarizing comparisons between SAP and ACS patients with regards to numbers of coronary risk factors and specific anti-anginal pharmacotherapy are summarized in Appendix Table 1.

Table 2.2 Clinical characteristics of the SAP and ACS patients

Clinical Determinants	*Stable Angina Pectoris (n =44)*	*Acute Coronary Syndrome (n=47)*
Men / Females, n	27/17	29/18
Age (Mean ± S.D), years	65 ± 10	68 ± 10
Diabetes, n (%)	16 (36)	14 (30)
Hypertension, n (%)	25 (57)	22 (47)
Hypercholesterolaemia, n (%)	23 (52)	18 (38)
Current smokers, n (%)	4 (10)	10 (21)
Medications		
Aspirin, n (%)	38 (86)	39 (83)
Prophylactic nitrates, n (%)	27 (61)	47 (100) *
ACE Inhibition, n (%)	14 (32)	10 (21)
SH-donors, n (%)	10 (23)	15 (32)
Perhexiline, n (%)	18 (41) *	3 (6)
Statins, n (%)	10 (23)	8 (17)
Ca^{2+} Antagonists, n (%)	24 (55)	29 (26)
β-adrenoceptor antagonists, n (%)	13 (30)	10 (21)

*S.D. standard deviation; Diabetes non-insulin dependent diabetes mellitus. *Significant differences between the subject cohorts. For a further summary on the differences in proportions of risk factors or anti-anginal pharmacotherapies see Appendix table 1. In the SAP cohort, 10 (23%) of patients were receiving captopril and were therefore counted as receiving a SH-donor on top of ACE inhibition.*

[2.3.4.2] Platelet response to ADP

Platelet response to ADP (1μM) for the cohorts of subjects examined was assessed according to gender and aspirin pharmacotherapy with the results summarized in Table 2.3. Firstly, each subject cohort was examined to see if the data conformed to a Gaussian distribution. Within the NV subgroup, both the male and female subjects taking aspirin contained insufficient numbers to assess normality. However, data from those NVs not taking aspirin were normally distributed (Kolmogorov-Smirnov: Males no ASA KS = 0.11, p = ns; Females no ASA KS = 0.15, p = ns). Within the SAP subject populations there were also insufficient numbers of subjects within the male and female subgroups not receiving aspirin and within the female ACS cohort not on aspirin to examine normality. However, data within all the other subgroups were shown to be normally distributed (Kolmogorov-Smirnov; SAP: Females ASA KS = 0.15, p = ns; Males ASA KS = 0.12, p = ns. ACS patients Females ASA KS = 0.15, p = ns, Males ASA KS = 0.11, p = ns, no ASA KS = 0.11, p = ns).

Table 2.3 Platelet aggregability in response to ADP

	Normal Volunteers		*Stable Angina Pectoris Patients*		*Acute Coronary Syndrome Patients*	
Subjects	No ASA	ASA	No ASA	ASA	No ASA	ASA
Males	9.8 ± 1.0 (13)	7.8 ± 1.0 (3)	13.7 ± 1.0 (4)	8.6 ± 0.7 (23)	14.2 ± 4.1 (5)	10.9 ± 0.9 (24)
Females	11.8 ± 1.0 (16)	11.3 ± 1.2 (4)	12.5 ± 17.6 (2)	11.7 ± 0.8 (15)	17.3 ± 4.0 (3)	14.0 ± 1.0 (15)

Samples obtained from NVs, SAP patients and ACS patients. Patient groups were separated according to gender and to those receiving or not receiving aspirin pharmacotherapy (± ASA). Numbers of subjects are indicated in parentheses. Data for female SAP patients not receiving aspirin is displayed as the mean ± S.D because of the low sample size.

Having established that the data for the majority of the subject populations conformed to a Gaussian distribution, a 3-way ANOVA was performed in order to assess the differences in platelet aggregability across the subject populations, across genders and between those subjects receiving and not receiving aspirin. As displayed within the 3-way ANOVA contingency table, there was a significant difference in platelet aggregability between the subject groups (Bartlett's statistic = 19.3, p = 0.056). Platelets from female subjects were significantly more aggregable than those obtained from their male counterparts. Platelets from subjects treated with aspirin tended to be significantly less aggregable than those not treaded with aspirin. There was also no significant interaction between any of the determinants. Utilizing Bonferroni's post hoc multiple comparison test, it was found that

male SAP patients treated with aspirin were significantly less aggregable than female ACS patients that were treaded with aspirin ($p < 0.05$). No other significant differences between any other combination of determinant was found.

Table 2.4 Platelet aggregability across subject populations, genders and aspirin pharmacotherapy, three-way ANOVA contingency table

Determinants	*F*	*p*
Subject group	5.18	0.007
Gender	4.90	0.028
Aspirin pharmacotherapy	6.05	0.015
Interactions		
Subject group x gender	0.42	0.66
Subject group x aspirin pharmacotherapy	0.39	0.68
Gender x aspirin pharmacotherapy	0.86	0.35
Subject group x gender x aspirin pharmacotherapy	0.37	0.69

Subject group = NVs, SAP patients and ACS patients.

Platelet aggregability in ACS patients

Platelet response to ADP (1μM) in the cohort of ACS patients was assessed according to the final diagnosis of either UAP or a NQAMI. There were insufficient numbers of ACS patients enrolled in this study to separate platelet response to ADP according to both gender and aspirin pharmacotherapy for each subject cohort. Accordingly ACS patients were categorized by disease state and aspirin pharmacotherapy only (results summarized in Table 2.4). By 2-way ANOVA there was no significant difference in platelet response to ADP within the ACS cohort for patients diagnosed with UAP vs NQAMI ($F = 0.03$, $p = 0.87$) or between subjects treated with/without aspirin ($F = 2.6$, $p = 0.11$; Bartlett's statistic = 5.96, $p = 0.11$). There was also no significant interaction between these two determinants (Disease state x aspirin pharmacotherapy $F = 0.19$, $p = 0.66$).

Table 2.5 Platelet aggregability in response to ADP in UAP and NQAMI patients

	Unstable Angina Pectoris Patients	*Non Q-wave Myocardial Infarction Patients*
No Aspirin	15.0 ± 3.5 (6)	16.4 ± 6.7 (2)
Aspirin	12.3 ± 0.8 (28)	11.6 ± 1.4 (11)

Blood samples were obtained from ACS patients and were characterized according to final diagnosis (UAP or NQAMI) and aspirin pharmacotherapy (± ASA). UAP vs NQAMI ($p = 0.87$, $F = 0.025$) and ± aspirin ($p = 0.11$, $F = 2.6$) interaction ($p = 0.19$ $F = 0.66$).

[2.3.4.3] Inhibition of Platelet Aggregation by sodium nitroprusside and nitroglycerine

In vitro addition of either SNP (10μM) or NTG (100μM) inhibited platelet aggregation in blood samples obtained from NVs, patients with SAP and in ACS, but to different extents. A representative aggregogram depicting whole blood platelet aggregation in response to ADP (1μM) and platelet responsiveness to SNP (10μM) and NTG (100μM) from a male normal volunteer is shown in Figure 2.3.1.

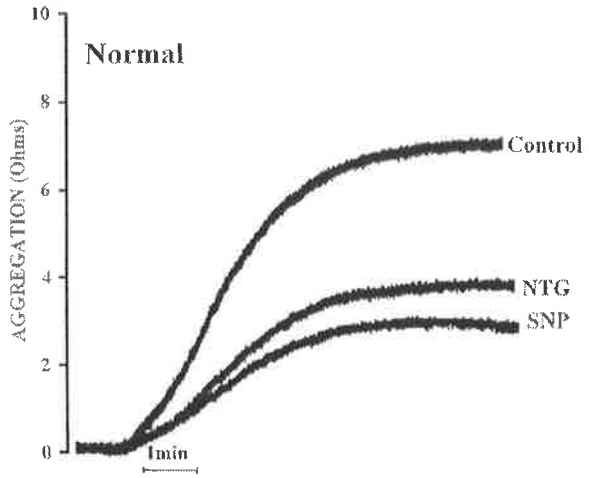


Figure 2.3.1 Platelet aggregability and responsiveness to donors of nitric oxide

Representative aggregation tracing for the degree of inhibition of ADP (1μM) induced aggregation by SNP (10μM) and NTG (100μM) in whole blood samples obtained from a male NV.

Platelet responsiveness to SNP

Having already established that there was a significant difference in platelet aggregability across the genders that are also influenced by a variable aspirin pharmacotherapy, platelet responsiveness to SNP was also assessed across the genders and for aspirin use.

Firstly and as displayed in Appendix Table 2, the majority of data populations conformed to a Gaussian distribution as determined using the Kolmogorov-Smirnov test. Then utilizing a 3-way ANOVA it was found that platelet responsiveness differed significantly between the disease states only. There was no significant difference between the genders, between those who were receiving and not receiving aspirin and across the various interactions of the three determinants. For a further summary of the levels of significance for each determinant see Appendix Table 3 (Bartlett's statistic = 13.9, $p = 0.23$). Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between any combination of determinant.

Given there was no significant influence on platelet responsiveness to SNP by gender and aspirin use, the results were pooled for each of the three subject populations. Pooled populations of data conformed to a Gaussian distribution (Kolmogorov-Smirnov: NV KS = 0.12, p = ns; SAP KS = 0.095, p = ns; ACS KS = 0.13, p = ns). The standard deviations between each subject cohort were also not significantly different (Bartlett's statistic = 1.47, p = 0.47).

SNP inhibited whole blood platelet aggregation by 51 ± 3.2% in samples obtained from the cohort of NVs. Mean platelet responsiveness to SNP in both the SAP and ACS patient cohorts (41.5 ± 3.2 and 30.6 ± 2.9 respectively) was significantly attenuated compared to that of the NV population (1-way ANOVA: $F = 9.8$, $p < 0.01$). Platelet responsiveness to SNP within the SAP cohort was significantly greater than that of ACS patients ($p < 0.05$ by Bonferroni's post hoc multiple comparison test) and significantly less than the mean platelet responsiveness obtained from the cohort of NVs ($p < 0.05$ by Bonferroni's post hoc multiple comparison test). Figure 2.3.2 illustrates the differences in platelet responsiveness to SNP (10μM) between the subject cohorts.

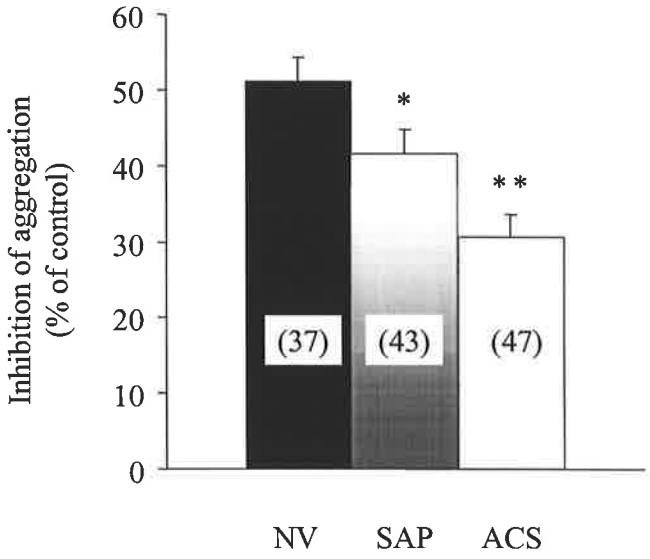


Figure 2.3.2 Platelet responsiveness to SNP

*Inhibition of ADP-induced platelet aggregation by SNP (10μM) in whole blood samples obtained from a cohort of NVs, patients with SAP and patients with ACS. Numbers of subjects analyzed in each group are indicated within the bars. 1-way ANOVA, F = 9.8, p < 0.01. Bonferroni's post hoc multiple comparison test: ** p < 0.01, * p < 0.05 vs. platelet responsiveness to SNP in NVs.*

Platelet responsiveness to NTG

The *in vitro* addition of NTG (100μM) was also shown to reduce the extent of platelet aggregation in whole blood in samples obtained from the cohorts of subjects examined. The influence of aspirin pharmacotherapy on platelet responsiveness to NTG (100μM) was not assessed as there were insufficient numbers of SAP subjects not receiving aspirin. Accordingly platelet responsiveness to NTG (100μM) was examined across the disease states (NVs, SAP patients and ACS patients) and gender. Utilizing the Kolmogorov-Smirnov test, all subject populations with sufficient numbers to perform the test, conformed to a Gaussian distribution. NVs female KS = 0.21, p = ns, males n = 4 (insufficient numbers); SAP patients female KS = 0.19, p = ns, males n = 3 (insufficient numbers); ACS patients females n = 4 (insufficient numbers), males KS = 0.23, p = ns.

By 2-way ANOVA there was a significant difference between the disease states and genders regarding platelet responsiveness to NTG (100μM). There was no significant interaction between the two determinants (2-way ANOVA: Disease state F = 6.1, p < 0.01; Gender F = 13.4, p < 0.01; Disease state x Gender F = 0.19, p = 0.83; Bartlett's statistic = 3.48, p = 063).

Utilizing Bonferroni's post hoc multiple comparison test, platelets from female ACS patients were found to be significantly less responsive to the anti-aggregatory effects of NTG (100μM) than those of their male counterparts (35.4 ± 4.7 (5) vs. 56.8 ± 4.4 (8) respectively, $p < 0.01$ by unpaired *t*-test). Results summarizing platelet responsiveness to NTG across the subject cohorts and between the genders are shown in Figure 2.3.3.

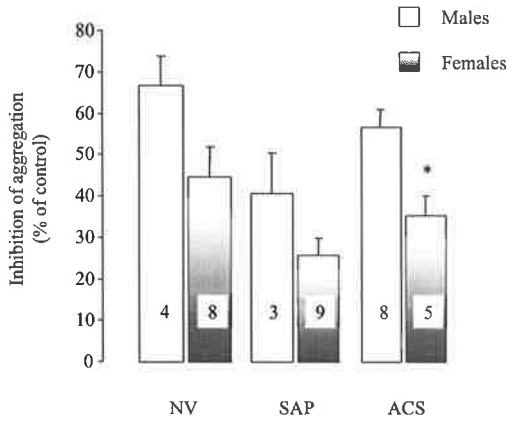


Figure 2.3.3 Platelet responsiveness to NTG

*Inhibition of ADP-induced platelet aggregation by NTG (100μM) in whole blood samples obtained from a series of NVs, patients with SAP and ACS. Platelet responsiveness to NTG was separated according to gender for each cohort of subjects examined. Numbers of subjects examined in each category are indicated within the bars. 2-way ANOVA; Disease state $F = 6.1$, $p < 0.01$, Gender $F = 13.4$, $p < 0.01$; Disease state x Gender $F = 0.19$, $p = 0.83$. Bonferroni's post hoc multiple comparison test: *$p < 0.01$ vs male platelet responsiveness to NTG (100μM).*

[2.3.4.4] Mechanism(s) of platelet hypo-responsiveness to donors of nitric oxide

Attenuated platelet responsiveness to nitric oxide may reflect increase clearance of nitric oxide by superoxide. To examine possible involvement of superoxide in the phenomenon of a reduced responsiveness to nitric oxide, the extra-cellular scavenger of superoxide, SOD, was used. In order to prevent any interference from hydrogen peroxide (H_2O_2), which is generated during SOD-catalyzed dismutation of superoxide (Fridovich, 1995), catalase was added in combination with SOD.

In a series of initial experiments to determine concentrations of SOD and catalase to utilize, the effect of SOD/catalase (90Units/ml or 300 Units/ml each) on the extent of ADP (1μM) induced platelet aggregation was examined in six subjects (2 SAP and 4 ACS patients). Data within each experimental cohort was demonstrated to conform to a Gaussian distribution (Control KS = 0.19, p = ns; SOD/catalase 90U/ml KS = 0.23, p = ns; SOD/catalase 300U/ml KS = 0.17, p = ns). All data populations were effectively matched for paired analysis (control

versus SOD/catalase 90U/ml, 300U/ml, $p < 0.01$). As shown in Figure 2.3.4 upper panel, SOD/catalase (90 Units/ml for each enzyme) had no significant effect on the extent of ADP (1μM) induced platelet aggregation (paired *t*-test $t = 1.57$, $p = 0.18$). In the same experimental samples, 300 Units/ml for each enzyme, significantly inhibited the extent of ADP (1μM) induced platelet aggregation (paired *t*-test $t = 7.8$, $p < 0.01$). Therefore 300 Units/ml for each enzyme was utilized in all subsequent experiments.

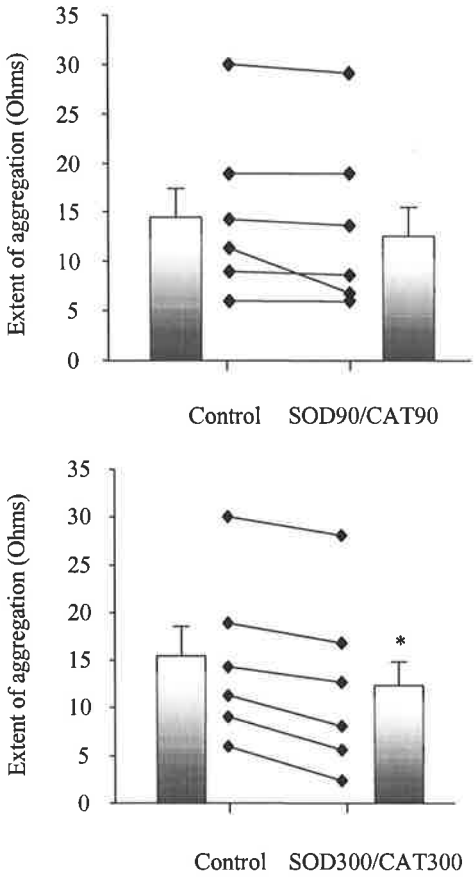


Figure 2.3.4 Effect of SOD/catalase on extent of ADP-induced platelet aggregation

Extent of ADP-induced (1μM) platelet aggregation in the presence of superoxide dismutase and catalase (SOD/CAT, 90U/mL upper panel and 300 Units/mL for each enzyme, lower panel) in six subjects (2 SAP patients and 4 ACS patients). Upper panel $p = 0.17$, lower panel $p < 0.01$ (paired t-test).

[2.3.4.5] Mechanism(s) of action

[2.3.4.5.1] Aggregability

The effects of SOD in combination with catalase (300Units/mL each) added 1 minute prior to the addition of ADP (1μM) were examined in 13 NVs (7 females, 6 males), 7 patients with SAP (3 females, 4 males) and 15 ACS patients (6 females, 9 males). Data within each subject cohort was found to be normally distributed (NV: control KS 0.13, p = ns, SOD/catalase KS

= 0.13, p = ns; SAP: control KS = 0.21, p = ns, SOD/catalase KS = 0.25, p = ns; ACS: control KS = 0.11, p = ns; SOD/catalase KS = 0.15, p = ns).

By 3-way repeated measures ANOVA (repeated measures was used as the control to SOD/catalase platelet aggregability comparison was paired) there was a significant difference in aggregability between genders and a trend towards a significant difference across the control to SOD/catalase treatment arm (Table 2.6; Bartlett's statistic = 4.2, p = 0.18). Interestingly there was no significant difference in platelet aggregability between the three subject populations, unlike that observed within section 2.3.4.2 ,table 2.3.

Table 2.6 Three-way repeated measures ANOVA contingency table

Determinants	*F*	*p*
Gender	13.3	< 0.01
Control/treatment	3.5	0.072
Subject	0.72	0.49
Interactions		
Gender x control/treatment	0.5	0.48
Gender x Subject	4.48	0.02
Control/treatment x Subject	0.42	0.65
Gender x Control/treatment x Subject	1.47	0.24

Control/treatment = platelet aggregability with ADP (1μM) alone and treatment = platelet aggregability in combination with SOD/catalase (300U/mL). Subjects NVs, SAP patients and ACS patients.

Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between any combination of points. However, by treating each subject population separately it was observed that the addition of SOD/catalase prior to induction of aggregation caused a number of different effects.

[2.3.4.5.2] Normal volunteers

By 2-way repeated measures ANOVA there was no significant difference in the extent of platelet aggregability between the genders. However, there were significant differences in the extent of aggregation between the control and treatment arms with a trend towards a significant interaction between the two determinants (2-way repeated measures ANOVA: Gender F = 0.09, p = 0.76; control/treatment group F = 12.4, p < 0.01; gender x control/treatment group F = 3.17, p = 0.098; Bartlett's statistic = 1.89, p = 0.59). Utilizing

Bonferroni's post hoc multiple comparison test, the extent of platelet aggregability in the presence of SOD/catalase for males was significantly reduced compared to their female counterparts, a result that goes some way to explain the significant change in platelet aggregability post SOD/catalase administration within this subject cohort. For a further illustration see Figure 2.3.5 upper panel.

[2.3.4.5.3] Stable angina pectoris patients

By 2-way repeated measures ANOVA, and as described for the NV cohort, there was no significant difference in platelet aggregability between the genders within the SAP cohort of patients. However, there was a significant difference in platelet aggregability post administration of SOD/catalase, with no significant interaction between the two determinants (2-way repeated measures ANOVA: Gender $F = 1.85$, $p = 0.23$; Control/treatment group $F = 15.8$, $p = 0.011$; gender x control/treatment group $F = 0.0009$, $p = 0.98$; Bartlett's statistic = 5.09, $p = 0.16$). Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between any combination of data points. For a further illustration see Figure 2.3.5 middle panel.

[2.3.4.5.4] Acute coronary syndrome subjects

By 2-way ANOVA and unlike the results obtained for the NVs and SAP patient cohorts, there was a significant difference in platelet aggregability between the genders and between the two determinants of the ACS subject population. However, there was no overall significant difference between the control and treatment group regarding the effects of SOD/catalase on platelet aggregability (2-way repeated measures ANOVA: gender $F = 6.4$, $p = 0.027$; control/treatment group $F = 0.12$, $p = 0.73$; gender x control/treatment group $F = 9.9$, $p < 0.01$; Bartlett's statistic = 7.3, $p = 0.063$). Utilizing Bonferroni's post hoc multiple comparison test there was also no significant difference between any combination of data points.

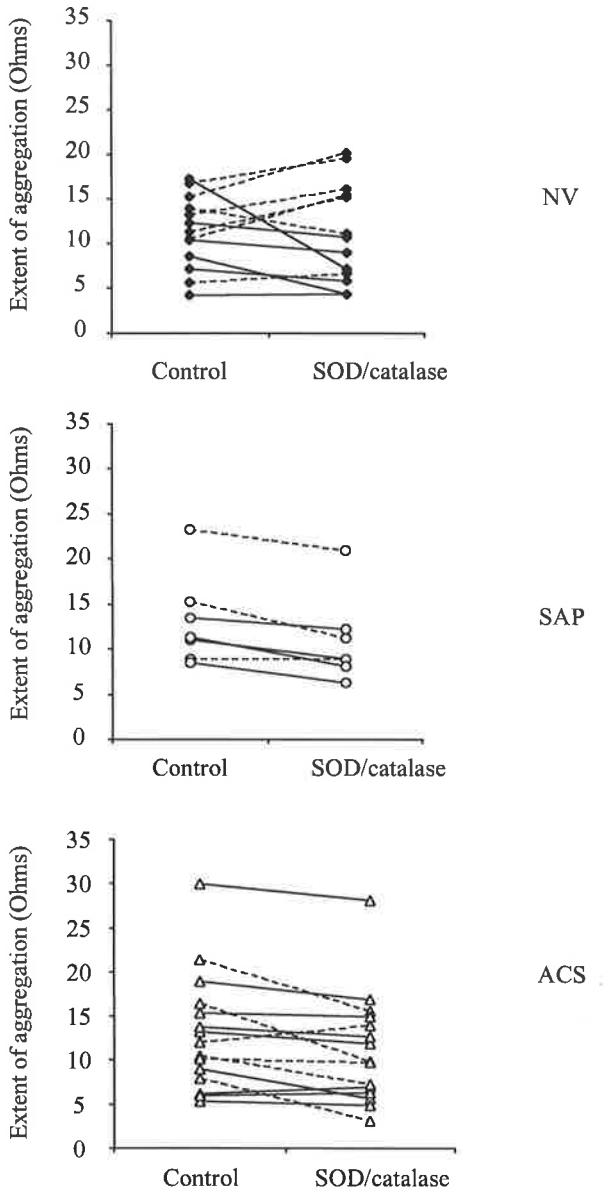


Figure 2.3.5 Effect of SOD/catalase on the extent of ADP-induced platelet aggregation

The extent of ADP (1μM)-induced platelet aggregation in the presence of SOD/catalase (300U/mL) for a cohort of NVs upper panel; SAP patients, middle panel; ACS patients, lower panel. ***Upper panel****: NVs Gender F = 0.09, p = 0.76; control/treatment group F = 12.4, p < 0.01; gender x control/treatment group F = 3.17, p = 0.098;* ***Middle panel****: SAP patients Gender F = 1.85, p = 0.23; Control/treatment group F = 15.8, p = 0.011; gender x control/treatment group F = 0.0009, p = 0.98;* ***Lower panel****: ACS patients gender F = 6.4, p = 0.027; control/treatment group F = 0.12, p = 0.73; gender x control/treatment group F = 9.9, p < 0.01. Solid line = males; Dotted line = females.*

[2.3.4.5.5] Inter-relationship between platelet aggregability and the degree of change post administration of SOD/catalase

Having demonstrated that the addition of SOD/catalase has a significant *in vitro* anti-aggregatory effect on platelets from patients with SAP or an ACS, the relationship between change resulting from SOD/catalase administration compared with initial platelet aggregability, was examined. This relationship was examined in order to test the hypothesis that platelet hyper-aggregability is associated with high superoxide concentrations.

Considering patients with a history of angina (SAP/ACS) separately from the NV cohort no significant correlation was observed with the angina or NV cohort regarding the initial extent of platelet aggregation or change in aggregation post administration of SOD/catalase (regression analysis "angina": r = -0.32, p = 0.15, run test p = 0.79; NV r = -0.11, p = 0.73, run test p = 0.87; Figure 2.3.6: left panel = angina patients, right panel = NVs). This result suggests that superoxide has no role in the phenomenon of platelet hyper-aggregability, but rather a more general role in the regulation of platelet function in all patients with SAP or ACSs, as a significant inhibition of aggregation with SOD/catalase was only observed in these groups (Figure 2.3.5).

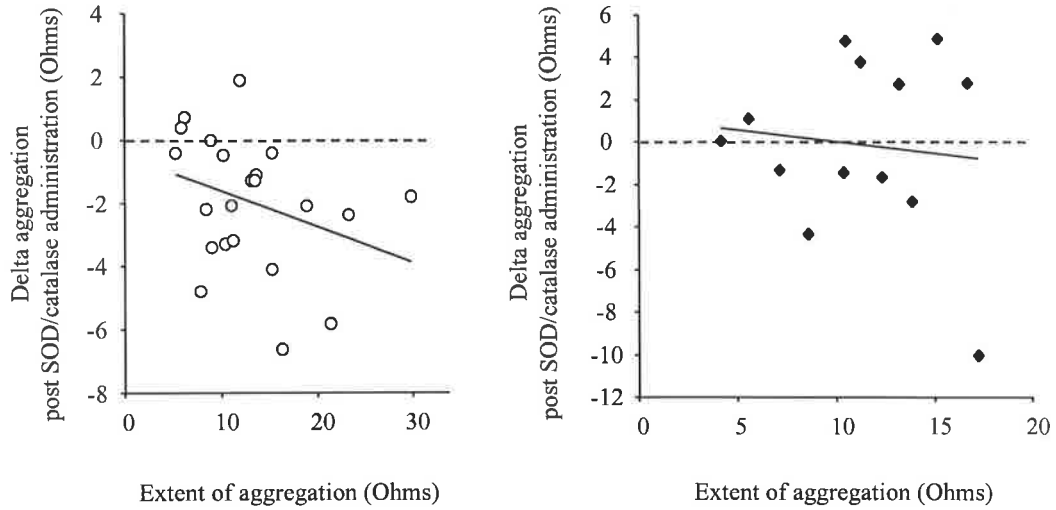


Figure 2.3.6 Extent of ADP-induced platelet aggregation correlated with change in platelet aggregation post addition of SOD/catalase

The relationship between the extent of platelet aggregability and change in aggregability post SOD/catalase administration was examined in subjects with a history of angina (left panel) and NVs (right panel). Regression analysis "angina" r = -0.32, p = 0.15, run test p = 0.79; NVs r = -0.11, p = 0.73, run test = 0.87. Dotted line indicates zero.

[2.3.4.5.6] Platelet responsiveness to SNP

Data populations representing the degree of platelet responsiveness to SNP (10μM) alone or in combination with SOD/catalase (300U/mL each enzyme) were firstly examined to see if they conformed to a Gaussian distribution. This was performed for all three subject groups (NVs n = 13, SAP n = 7, ACS n = 15) with all data populations being normally distributed. (NVs: SNP KS = 0.24, p = ns; SNP + SOD/catalase KS = 0.19, p = ns; SAP patients: SNP KS = 0.26, p = ns; SNP + SOD/catalase KS = 0.23, p = ns; ACS patients: SNP KS = 0.16, p = ns; SNP + SOD/catalase KS = 0.14, p = ns).

Having established that the data obtained conform to Gaussian distribution, a 2-way repeated measures ANOVA was performed. As displayed in Figure 2.3.7, there was no significant difference in platelet responsiveness to SNP post administration of SOD/catalase. However, there was a significant difference between the three subject populations, with no significant interaction between the two determinants (2-way repeated measures ANOVA: treatment group $F = 0.027$, $p = 0.87$; disease state $F = 12.8$, $p < 0.01$; treatment group x disease state $F = 0.45$, $p = 0.64$). Utilizing Bonferroni's post hoc multiple comparison test, platelet responsiveness to SNP within the ACS subject population was significantly less than that of the SAP subject cohort ($p < 0.05$).

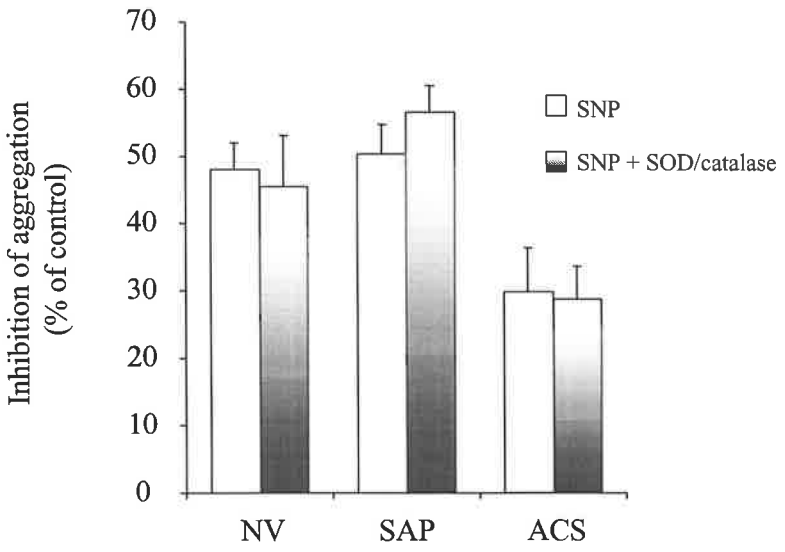


Figure 2.3.7 Effects of SOD/catalase on platelet responsiveness to SNP

The effects of SOD/catalase in combination with SNP (10μM) compared to SNP alone was examined in a series of NVs, SAP patients and ACS patients. 2-way repeated measures ANOVA: treatment group $F = 0.027$, $p = 0.87$; disease state $F = 12.8$, $p < 0.01$; treatment group x disease state $F = 0.45$, $p = 0.64$. Treatment group = SNP alone or SNP in combination with SOD/catalase.

[2.3.4.5.7] Inter-relationship between SNP responsiveness and the degree of change in SNP responsiveness post SOD/catalase administration

After demonstrating that the addition of SOD/catalase had no significant effect overall on the extent of platelet responsiveness to SNP in a range of subject cohorts, the ability of SOD/catalase to restore platelet responsiveness to SNP within a platelet sample was examined further. Accordingly baseline SNP responsiveness was examined to see if it correlated with change in platelet responsiveness to SNP post administration of SOD/catalase.

Treating patients with angina (SAP/ACS) separately from the cohort of NVs, a significant correlation was observed in the angina cohort between the baseline SNP responsiveness and the change in SNP responsiveness post administration of SOD/catalase (regression analysis: $r = -0.55$, $p = 0.0085$, run test $p = 0.74$; upper left panel of Figure 2.3.8). This result implies a bidirectional effect (lower panel of 2.3.8) of superoxide in determining the extent of platelet responsiveness to SNP. That is, inhibition of the NO response in subjects with a poor initial platelet responsiveness to SNP, but a relative augmentation in subjects with a normal platelet responsiveness to SNP (Unpaired *t*-test $t = 2.7$, $p = 0.012$). Data from the cohort of NVs demonstrated no correlation between baseline SNP responsiveness and change in SNP responsiveness post SOD/catalase administration (regression analysis: $r = 0.32$, $p = 0.28$, run test $p = 0.29$; upper right panel), indicating possible heterogeneity of superoxide's effect between patients with angina and NVs. However, only 4 NV subjects had initial SNP responses < 50 % inhibition.

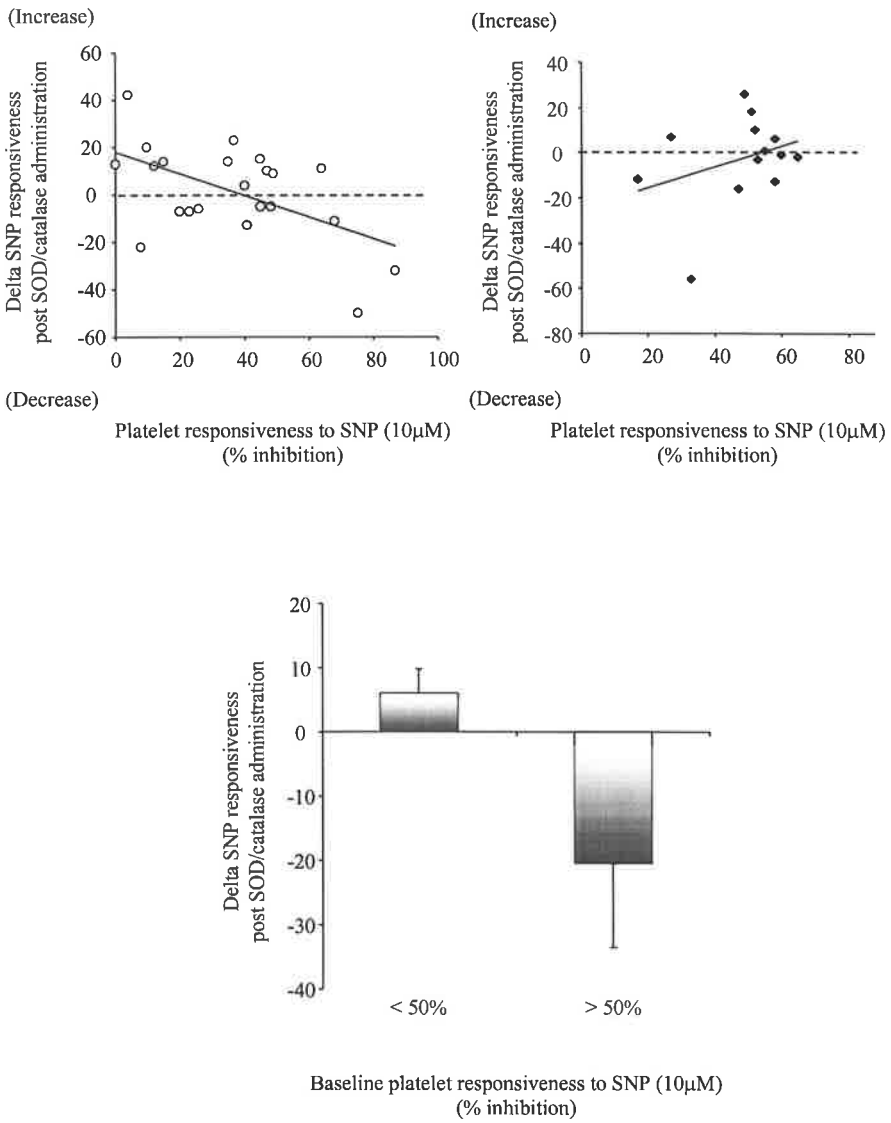


Figure 2.3.8 Platelet responsiveness to SNP correlated with change post SOD/catalase administration

The relationship between platelet responsiveness to SNP (10μM) and delta SNP responsiveness post SOD/catalase administration was examined in patients with angina (SAP/ACS) upper left panel and NV's upper right panel. Lower panel; Bidirectional effect of superoxide (Unpaired t-test t = 2.7,p = 0.012). Regression analysis angina patients: r = -0.55, p = 0.0085, run test p = 0.74; NV's r = 0.32, p = 0.28, run test p = 0.29). Dotted line = zero.

[2.3.5] Discussion

In the current study, platelets from patients with SAP or ACS were hyper-aggregable towards ADP (1μM) compared with those from a cohort of NVs. Furthermore, the addition of SOD/catalase to a platelet suspension from either SAP or ACS patients significantly inhibited platelet aggregation, a phenomenon that was not observed within the cohort of NVs.

Comparing the extent of platelet aggregation with the degree of change in platelet aggregation post SOD/catalase administration, no significant relationship existed for all subjects examined. These results suggest that superoxide plays a role in platelet aggregation in subjects with a history of angina rather than in the phenomenon of platelet hyper-aggregability as such.

The current study has also demonstrated that the *in vitro* anti-aggregatory effects of both SNP (10μM) and NTG (100μM) were significantly attenuated in both the SAP and ACS patient cohorts. This apparent platelet hypo-responsiveness towards donors of nitric oxide, representing a resistance towards their anti-aggregatory effects, was overall found not to be affected by scavenging of the superoxide radical. However, when the subject populations were divided into those with/without angina, a significant inverse relationship existed within the angina cohort for the extent of platelet responsiveness to SNP and the extent of change in SNP responsiveness post superoxide scavenging. A relationship that was not observed in the NV cohort. These results imply a significant role for superoxide in the phenomenon of nitric oxide resistance, such as was apparent among subjects with a history of angina pectoris.

Platelet hyper-aggregability

Phenomenon

As illustrated in Table 2.3, platelets from patients with either SAP or an ACS were hyper-aggregable to ADP (1μM), despite treatment with a number of anti-anginal agents known to possess anti-aggregatory effects. Furthermore, females and those subjects not undergoing aspirin pharmacotherapy were also hyper-aggregable compared to their male or aspirin consuming counterparts. A result that is agrees with the observations of others (Swahn and Wallentin, 1987). There was also no significant difference between subjects with a final diagnosis of either UAP or NQAMI regarding the extent of platelet response to ADP (1μM).

Mechanism/s

In the current study we examined whether platelet hyper-aggregability, observed within the cohort of SAP and ACS patients, resulted from the actions of superoxide/hydrogen peroxide. As described in section C.12 of chapter 1, there is considerable evidence throughout the literature demonstrating that ROS are associated with platelet hyper-aggregability (Iuliano *et al.*, 1997; Iuliano *et al.*, 1994; Iuliano *et al.*, 1991; Leo *et al.*, 1997; Pratico *et al.*, 1991).

In the current study scavenging of superoxide/hydrogen peroxide by SOD/catalase caused a significant inhibition of platelet aggregation within the SAP and ACS subject cohorts. The addition of SOD/catalase had no significant effect on the extent of platelet aggregation within the NV subject cohort (upper panel of Figure 2.3.5). By 2-way repeated measures ANOVA a significant difference between the control and treatment groups was observed. However, given there was no significant gender-related differences in the extent of platelet aggregation for this subject cohort, a paired *t*-test was performed with no difference between the control and SOD/catalase treatment arms being found (paired *t*-test $t = 0.94$, $p = 0.079$).

The addition of SOD/catalase to whole blood samples obtained from SAP patients was demonstrated to cause a significant inhibition of aggregation (middle panel of Figure 2.3.5). By 2-way repeated measures ANOVA a significant difference between the control and treatment groups was observed, a result that was confirmed using a paired *t*-test on the data from both male and female subjects (paired *t*-test $t = 4.4$, $p < 0.01$). Data demonstrating this phenomenon formed part of the published results (Chirkov *et al.*, 1999) demonstrating that the addition of SOD/catalase to a whole blood sample obtained from SAP patients causes a ~25% inhibition of platelet aggregation.

Unlike the results observed within the SAP patient cohort and by 2-way repeated measures ANOVA, there was no significant difference between the control and SOD/catalase treatment arms for the ACS patients (Lower panel of Figure 2.3.5).

Given the hypothesis that superoxide, serving as a pro-aggregant, was responsible for the phenomenon of platelet hyper-aggregability, one may assume that the extent of platelet aggregation would be a function of the degree of inhibition of platelet aggregation post addition of SOD/catalase. Combing the subject cohorts into those subjects with/without a history of angina pectoris (Figure 2.3.6), neither population showed a relationship between the baseline extent of platelet aggregation and the extent of change in platelet aggregation post administration of SOD/catalase. This result implies that superoxide is not directly involved in the phenomenon of platelet hyper-aggregability but rather it plays a critical role in platelet function in subjects with a history of angina pectoris, given that a significant inhibition of platelet aggregation with SOD/catalase was observed within the SAP and ACS patients (Figure 2.3.5), but not in the NV cohort.

Evidence throughout the literature suggests that platelets, apart from being affected by superoxide from various sources (Pratico *et al.*, 1993), have the ability to generate superoxide (Freedman and Keaney, 1999), whether by a NAD(P)H oxidase (Seno *et al.*, 2001) or arachidonic acid (Caccese *et al.*, 2000) dependent mechanisms. It therefore remains possible that components of the generation of superoxide, inducing platelet hyper-aggregability, are intra-platelet in origin. This hypothesis may therefore explain an absence of a significant relationship between the extent of platelet aggregation and the degree of change in platelet aggregability post (extracellular) superoxide scavenging. Investigating this hypothesis was beyond the scope of the current study. As described in the introduction of this chapter, EC-SOD scavenges superoxide only at an extra-cellular level (Fridovich, 1995). Given SOD/catalase was demonstrated to significantly inhibit the extent of platelet aggregation within samples obtained from subjects with CAD but not in NVs, the results suggest that extra-cellular superoxide plays a role in the phenomenon of platelet hyper-aggregability. Determination of the relative importance of superoxides function at an intra-platelet compared to an extracellular level in the phenomenon of platelet hyper-aggregability would require the utilization of a relatively selective intracellular superoxide "scavenging" system.

Indirect effects of superoxide causing platelet hyper-aggregability

Rather than viewing superoxide as having direct effects on platelets (either intra or extra-cellular) causing hyper-aggregability, the hypothesis that increased production of superoxide may scavenge endogenously derived (EDRF/NO) nitric oxide, effectively shifting the balance in favor of a pro-aggregatory environment, may also serve as a potential mechanism for platelet hyper-aggregability. An examination of this hypothesis is not possible with the current experimental design. However, there is evidence within the literature that removal of nitric oxide induces platelet hyper-aggregability and comes from experiments in which the NOS inhibitor *L*-N^G-nitro-arginine methyl ester (*L*-NAME) was added to a platelet preparation effectively depriving the platelet of nitric oxide (Freedman *et al.*, 1997). Platelets from NVs pre-incubated with *L*-NAME were significantly more aggregable than control platelets (Freedman *et al.*, 1997). Despite not examining the potential mechanisms behind a decreased nitric oxide release from activated platelets (oxidative stress was postulated as a mechanism within the discussion), Freedman *et al* (1998) also demonstrated that impaired platelet production of nitric oxide predicts the presence of an ACS.

As described within section C.12.1.4 of chapter 1, superoxide reacts with nitric oxide to generate peroxynitrite in a reaction that is faster than that with SOD, effectively removing nitric oxide from the system. However, evidence in the literature suggests that peroxynitrite can serve as both a pro-or anti-aggregant (Brown *et al.*, 1998; Moro *et al.*, 1994; Moro *et al.*, 1995; Yin *et al.*, 1995).

Platelet hypo-responsiveness to donors of nitric oxide (nitric oxide resistance)

On two separate occasions Chirkov *et al* (1993,1996) demonstrated a diminished ability of NTG and SNP to cause the reversal of platelet aggregation in samples obtained from patients with SAP in comparison to NVs. In the current study and utilizing whole blood samples from patients with SAP, the extent of platelet responsiveness to both SNP and NTG was significantly attenuated compared to that of NVs, when either nitric oxide donor was added prior to induction of platelet aggregation. Data from the SAP and NV cohorts was used to form part of a published study (Chirkov *et al.*, 1999). Extending these published observations further, the degree of *in vitro* platelet responsiveness to both SNP and NTG for platelets from ACS patients was also found to be significantly attenuated compared to that of platelets obtained from a cohort of NVs (Figure 2.3.3).

Role of superoxide in platelet hypo-responsiveness to donors of nitric oxide

The hypothesis that superoxide scavenges nitric oxide resulting in platelet hyper-aggregability may also serve as a potential mechanism for the phenomenon of platelet hypo-responsiveness to nitric oxide. Reduced responsiveness to nitric oxide due to the actions of superoxide has also served as a hypothesis explaining a dysfunction at the vascular level (Indik *et al.*, 2001; Lopez-Lopez *et al.*, 2001; MacCarthy *et al.*, 2001).

Within a cohort of NVs the addition of SOD/catalase in combination with SNP (10μM) to whole blood samples had no significant effect on the extent of inhibition of platelet aggregation (Figure 2.3.7). In blood samples from patients with SAP or ACS the addition of SOD/catalase also had no overall effect on the extent of platelet responsiveness to SNP (Figure 2.3.7). However, in SAP/ACS patients a significant inverse relationship was found between the degree of baseline SNP responsiveness and the extent of change in SNP responsiveness post SOD/catalase administration (Figure 2.3.8). Thus, a "poor" baseline platelet responsiveness to SNP was restored following superoxide scavenging, a phenomenon

that was not observed within the cohort of NVs. Moreover, in those patients with a history of angina pectoris and a normal baseline SNP responsiveness, the administration of SOD/catalase caused a reduction in the anti-aggregatory effects of SNP, implying a bidirectional effect of superoxide removal that produces no overall effect when each patient population is examined individually (Figure 2.3.7). Data from both the SAP and NV cohorts was used to form the basis of a published study demonstrating that the addition of SOD/catalase to samples from SAP subjects only, significantly increased the extent of the anti-aggregatory effect of SNP (Chirkov *et al.*, 1999).

Alternative mechanism/s

A decrease in the extent of platelet responsiveness to both SNP and NTG as demonstrated herein may also be explained by a defect within the nitric oxide/cyclic GMP pathway that governs the regulation of platelets.

As described in section A.11.1 of chapter 1, the generation and decomposition of intra-platelet cGMP is dependent on platelet guanylate cyclase and on cyclic nucleotide phosphodiesterases (Waldman and Murad, 1987). Chirkov *et al* (1999), whilst investigating the phenomenon of a reduced platelet responsiveness to SNP, demonstrated that the amount of intra-platelet cGMP generated post administration of SNP in platelet samples obtained from SAP patients, was significantly less than that of results from NV, results that were in agreement with previous observations (Chirkov *et al.*, 1996). This reduction in cGMP within platelets from patients with SAP was then demonstrated not to be a reflection of an increase in phosphodiesterase activity. Inhibition of phosphodiesterase activity with the use of IMBX did not restore the impaired cGMP response to SNP in platelets from patients with SAP (Chirkov *et al.*, 1999).

Investigating further the phenomenon of reduced platelet responsiveness to donors of nitric oxide, Chirkov *et al* (1999) went on to examine the interaction of guanylate cyclase with nitric oxide utilizing the guanylate cyclase inhibitor 1*H*-[1,2,4] oxodiazolo [4,3,α] quinoxalin-1-one (ODQ). ODQ (1μM) significantly reduced the anti-aggregatory effects of both NTG and SNP in whole blood samples from NVs, an observation that was not witnessed in blood samples from patients with SAP. These results were therefore interpreted to imply a decrease in the sensitivity of guanylate cyclase to nitric oxide in platelets from SAP patients.

A possible problem with this conclusion emerged with the discovery that ODQ was not a selective inhibitor of guanylate cyclase as once thought (Feelisch *et al.*, 1999). In a comprehensive examination of the effects of ODQ on heme-containing enzymes that included guanylate cyclase, Feelisch *et al* (1999) demonstrated that, apart from its guanylate cyclase inhibitory effects, ODQ inhibited both basal and stimulated endothelial nitric oxide production. The extent of this inhibition was virtually identical with that observed with the NOS inhibitor L-NAME. Moreover, there is a suggestion that ODQ may be metabolically converted to a more potent NOS inhibitor. Within the same series of experiments Feelisch *et al* (1999), demonstrated that ODQ also affected NTG and SNP-mediated vasorelaxation by inhibiting their reductive bio-activation via the cytochrome P_{450} enzyme system. Counteracting these arguments for non-specificity of ODQ effects is evidence that ODQ (1μM) alone failed to have any significant effect on the extent of ADP-induced platelet aggregation in platelet samples from either SAP patients or NVs (Chirkov *et al.*, 1999). Moreover, concentrations of ODQ required to elicit a significant reduction in NOS activity were found to be >30μM (Feelisch *et al.*, 1999).

cGMP-independent effects of nitric oxide

Within the study performed by Chirkov *et al* (1999), the addition of ODQ (1μM) in combination with either SNP or NTG was demonstrated to reduce the anti-aggregatory effect significantly but not completely. These results therefore suggest that a cGMP-independent effect of nitric oxide may be an important component of the anti-platelet actions of nitric oxide donors. As mentioned in section A.11.1.1 of chapter 1, nitric oxide has the ability to inhibit platelet function independent of its interaction with guanylate cyclase/cGMP.

Nitric oxide, through its interaction with superoxide, forms peroxynitrite that readily diffuses across the platelet cytosol and inhibits the formation and function of cyclooxygenase-1 and 2 by a nitrotyrosine-dependent mechanism (Boulos *et al.*, 2000). As described in section C.13.3, aspirin permanently acetylates both the cyclo-oxygenase-1/2 enzymes within platelets, thereby preventing the formation of TxA_2 (Vane and Botting, 1995). The results shown in the current study demonstrated that the use of aspirin, a known inhibitor of cyclo-oxygenase-1/2, was not a significant determinant of platelet responsiveness to nitric oxide either donated from SNP or NTG. It therefore seems unlikely that a significant contribution

of a dysfunction within the COX-1/2 pathway of cGMP-independent effects of nitric oxide serves as a major mechanism of a reduced responsiveness to nitric oxide at the platelet level.

[2.3.6] Study Limitations

The current study has a number of important limitations. Firstly the *in vitro* administration of either nitric oxide donor (SNP/NTG) and the apparent attenuation in platelet responsiveness towards these agents does not necessarily reflect accurately the extent of platelet resistance towards nitric oxide or donors there of *in vivo*.

The numbers of subjects utilized within this study were insufficient to delineate the relative contribution of common coronary risk factors and prescribed anti-anginal medications in the phenomena of platelet hyper-aggregability and platelet hypo-responsiveness to donors of nitric oxide. The latter forms the basis for a multivariate analysis performed within chapter 3 of this thesis. Potential risk factors that may influence the extent of platelet aggregation include hypercholesterolaemia (Aoki *et al.*, 1997b), diabetes (Davi *et al.*, 1990; Mandal *et al.*, 1993), smoking (Fusegawa *et al.*, 1999; Fusegawa and Handa, 2000) and hypertension (Lande *et al.*, 1987; Thomas *et al.*, 1992), all possible determinants that were evenly matched across the subject cohorts. Elevation of homocysteine levels may also be associated with impairment of tissue responses to nitric oxide (Tawakol *et al.*, 2002). The use of nitrates was not surprisingly more extensive within the ACS subject cohort. Despite this and evidence that organic nitrates are potent inhibitors of platelet function (Loscalzo, 1992), platelets from ACS patients tended to be significantly more aggregable than those from their SAP counterparts. Perhexiline use within the SAP subject cohort was also more common than in the ACS patient population. A direct anti-aggregatory effect of perhexiline has not been reported to date. However, there is evidence of increased bioavailability of nitric oxide with perhexiline therapy (Willoughby *et al.*, 2002).

The technique of ADP-induced whole blood impedance aggregometry precluded recruitment of ACS patients who were undergoing pharmacotherapy with either ADP or GPIIb/IIIa receptor antagonists. This was an important limitation of this study and one that effectively biased the ACS-subject population towards ACS patients that were being treated less aggressively.

Another important limitation of this study is the technique that scavenges superoxide and hydrogen peroxide through the use of SOD and catalase. A method used by many to assess the role of superoxide within particular disease states/conditions (Bauersachs *et al.*, 1999; Munzel *et al.*, 1995b), the addition of SOD/catalase is not an accurate method/measure of the relative contribution of superoxide. A more direct method of assessing the amount/contribution of superoxide to the above phenomena is explored within the following section of this chapter.

[2.3.7] Conclusions

Results reported in this chapter have demonstrated that platelets from patients with SAP or an ACS are hyper-aggregable and hypo-responsive to the anti-aggregatory effects of nitric oxide. Decreased platelet responsiveness to an exogenous source of nitric oxide implies diminution of responsiveness to endogenous nitric oxide. In turn diminution of responsiveness to nitric oxide may provide a potential basis for local or global increases in platelet aggregability associated with acute myocardial ischaemia and/or acute redox stress.

[2.4] Superoxide detection in whole blood

[2.4.1] Introduction

Reactive oxygen species

As described within chapter 1 section A.3.2 and C.3 evidence now exists suggesting that ROS play a functional role in the progression of the various cardiovascular disease states. The presence of superoxide and its reaction with nitric oxide to form peroxynitrite, effectively removing nitric oxide from the system, is now thought to contribute the phenomenon of endothelial dysfunction (Britten *et al.*, 1999; Harrison, 1997; Kanani *et al.*, 1999). ROS also play an integral role in the development of atherosclerosis, via oxidation of low-density lipoprotein (Jimi *et al.*, 1998) and are also thought to be responsible for the vascular smooth muscle cell proliferation associated with atherosclerosis development (Griendling and Ushio-Fukai, 1998). The generation and actions of ROS are also thought to play major roles in ischaemia/reperfusion injury (Ferrari *et al.*, 1998) and necrosis and/or apoptosis in cardiac myocytes (Anversa *et al.*, 1998).

Important ROS in mammalian cell systems include the superoxide anion ($O_2^{\cdot-}$), the hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl) and lipid radicals (Cai and Harrison, 2000; Droge, 2002; Iuliano *et al.*, 1997; Salvemini and Botting, 1993; Wattanapitayakul and Bauer, 2001). As the superoxide anion influences the subsequent formation of other ROS, a large amount of research interest has specifically focused on its role in various cardiovascular conditions/disease states.

Sources of reactive oxygen species in cardiovascular disease states

Potential enzymatic sources of superoxide include mitochondrial respiration, lipoxygenase, cyclooxygenase, cytochrome P_{450}'s, xanthine oxidase, nitric oxide synthase and the NAD(P)H oxidase enzyme system (Cai and Harrison, 2000; Droge, 2002).

As summarized in section A.3.2.1 of chapter 1 the activation of the NAD(P)H oxidase enzyme system, found in phagocytic cells (Babior, 1999) but also in the endothelium (Griendling *et al.*, 2000) and its subsequent generation of superoxide, has been implicated in the pathogenesis of endothelial dysfunction and vascular hypertrophy (Griendling *et al.*, 1994; Rajagopalan *et al.*, 1996; Wang *et al.*, 2001; Zhang *et al.*, 1999). Superoxide derived

from the endothelium may also play a role in the development of nitrate tolerance (Munzel *et al.*, 1995b). Released due to high intra-arteriolar pressure, superoxide reduces nitric oxide-mediated shear stress induced dilatation (Huang *et al.*, 1998). Moreover, in a canine model of *in vivo* platelet function Yao *et al* (1993) demonstrated a reduction in the frequency of cyclical flow variations following treatment with recombinant human copper-zinc SOD/human, manganese SOD or catalase. Infusion of xanthine-xanthine oxidase and/or hydrogen peroxide was demonstrated to induce these cyclical flow variations. Thus superoxide and nitric oxide exert opposite effects on cyclic platelet deposition in this model.

Platelets and superoxide

It was previously thought that platelets themselves produce superoxide in a continuous fashion that was independent of activation/aggregation (Marcus *et al.*, 1977). Baseline levels of superoxide, as detected by cytochrome *c* reduction and a nitroblue tetzolium assay, did not change upon exposure of platelets to various agonists. Freedman and Keaney (1999) determined that superoxide was released from platelets in an aggregation-dependent fashion, with Caccese *et al* (2000) recently demonstrating that superoxide and hydroxyl radical are released by aggregating platelets.

Numerous studies have investigated the influence of superoxide and other ROS on platelet adhesion/activation and aggregation (section C.12.1 of chapter 1). Salvemini *et al* (1989b) demonstrated that platelet activation is enhanced by incubation of platelets with sources of superoxide that included pyrogallol. Other ROS, such as hydrogen peroxide and hydroxyl radical, have also been shown to influence platelet activation/aggregation. Leo *et al* (1997) also demonstrated that platelets subjected to experimental models of ischaemia/reperfusion associated with oxidative stress undergo spontaneous aggregation.

Detection systems (Lucigenin)

Superoxide readily reacts with nitric oxide to form peroxynitrite with a rate constant of 2 x $10^{10}M^{-1}s^{-1}$. It also reacts with superoxide dismutase to generate H_2O_2 at a rate constant of 2.4 x $10^{9}M^{-1}s^{-1}$ (Boulos *et al.*, 2000). Given superoxide's limited life span, superoxide detection systems, as summarized within section 2.2.8 of this chapter, are required to compete with compounds that readily scavenge superoxide from cellular systems.

One such superoxide detecting compound that has been utilized by a number of investigators is the luminescent agent *bis N*-methylacridinium nitrate (lucigenin) (Caccese *et al.*, 2000; Gyllenhammar, 1987; Liochev and Fridovich, 1997; Munzel *et al.*, 1995b). Despite a number of controversies regarding its potential to undergo "redox cycling" and therefore elevating the level of superoxide (Liochev and Fridovich, 1998; Spasojevic *et al.*, 2000; Tarpey *et al.*, 1999; Vasquez-Vivar *et al.*, 1997), LDCL is still regarded widely as a reliable method of quantifying superoxide (Afanas'ev, 2001; Li *et al.*, 1999d).

Superoxide detection by lucigenin depends upon the reaction of superoxide with the reduced mono-cation radical of lucigenin (Faulkner and Fridovich, 1993). The *bis*-acridinium is reduced to the corresponding radical that is capable of reacting with superoxide to yield dioxetane which readily decomposes into two acridone compounds, one of which is electronically excited and emits a photon upon returning to a ground state (Faulkner and Fridovich, 1993).

In the previous section of this chapter the superoxide anion was demonstrated to play an important role in the phenomenon of whole blood platelet hyper-aggregability and to a lesser degree in hypo-responsiveness to nitric oxide. Most investigations examining the putative role of superoxide in modulating platelet function have been performed in isolated platelet preparations where contributions of superoxide from extra-platelet sources are largely excluded (Ambrosio *et al.*, 1994; Freedman and Keaney, 1999).

Utilizing lucigenin as a probe for superoxide the current study demonstrated:- 1) the presence of superoxide in an unstimulated whole blood sample; 2) generation and release of superoxide post induction of platelet aggregation. This study thereby highlights a method that allows further investigation of superoxide on platelet function in various clinical situations.

[2.4.2] Current study hypothesis

This study was designed to test the following *null* hypotheses in blood samples obtained from a cohort of normal volunteers.

Primary:

- *The administration of lucigenin to a whole blood sample obtained from a normal volunteer is associated with no detectable LDCL.*

Secondary:

- *Induction of platelet aggregation post administration of lucigenin fails to change the degree of detectable LDCL.*

- *There is no relationship between platelet aggregability and luminescent parameters.*

[2.4.3] Methods

[2.4.3.1] Subjects

Studies were performed on blood samples obtained from NVs not taking any medication that may influence platelet aggregation. Numbers of subjects used in individual experiments are indicated below (Results: Section 2.4.4). The study was approved by the North Western Adelaide Health Service Ethics of Human Research Committee. Written informed consent was obtained prior to study entry.

[2.4.3.2] Blood Sampling

Blood samples from NVs were collected and prepared according to the method described in section 2.3.3. All experiments were commenced within 5-10 minutes following blood collection.

[2.4.3.3] Preparation of platelets

For platelet-rich plasma studies, blood was centrifuged at 250*g* for 10 minutes at room temperature (RT) to obtain PRP. Platelet poor plasma (PPP) was prepared by further centrifugation of the remaining blood at 2500*g* for 20 minutes. Platelet counts were

performed on the STKS Coulter Counter (Coulter Electronics Inc) and the PRP was adjusted with PPP to a constant count of 250 000/μL.

Washed platelets were prepared by a method described previously (Iida *et al.*, 1993). Briefly, PRP was washed twice by centrifugation at 800*g* for 15 minutes (RT) in a solution containing 36mM citric acid, 5mM glucose, 5mM KCl, 90mM NaCl and prostaglandin E_1 (PGE_1) 1μM, pH 6.5. The final platelet pellet was then re-suspended in modified Tyrode's solution consisting of 11.9mM $NaHCO_3$, 0.555mM glucose, 2.68mM KCl, 137mM NaCl, 0.416mM NaH_2PO_4, 1mM $MgCl_2$ and 5mM Hepes, pH 7.35.

Blood samples devoid of platelets were prepared as follows. PRP was isolated as described above from a series of 10mL whole blood samples. The remaining red blood cell (RBC)/leukocyte fraction was further spun at 2500*g* for 10 minutes to remove any remaining PRP. PPP was then prepared from the PRP fraction as also described above. To the remaining RBC/leukocyte fraction either normal saline or PPP was added up to a final volume of 10mls.

[2.4.3.4] Preparation of neutrophils

Following removal of platelet-rich plasma, neutrophils were isolated by Lymphoprep density centrifugation. Briefly, HBSS was added in an equal volume to that of PRP removed. Lymphoprep was then underlain and spun at 500g for 30 minutes at RT. The neutrophil and RBC containing fraction was then washed twice with warm (30°C) lysis buffer containing 155.2mM NH_4Cl, 100μM Na_2EDTA dihydrate, 10mM $NaHCO_3$, pH 7.3 and spun at 500g for 10 minutes at room temperature. Neutrophils were then further washed twice with HBSS. Neutrophil counts were performed on the STKS Coulter Counter (Coulter Electronics Inc).

[2.4.3.5] Platelet Aggregation and Chemiluminescence assay for superoxide

Platelet aggregation and LDCL were monitored simultaneously using the dual channel Lumi-aggregometer (Model 560, Chrono-Log, Haverstown, PA, USA) equipped with a computer interface system (aggro/Link revision 4.71, Chrono-Log, Havestown, PA, USA) and a 486 IBM computer. Tests were performed at 37°C with a constant stirring speed of 900rpm. Samples to be tested were diluted 2-fold in physiological saline (0.9% NaCl) for whole blood and platelet-rich plasma, or modified Tyrode's for washed platelet preparations and HBSS for neutrophil preparations, to the final volume of 1mL and pre warmed for 5 minutes at 37°C. In

the whole blood samples lucigenin was added and chemiluminescence was monitored continuously until reaching a plateau approximately one-minute post lucigenin administration. Platelet aggregation for both whole blood and platelet-rich plasma was induced with either adenosine 5'-diphosphate (ADP) or adrenaline (final concentration as indicated).

[2.4.3.6] Aggregability and LDCL parameters

Platelet aggregation and LDCL were continuously monitored for 7 minutes. Platelet aggregation was recorded as electrical impedance in Ohms. LDCL was recorded in mV (milli-volts), as the maximum plateau amplitude 1 minute post lucigenin administration, and the maximum amplitude of the LDCL signal 7 minutes post platelet agonist addition (Baseline and aggregation-associated superoxide generation respectively; Figure 2.4.1). The rate of LDCL generation post induction of platelet aggregation was determined as the maximal slope of the luminescence curve post induction of platelet aggregation. The lag period between the induction of platelet aggregation and the commencement of the aggregation-associated increase in superoxide was recorded in seconds.

[2.4.3.7] Validation of the LDCL assay

Superoxide detection by lucigenin was verified by administration of superoxide dismutase (SOD) (300Units/ml). In control tests physiological saline (0.9% NaCl) was added in appropriate volumes. The effect of lucigenin on platelet aggregation was evaluated as a percentage, comparing the extent of maximal aggregation in the presence and absence of a range of concentrations of lucigenin.

[2.4.3.8] Chemicals

Adenosine 5'-diphosphate (ADP) sodium salt, superoxide dismutase (SOD) (from bovine erythrocytes), catalase (from bovine liver) was purchased as indicated in Section 2.3.3. bis-*N*-methylacridinium nitrate (lucigenin) was purchased from Sigma (St.Louis, MO, USA).

[2.4.3.9] Statistical Analysis

All data was firstly assessed for "normality" utilizing the Kolmogorov-Smirnov method. Log transformations of data were performed on non-Gaussian data. Differences between standard

deviations were examined utilizing Bartlett's statistic. Differences in the magnitude of the LDCL signal/rate and the lag period prior to the aggregation-associated release of superoxide for different concentrations of ADP was performed utilizing ANOVA followed by Bonferroni's post hoc multiple comparison test. Significance of correlation was determined by linear and non-linear regression analysis. Run tests were performed in order to assess linearity. Differences between regression curves were assessed using analysis of co-variance (ANCOVA). Statistically significant differences were limited to $p < 0.05$ with results being expressed as a mean ± S.E.M unless otherwise indicated. Statistical analysis was performed using the computer programs outlined in section 2.3.3.6 of chapter 2.

[2.4.4] Results

[2.4.4.1] Influence of lucigenin on the extent of platelet aggregation

Initially a series of experiments were performed to address the possible effects of lucigenin on the extent of ADP (1μM) induced platelet aggregation. Lucigenin's effect on the extent of platelet aggregation over a range of concentrations (12.5 – 250μM) was assessed in samples obtained from normal NVs (7 males, 3 females). Lucigenin was shown to both potentate and inhibit the extent of ADP (1μM) induced aggregation for certain concentrations of lucigenin. Data representing the effect of lucigenin on the extent of platelet aggregation were found to conform to a Gaussian distribution (Kolmogorov-Smirnov 12.5μM KS = 0.42, p = ns; insufficient numbers (n = 4) to test for normality within the 20 to 250μM data populations; Bartlett's statistic = 5.89, p = 0.21). By 1-way ANOVA there was a non-significant trend towards a variability in platelet responsiveness towards lucigenin over a range of lucigenin concentrations (1-way ANOVA F = 2.57, p = 0.075). Given the controversies regarding the use of lucigenin at high concentrations (Skatchkov *et al.*, 1999) and the observation that lucigenin at 12.5μM was shown to have less variable effects on the extent of platelet aggregation in these subjects and was also shown to detect both baseline and aggregation-associated superoxide release (below), this concentration of lucigenin was therefore used in all subsequent experiments in this section.

[2.4.4.2] Superoxide detection in whole blood pre and post platelet aggregation

When examining the effect of variable concentrations of lucigenin on ADP (1μM) induced platelet aggregation, lucigenin was shown to detect a baseline superoxide level ("Baseline

LDCL" Figure 2.4.1). The addition of ADP (1μM) 1 minute post lucigenin administration and following attainment of a steady state LDCL signal, induced both platelet aggregation and an aggregation-associated increase in superoxide ("Aggregation-associated LDCL" Figure 2.4.1). The addition of adrenaline (1μM) as an alternative platelet agonist, initiated both platelet aggregation and an aggregation-associated increase in superoxide, similar to that observed following ADP (1μM) administration (n = 3 utilizing lucigenin (125μM)). The aggregation-associated increase in LDCL peaked at approximately the same time of maximal aggregation (7 min for ADP 1μM). This aggregation-associated increase in superoxide level was shown to decay back towards baseline levels approximately 25 minutes post induction of platelet aggregation. As depicted in Figure 2.4.1, there was a consistent lag period of approximately 30 seconds between the induction of platelet aggregation and the commencement of the aggregation-associated increase in LDCL.

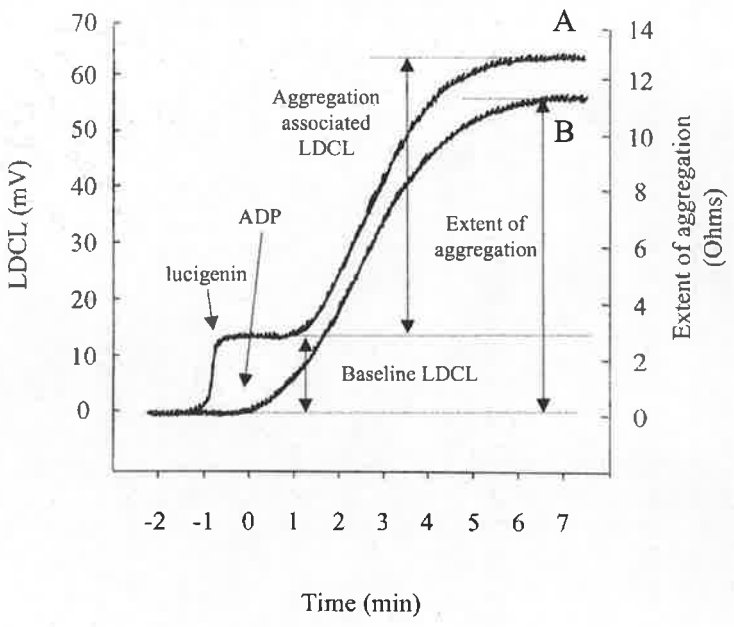


Figure 2.4.1 Superoxide detection in whole blood pre and post platelet aggregation

Representative tracings for baseline and aggregation-associated superoxide generation in a whole blood sample obtained from a healthy male subject. Administration of lucigenin (12.5μM) immediately detects baseline superoxide levels. Induction of platelet aggregation 1 minute post lucigenin administration by ADP (1μM) initiates further release of superoxide after a lag of approximately 30 seconds. Curve A denotes the luminescence trace and curve B denotes the aggregation trace.

[2.4.4.3] Validation of the superoxide detection and release post induction of whole blood platelet aggregation

In order to validate that the LDCL signals detected post lucigenin administration and induction of platelet aggregation were produced by superoxide, superoxide dismutase (SOD 300U/mL) was added to whole blood samples at various times throughout the reaction. As illustrated in the upper panel of Figure 2.4.2 there was no significant effect of SOD on the extent of ADP (1μM) induced platelet aggregation following the addition of SOD either 1 minute prior to the addition of lucigenin (solid line) or at the point of maximal release of superoxide post induction of platelet aggregation (approximately 7-minutes post ADP addition; dotted line). Confirming the hypothesis that extracellular superoxide was responsible for the observed increases in LDCL, the addition of SOD 1 minute prior to the administration of lucigenin or induction of platelet aggregation completely suppressed the baseline and aggregation-associated LDCL signals (solid line) (lower panel of Figure 2.4.2). Furthermore, the administration of SOD at the point of maximal aggregation-associated LDCL caused an immediate reduction in LDCL, to a level of that approaching the baseline LDCL signal (dotted line).

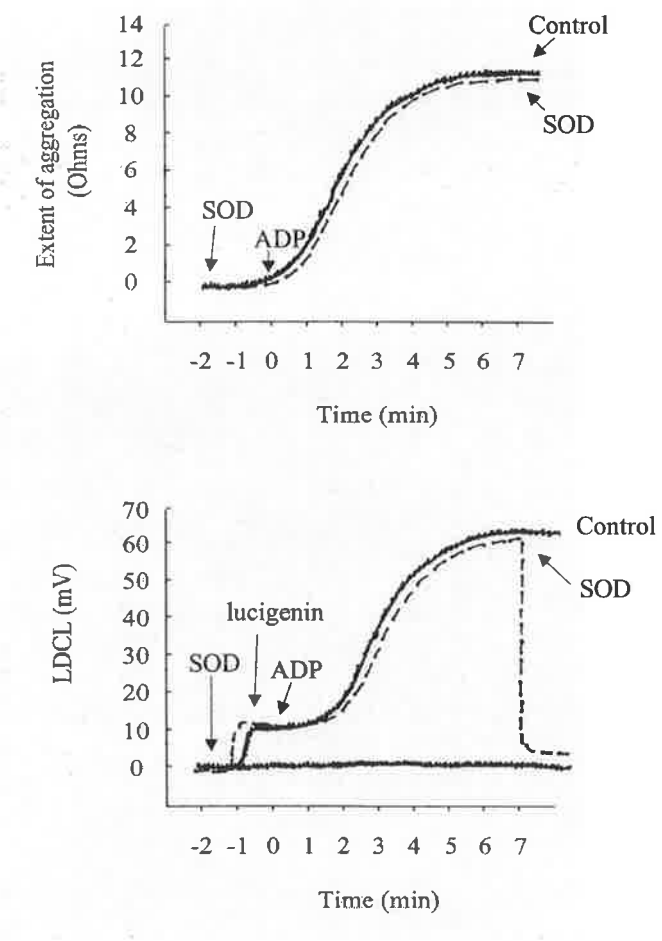


Figure 2.4.2 Inhibition of baseline and aggregation-associated superoxide generation by SOD

Upper panel: *Effects of SOD (300U/mL) on the extent of platelet aggregation when administered either prior to addition of lucigenin or at the point of maximal aggregation-associated LDCL (dotted line) (7-minutes post induction of platelet aggregation).* ***Lower panel****: Effects of SOD on LDCL either prior to administration of lucigenin that results in no significant LDCL signal or at the point of maximal aggregation-associated LDCL (dotted line), that results in a reduction of the LDCL signal. The "control" LDCL trace refers to the baseline and aggregation-associated LDCL generated in the presence of lucigenin and ADP but the absence of SOD.*

[2.4.4.4] Relationship between baseline and aggregation-associated LDCL

In a series of blood samples from eight males and four females, the relationship between the extent of baseline LDCL (lucigenin 12.5μM) and aggregation-associated LDCL (1μM) was examined. Given samples from both genders were examined and that the relationship between the baseline and the extent of aggregation-associated LDCL may differ significantly between subjects, the data were firstly analyzed by ANCOVA to determine if the relationship differed significantly between the individuals.

Utilizing ANCOVA there was no significant difference between subjects regarding the relationship between the baseline and aggregation-associated LDCL despite a trend towards one (ANCOVA $F = 1.98$, $p = 0.077$). Accordingly all the data were pooled and subsequently found to conform to a Gaussian distribution (Kolmogorov-Smirnov; Baseline LDCL KS = 0.12, p = ns; Aggregation-associated LDCL KS = 0.16, p = ns).

By regression analysis and as displayed in Figure 2.4.3 the extent of baseline LDCL correlated moderately with aggregation-associated LDCL (regression analysis $r = 0.54$, $p < 0.01$, run test $p = 0.095$).

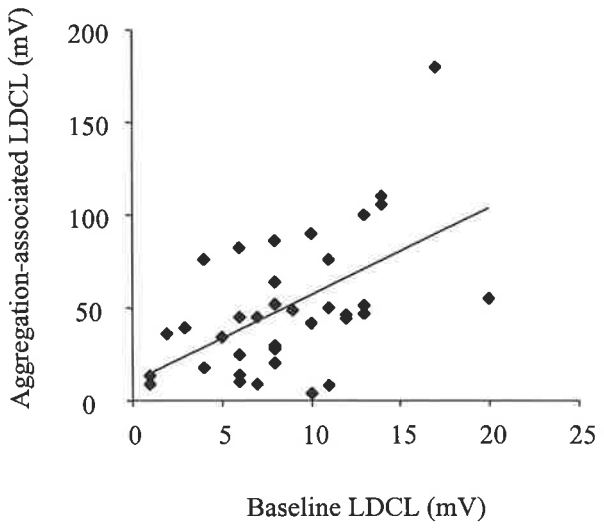


Figure 2.4.3 Relationship between the extent of baseline and aggregation-associated LDCL

The relationship between the extent of baseline and aggregation-associated LDCL was examined in samples from twelve NVs (8-male/4 female). By regression analysis $r = 0.54$, $p < 0.01$, run test $p = 0.095$.

[2.4.4.5] The relationship between the extent of platelet aggregation and the degree of aggregation-associated increase in LDCL

Within a series of blood samples obtained from five NVs (4-males/1 female) the relationship between the extent of platelet aggregation in response to a range of concentrations of ADP (0.5, 1, 2.5 and 5.0μM) and aggregation-associated LDCL was examined. The extent of platelet aggregation and aggregation-associated LDCL with each concentration of ADP was assessed at least in duplicate. As blood deteriorated with time, it was possible to process only four concentrations of ADP.

Data representing the extent of platelet aggregation and aggregation-associated LDCL were pooled according to the concentration of ADP used to initiate aggregation and then assessed for normality. Utilizing the Kolmogorov-Smirnov method all data populations representing either aggregability or LDCL conformed to a Gaussian distribution apart from the pooled population of ADP (0.5μM) induced platelet aggregation. (Kolmogorov-Smirnov: Aggregability 0.5μM ADP KS = 0.39, $p < 0.01$; 1μM ADP KS = 0.25, p = ns; 2.5μM ADP KS = 0.17, p = ns; 5.0μM ADP KS = 0.19, p = ns. LDCL 0.5μM ADP KS = 0.12, p = ns; 1μM ADP KS = 0.18, p = ns; 2.5μM ADP KS = 0.15, p = ns; 5.0μM ADP KS = 0.31, p = ns).

Given that the data for the ADP (0.5μM)-induced platelet aggregation failed to conform to a Gaussian distribution, the total data population, representing the extent of platelet aggregation to all ADP concentrations, underwent a log transformation. Post log transformation data for each ADP concentration conformed to a Gaussian distribution (Kolmogorov-Smirnov: Aggregability ADP 0.5μM KS = 0.32, p = ns; ADP 1.0μM KS = 0.17, p = ns; ADP 2.5μM KS = 0.19, p = ns; ADP 5μM KS = 0.27, p = ns).

The relationships between the extent of platelet aggregation and aggregation-associated LDCL for each concentration of ADP used did not significantly differ (ANCOVA F = 1.1, p = 0.35). Accordingly, all the data were pooled (n = 64). Upon pooling data representing the log transformed extent of platelet aggregation and the extent of aggregation-associated LDCL did not conform to a Gaussian distribution (Kolmogorov-Smirnov; log transformed extent of platelet aggregation KS = 0.21, $p < 0.01$; Aggregation-associated LDCL KS = 0.18, p = 0.037). Accordingly a Spearman rank correlation test for non-parametric data was performed

and as illustrated in Figure 2.4.3 a significant exponential relationship existed between the extent of log transformed platelet aggregation and aggregation-associated LDCL (Spearman rank = 0.67, $p < 0.01$).

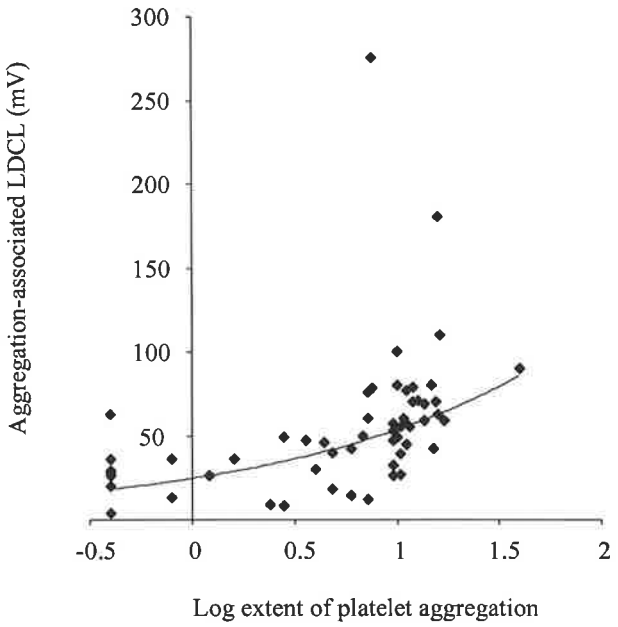


Figure 2.4.4 Platelet aggregability and aggregation-associated LDCL

The extent of platelet aggregation and aggregation-associated LDCL generated utilizing a range of concentrations of ADP (0.5 – 5.0μM) were examined in a series of blood samples obtained from a cohort of NVs (Spearman rank = 0.67, $p < 0.01$).

Aggregability and aggregation-associated LDCL:- ADP (1μM)

Having demonstrated that aggregation-associated LDCL is directly related to the extent of platelet aggregation, this relationship was examined further for the ADP (1μM) data population only. Utilizing ANCOVA the relationship between the extent of platelet aggregation and aggregation-associated LDCL for each of the five subjects analyzed were found not to significantly differ from each other (ANCOVA $F = 0.52$, $p = 0.72$). Accordingly all data were then pooled. By regression analysis, the extent of platelet aggregation was strongly correlated with aggregation-associated LDCL utilizing ADP (1μM) (Figure 2.4.4) (regression analysis $r = 0.79$, $p < 0.01$, run test $p = 0.7$).

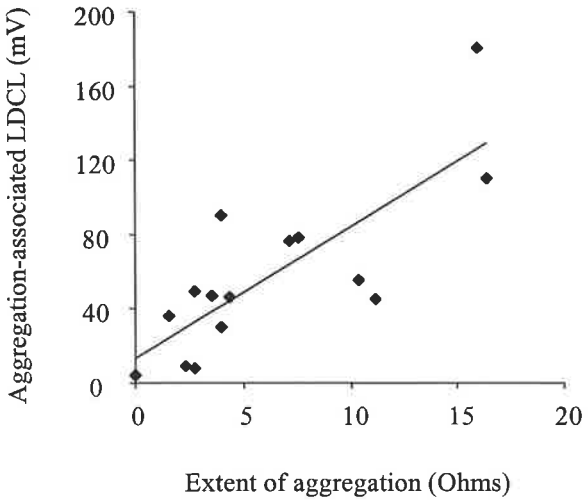


Figure 2.4.5 The extent of platelet aggregation and aggregation-associated LDCL

Within the ADP (1μM) sub-population of data the relationship between the extent of platelet aggregation and aggregation-associated LDC was examined. By regression analysis a significant positive relationship existed between the extent of platelet aggregation and aggregation-associated LDCL (regression analysis r = 0.79, p < 0.01).

[2.4.4.6] The relationship between the extent of aggregation-associated LDCL and the rate of superoxide generation.

Utilizing the aggro/Link (revision 4.7.1 Chrono-Log, Haverstown PA, USA) the rate of superoxide generation could be determined (measured as the maximal slope of the LDCL curve post ADP administration). Accordingly the relationship between the extent of aggregation-associated LDCL and the rate of its generation was examined within the cohort of five NVs. As indicated earlier (section 2.4.4.4), the aggregation-associated LDCL was initiated by the addition of ADP in a range of concentration (0.5 – 5.0μM). Therefore the relationship between the extent of aggregation-associated LDCL and the rate of its generation for the five NVs was firstly analyzed according to ADP concentrations used.

Data representing the extent of aggregation-associated LDCL and the rate of its generation conformed to a Gaussian distribution (Kolmogorov-Smirnov: Aggregation-associated LDCL shown in section 2.4.4.4; Rate of aggregation-associated LDCL 0.5μM ADP KS = 0.22, p = ns; 1μM ADP KS = 0.15, p = ns; 2.5μM ADP KS = 0.14, p = ns; 5.0μM ADP KS = 0.16, p = ns).

The relationship between the extent of aggregation-associated LDCL and the rate of its generation for each concentration of ADP used were shown not to significantly differ (ANCOVA F = 0.29, p = 0.83). Upon pooling that data from each ADP sub-population however, data representing the extent of aggregation-associated LDCL were shown not to conform to a Gaussian distribution (Kolmogorov-Smirnov, pooled population of aggregation-associated LDCL data KS = 0.18, p = 0.04; Rate of generation KS = 0.1, p = ns). Accordingly a log transformation of the pooled aggregation-associated LDCL data was performed (KS post log transformation = 0.14, p = ns).

Using non-linear regression analysis, the relationship between the log extent of aggregation-associated LDCL and the rate of its generation conformed to a sigmoid curve (upper panel of Figure 2.4.6).

Aggregation-associated LDCL and rate of generation:- ADP (1μM)

Having demonstrated that aggregation-associated LDCL strongly correlates with the rate of its generation this relationship was examined further within the ADP (1μM) sub-population. Utilizing ANCOVA, the relationship between the extent of aggregation-associated LDCL and the rate of its generation for each of the five subjects analyzed was not significantly different (ANCOVA F = 3.4, p = 0.065), therefore data from each subject were pooled. By regression analysis the extent of aggregation-associated LDCL induced by ADP (1μM) was shown to strongly correlate with the rate of its generation (regression analysis r = 0.95, p < 0.01, run test p = 0.077). For a further summary see the lower panel of Figure 2.4.6.

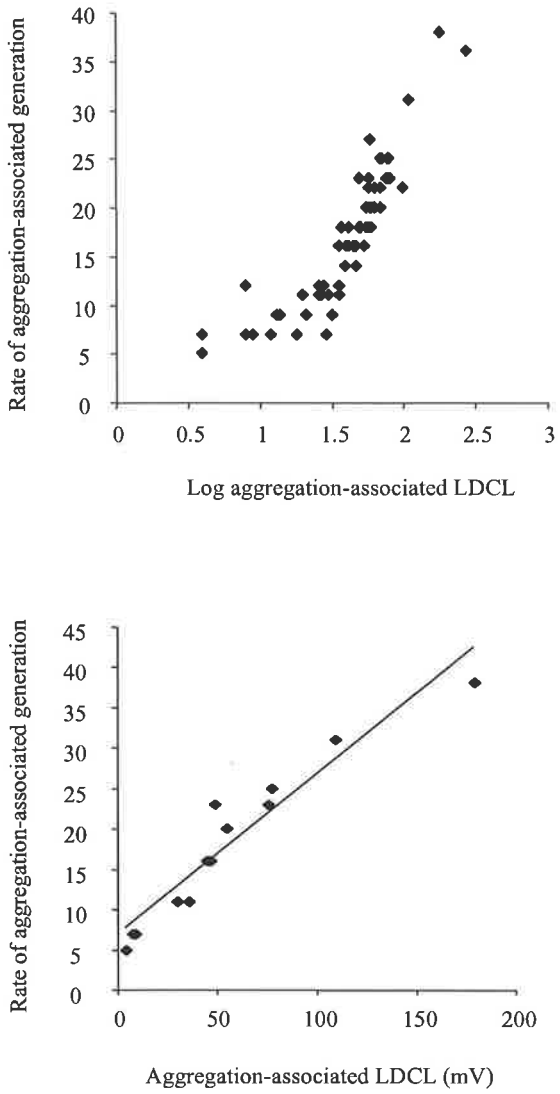


Figure 2.4.6 Aggregation-associated LDCL and its relationship to rate of generation

Aggregation-associated LDCL strongly correlates with the rate of its generation: ***Upper panel****: The relationship between the extent of aggregation-associated LDCL and the rate of its generation was assessed in blood samples obtained from five NVs utilizing a range of concentrations of ADP (non-linear regression analysis).* ***Lower panel****: within the ADP (1 μM) sub-population, a strong inter-relationship between the extent of aggregation-associated LDCL and the rate of its generation was found (regression analysis* $r = 0.95$, $p < 0.01$, *run test* $p = 0.077$*).*

[2.4.4.7] Platelet aggregation and its relationship to the rate of aggregation-associated LDCL generation.

Having established that there was a close relationship between the extent of aggregation-associated LDCL and the rate of its generation, the relationship between the extent of platelet aggregation and the rate of aggregation-associated LDCL was examined.

The data populations representing the rate of aggregation-associated LDCL for each individual ADP concentration were previously shown to conform to a Gaussian distribution. However, the extent of platelet aggregation within the ADP (0.5μM) sub-population was not Gaussian (Kolmogorov-Smirnov 0.5μM ADP KS = 0.39, $p < 0.01$). Accordingly, a log transformation of all the platelet aggregation data was performed.

For each concentration of ADP used, the relationships between the extent of log transformed platelet aggregation and the rate of aggregation-associated LDCL generation were not significantly different (ANCOVA $F = 2.26$, $p = 0.092$). Accordingly all the data regarding the relationship between the extent of platelet aggregation and the rate of the aggregation-associated LDCL generation for each ADP concentration were pooled. However, upon pooling the data, the distribution of log-transformed data was no longer Gaussian (Kolmogorov-Smirnov KS = 0.21, $p = 0.011$). Utilizing the original aggregability data that were shown to conform to a Gaussian distribution upon pooling, the relationship between the extent of platelet aggregation and the rate of aggregation-associated LDCL was examined utilizing regression analysis (Kolmogorov-Smirnov pooled aggregability data (un-transformed) KS = 0.14, p = ns; rate of LDCL generation KS = 0.1, p = ns). As displayed in the upper panel of Figure 2.4.7 the extent of platelet aggregation correlated with the rate of aggregation-associated LDCL generation (regression analysis $r = 0.61$, $p < 0.01$, run test $p = 0.41$).

Platelet aggregation and rate of aggregation-associated LDCL:- ADP (1μM)

The relationship between the extent of platelet aggregation and the rate of aggregation-associated LDCL was then analyzed further within the ADP (1μM) sub-population of data. Firstly, data representing both the extent of platelet aggregation and the rate of aggregation-associated LDCL were previously shown to conform to a Gaussian distribution. Secondly, utilizing ANCOVA it was observed that the relationship between the extent of platelet aggregation and the rate of aggregation-associated LDCL for each subject was not significantly different (ANCOVA $F = 0.96$, $p = 0.48$). The extent of platelet aggregation induced by ADP (1μM) correlated with the rate of aggregation-associated LDCL generation (lower panel of Figure 2.4.7) (regression analysis $r = 0.77$, $p < 0.01$, run test $p = 0.79$).

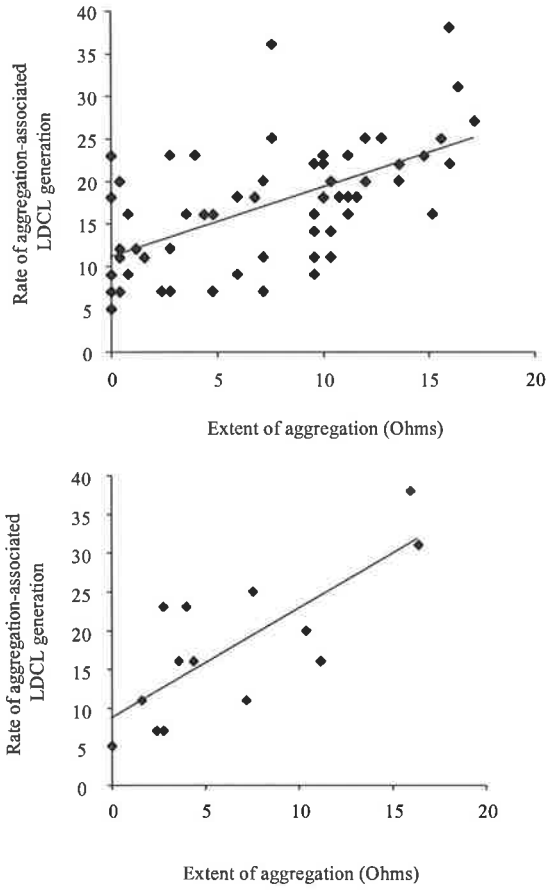


Figure 2.4.7 Platelet aggregability and the rate of aggregation-associated LDCL generation

The extent of platelet aggregation correlated with the rate of aggregation-associated LDCL generation. ***Upper panel****: The relationship between the extent of platelet aggregation and the rate of aggregation-associated LDCL generation was assessed in blood samples obtained from five NVs utilizing a range of concentrations of ADP (regression analysis $r = 0.61$, $p < 0.01$).* ***Lower panel****: Within the ADP (1μM) sub-population a significant relationship between the extent of platelet aggregation and the rate of aggregation-associated LDCL generation was found (regression analysis $r = 0.77$, $p < 0.01$, run test $p = 0.79$).*

[2.4.4.8] The rate of platelet aggregation and its relationship to the rate of aggregation-associated LDCL.

Having established a close relationship between platelet aggregation and the aggregation-associated increase in LDCL, we examined whether the rate of platelet aggregation (as determined by the maximal slope) was correlated with the rate of aggregation-associated LDCL generation.

Data representing the rate of platelet aggregation in the five NVs for a range of concentrations of ADP (0.5-5μM) conformed to a Gaussian distribution (Kolmogorov-Smirnov; 0.5μM ADP KS = 0.28, p = ns; 1.0μM ADP KS = 0.23, p = ns; 2.5μM ADP KS =

0.24, p = ns; 5.0μM ADP KS = 0.24, p = ns). Data representing the rate of aggregation-associated LDCL generation were previously shown to conform to a Gaussian distribution.

The relationships between the rate of platelet aggregation and the rate of the aggregation-associated LDCL for each concentration of ADP used did not significantly differ from each other (ANCOVA F = 0.79, p = 0.5). Accordingly all data across each concentration of ADP were pooled. The rate of platelet aggregation correlated with the rate of aggregation-associated LDCL generation (upper panel of Figure 2.4.8) (regression analysis r = 0.48, p < 0.01, run test p = 0.99).

Rate of platelet aggregation and rate of aggregation-associated LDCL:- ADP (1μM)

This relationship between the rate of platelet aggregation and the rate of aggregation-associated LDCL was explored further in the ADP (1μM) sub-population of data. Utilizing ANCOVA it was demonstrated that the relationship between the rate of platelet aggregation and the rate of aggregation-associated LDCL for each individual subject (n = 5) was not significantly different (ANCOVA F = 1.27, p = 0.35). Using regression analysis the rate of platelet aggregation induced by ADP (1μM) correlated with the rate of aggregation-associated LDCL generation (lower panel of Figure 2.4.8) (regression analysis r = 0.74, p < 0.01, run test p = 0.65).

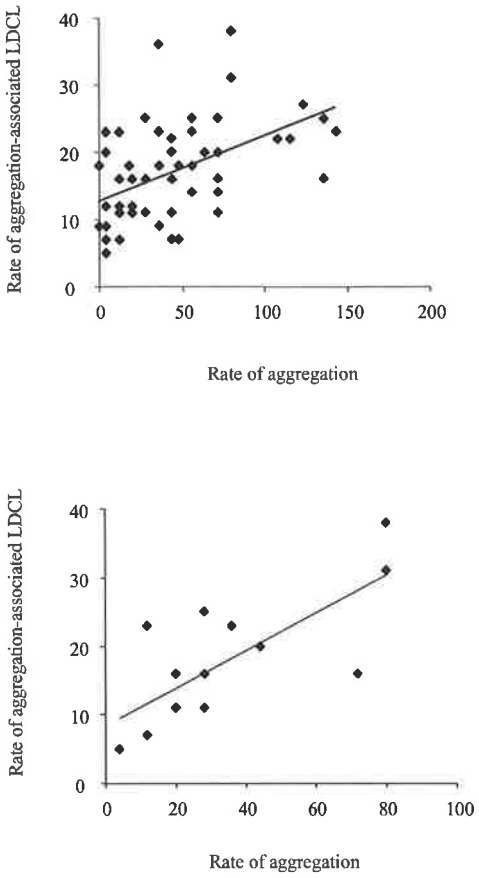


Figure 2.4.8 Rate of platelet aggregability and the rate of aggregation-associated LDCL

The rate of platelet aggregation correlates with the rate of aggregation-associated LDCL generation. ***Upper panel****: The relationship between the rate of platelet aggregability and the rate of aggregation-associated LDCL was examined in a series of blood samples obtained from five NVs utilizing a range of concentrations of ADP (regression analysis $r = 0.48$, $p < 0.01$).* ***Lower panel****: Within the ADP (1μM) sub-population a significant relationship between the extent of platelet aggregation and the rate of aggregation-associated LDCL generation was found (regression analysis $r = 0.74$, $p < 0.01$, run test $p = 0.65$).*

[2.4.4.9] Lag period

As discussed within section 2.4.4.2, the addition of ADP to a whole blood sample 1-minute post administration of lucigenin induced not only platelet aggregation but also an increase in LDCL. The induction of platelet aggregation preceded the commencement of the second phase increase in LDCL. This apparent lag period, defined as the time between an increase in LDCL and an increase in platelet aggregation, was explored further, with blood samples obtained from five NVs utilizing a range of ADP concentrations (0.5-5.0μM).

The data populations representing the lag period between the induction of platelet aggregation and the commencement of the increase in aggregation-associated LDCL for each ADP concentration were found to conform to Gaussian distributions (Kolmogorov-Smirnov:

Lag period 0.5μM ADP KS = 0.13, p = ns; 1.0μM ADP KS = 0.11, p = ns; 2.5μM ADP KS = 0.13, p = ns; 5.0μM ADP KS = 0.24, p = ns). The differences between the standard deviations of each data population were not significantly different (Bartlett's statistic = 0.91, p = 0.53).

Utilizing 1-way ANOVA, a significant difference in the lag period existed across the concentrations of ADP used (1-way ANOVA $F = 5.4$, $p < 0.01$). Utilizing Bonferroni's post hoc multiple comparison test, the lag period between the induction of platelet aggregation and the commencement of the aggregation-associated LDCL was significantly greater within the ADP (0.5μM) group than both the ADP (2.5μM) ($t = 3.3$, $p < 0.05$) and ADP (5.0μM) ($t = 3.6$, $p < 0.01$) data populations. For a further summary see Figure 2.4.9.

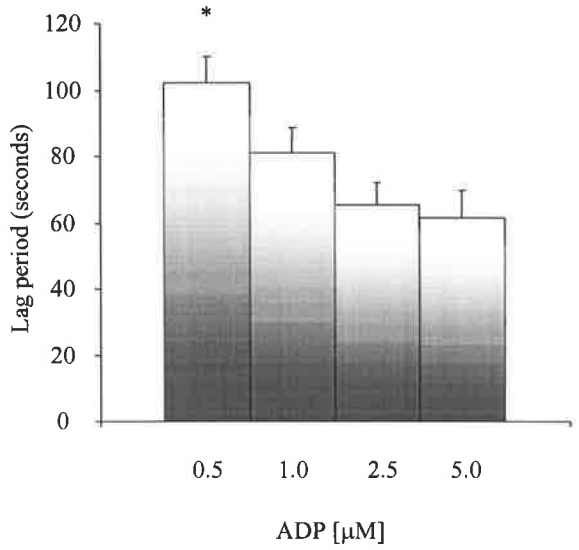


Figure 2.4.9 Concentration of ADP and the lag period

Duration of the lag period between the induction of platelet aggregation and the commencement of the aggregation-associated increase in LDCL is related to the concentration of ADP used to initiate aggregation. 1-way ANOVA $F = 5.4$, $p < 0.01$. Bonferroni's post hoc multiple comparison test ADP 0.5μM $p < 0.05$ vs 2.5μM, $p < 0.01$ vs 5.0μM.

Having established that a lag period exists between the induction of platelet aggregation and the commencement of the aggregation-associated LDCL, the duration of which is a function of the concentration of ADP used, the lag period was examined further to see if it correlated

with:- 1) the extent of platelet aggregation prior to commencement of the aggregation-associated LDCL and 2) with the maximal extent of aggregation-associated LDCL.

Lag period and extent of platelet aggregation

Firstly the extent of platelet aggregation prior to the commencement of the aggregation-associated increase in LDCL was expressed as a percentage of the final degree of platelet aggregation for each concentration of ADP used. The relationship between the duration of the lag period and the extent of platelet aggregation prior to commencement of the aggregation-associated increase in LDCL was not significantly different for each concentration of ADP (ANCOVA $F = 1.16$, $p = 0.33$). Accordingly all data were pooled and demonstrated to conform to a Gaussian distribution (Kolmogorov-Smirnov; lag period KS = 0.1, p = ns; aggregation KS = 0.15, p = ns). By regression analysis a significant positive relationship existed between the duration of the lag period and the extent of platelet aggregation prior to the commencement of the aggregation-associated LDCL (upper panel of Figure 2.4.10) (regression analysis $r = 0.76$, $p < 0.01$, run test $p = 0.56$). The longer the lag period the greater the extent of platelet aggregation prior to the commencement of the aggregation-associated LDCL.

Lag period and extent of aggregation-associated LDCL

Having demonstrated that the duration of the lag period was influenced by the concentration of ADP and was correlated with the extent of platelet aggregation prior to the increase in LDCL, we also examined whether the lag period was correlated with the extent of post aggregation-associated LDCL. Utilizing ANCOVA the relationship between the lag period and the extent of aggregation-associated LDCL for each of the four concentrations of ADP used were not significantly different (ANCOVA $F = 0.96$, $p = 0.42$). Accordingly all the data for the ADP concentration were pooled and found to conform to a Gaussian distribution (Kolmogorov-Smirnov; lag period KS = 0.096, p = ns; Extent of aggregation-associated LDCL KS = 0.054, p = ns). By regression analysis, an inverse relationship existed between the lag period and the extent of aggregation-associated LDCL (lower panel of Figure 2.4.10) (regression analysis $r = -0.29$, $p = 0.025$, run test $p = 0.76$). The shorter the lag period the greater the extent of aggregation-associated LDCL.

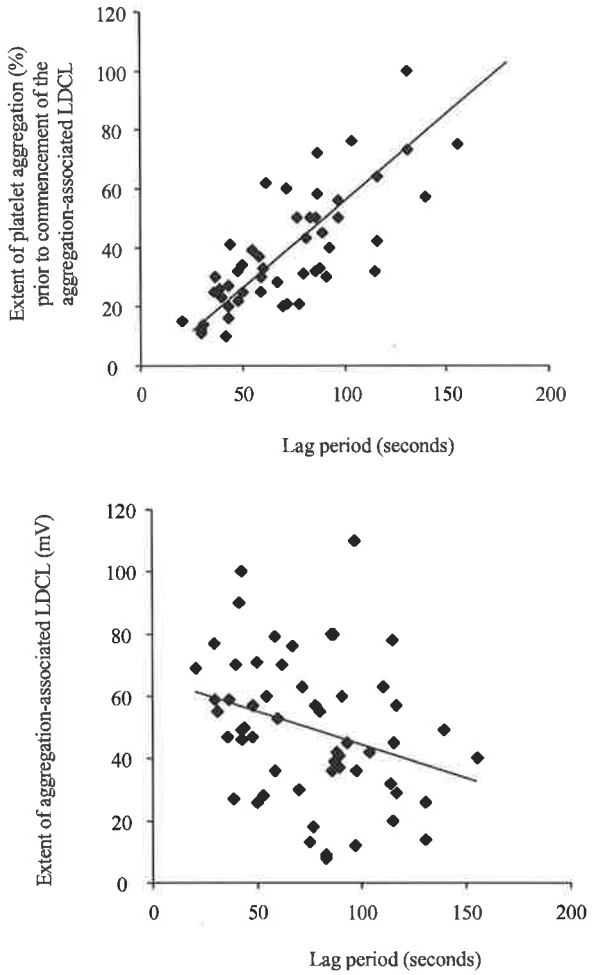


Figure 2.4.10 Lag period as a function of platelet aggregation and aggregation-associated LDCL

The lag period prior to the commencement of the increase in aggregation-associated LDCL and post induction of platelet aggregation positively correlates with the extent of platelet aggregation detectable prior to the increase in LDCL (Upper panel) and negatively correlates with the final amount of aggregation-associated LDCL (Lower panel). ***Upper panel;*** *regression analysis $r = 0.76$, $p < 0.01$.* ***Lower panel;*** *regression analysis $r = -0.29$, $p = 0.025$.*

[2.4.4.10] Identification of the source of superoxide

To identify the primary source of the aggregation-associated increase in superoxide, a series of blood cell isolation experiments were performed. As illustrated in the upper panel of Figure 2.4.11, a baseline LDCL signal was observed in the platelet-rich and platelet-poor preparations, with no baseline LDCL signal being detected with a washed platelet preparation. Administration of ADP (1μM) to a PRP, PPP or washed platelet preparation was not followed by an increase in the aggregation-associated superoxide levels over those observed following baseline steady state stabilization. As illustrated in the lower panel of

Figure 2.4.11, the addition of ADP (1μM) induced platelet aggregation in the PRP and washed platelet preparation but not in the PPP preparation, as expected.

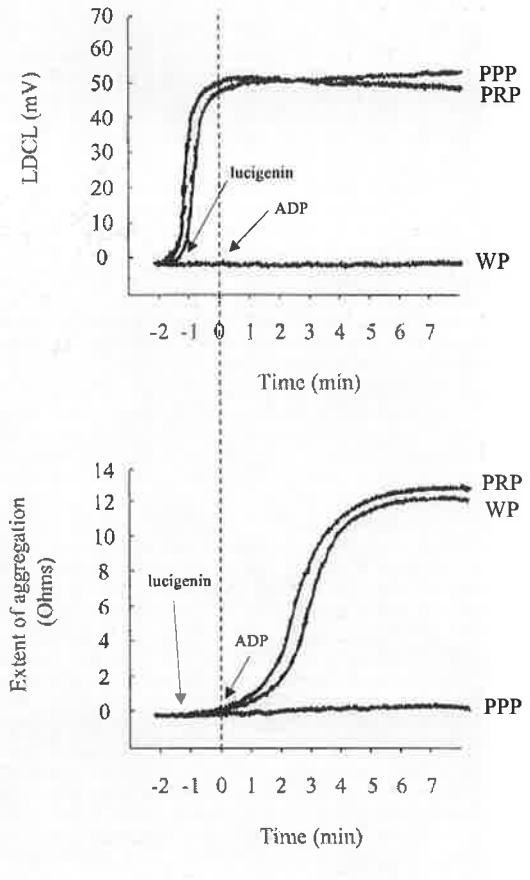


Figure 2.4.11 Luminescence and aggregation curves for PRP/PPP and washed platelet preparations

A representative diagram of the LDCL and platelet aggregation curves generated from various blood preparations. ***Upper panel:*** *LDCL traces for a series of platelet preparations that included platelet-rich plasma (PRP), platelet-poor plasma (PPP) and washed platelet preparations (WP). No aggregation-associated increases in LDCL were observed in any preparations.* ***Lower panel:*** *Aggregation traces for the series of platelet preparations that included PRP, PPP and WP preparations. Platelet aggregation post addition of ADP (1μM) was only observed within the PRP and WP preparations as expected.*

[2.4.4.11] Further identification of the source of superoxide

In a separate series of experiments platelets were removed from a whole blood sample to determine the influence of platelets on the release of the aggregation-associated superoxide generation. Addition of lucigenin to a sample devoid of platelets detected baseline superoxide signal ~25% greater than that of whole blood samples. No differences in the baseline superoxide levels were observed for the two methods of re-suspension of the RBC/leukocyte

fraction (normal saline or PPP; upper panel of Figure 2.4.12). Addition of ADP (1μM) 1-minute post lucigenin administration failed to induce platelet aggregation (lower panel of Figure 2.4.12) as well as an aggregation-associated increase in superoxide in both samples devoid of platelets (lower panel Figure 2.4.12).

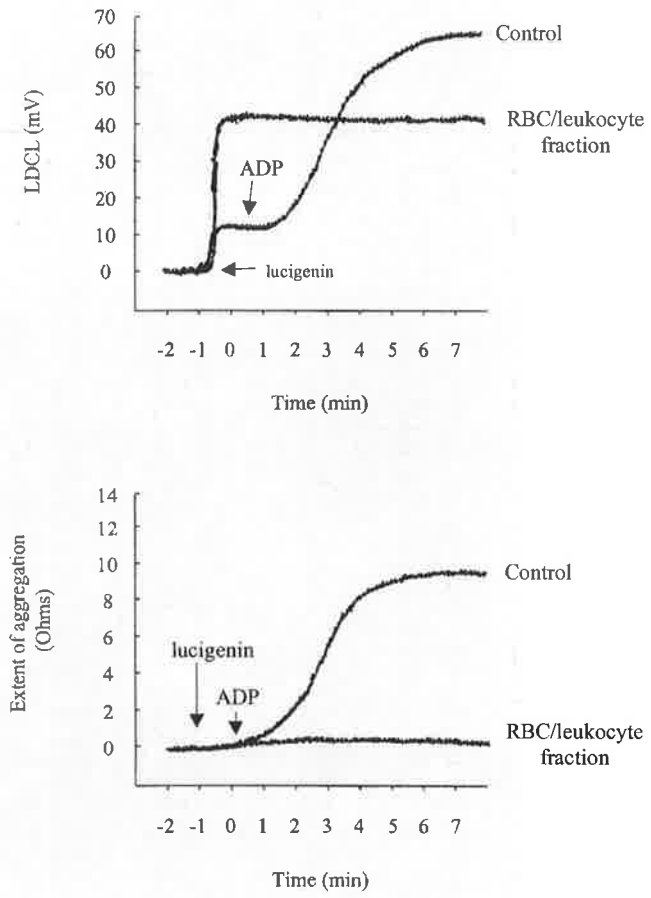


Figure 2.4.12 Luminescence and aggregation curves from a series of platelet preparations

Upper panel: Representative LDCL traces for preparations devoid of platelets, RBC/leukocyte fraction was re-suspended in either normal saline or PPP, compared to control whole blood samples. Lower panel: Aggregation traces for preparations devoid of platelets. As expected no ADP (1μM) induced aggregation was observed within the RBC/leukocyte preparation.

[2.4.4.12] Neutrophil preparations

A number of studies have demonstrated that platelets and neutrophils readily interact with each other (Moon *et al.*, 1990; Nagata *et al.*, 1993; Salvemini *et al.*, 1989a). Utilizing the luminescence methods developed within the current study pure neutrophil preparations were examined for their ability to mediate LDCL.

As depicted in Figure 2.4.13, addition of lucigenin to a neutrophil preparation (final concentration 3 x10^6/mL) caused an immediate increase in LDCL, similar to that which was

observed within a whole blood or PRP/PPP preparation, a signal that was inhibited by the addition of SOD (300U/mL). Addition of either ADP or adrenaline in a range of concentrations (0.5-20μM), 1 minute post lucigenin administration to a neutrophil preparation failed to induce any additional release of superoxide (Figure 2.4.13: LDCL trace A (ADP (5μM) shown)). As a control for neutrophil capacity to release superoxide, the addition of fMLP (1μM) 1 minute post lucigenin administration to the neutrophil preparation stimulated an immediate release of superoxide that peaked sharply at approximately 3-minutes post fMLP addition and decayed to a level approaching the baseline LDCL signal. Furthermore, the extent of superoxide release following fMLP (1μM) addition was significantly diminished by pre-incubation with SOD within the neutrophil preparation.

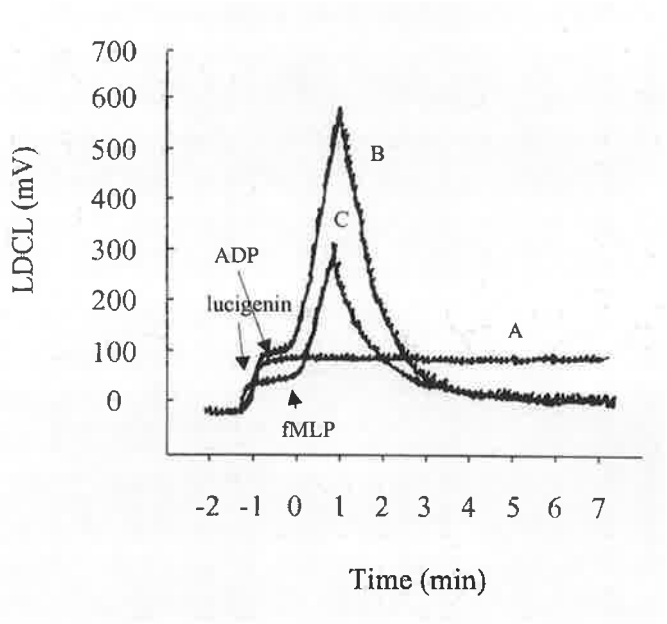


Figure 2.4.13 Luminescence curve generated from neutrophil preparations

A representative diagram of the LDCL signal generated from the addition of either A) ADP (5μM), or B) fMLP (1μM) 1 minute post lucigenin addition to a neutrophil preparation. C) Inhibition of baseline and fMLP (1μM) induced release of superoxide by SOD (300U/mL). The luminescence curves were representative of results obtained in three separate experiments. Inhibition of the baseline LDCL by SOD is not shown.

[2.4.5] Discussion

In the current study, a LDCL based method of assessing the amount of detectable superoxide within an unstimulated/stimulated whole blood sample was established. LDCL within a whole blood sample was observed post administration of lucigenin. Addition of ADP induced both platelet aggregation and an aggregation-associated increase in LDCL, a luminescent signal that was blocked and/or inhibited by the addition of the extracellular superoxide

scavenger superoxide dismutase. Delineating the properties of these luminescence signals, it was observed that the extent of the aggregation-associated LDCL was a function of baseline LDCL. The extent of platelet aggregation was found to correlate with the extent of aggregation-associated LDCL/the rate of aggregation-associated LDCL generation indicating a role for superoxide in the aggregation process.

Mechanism of superoxide generation (Baseline and aggregation-associated LDCL)

Baseline LDCL

In the current study addition of lucigenin to an unstimulated whole blood sample obtained from a NV detected the presence of superoxide. This baseline lucigenin signal was shown to plateau out soon after its administration (Figure 2.4.1). Addition of superoxide dismutase prior to and post administration of lucigenin either prevented or inhibited the luminescent signal (Figure 2.4.2), indicating that it was superoxide in origin. The extent of baseline LDCL was demonstrated to correlate with aggregation-associated LDCL.

Previous methods used to detect the generation of ROS by leukocytes in particular have used various stimulants, such as Bacto latex particles, opsonized zymosan, PMA and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP; section 2.4.4.12) (Christiansen, 1988; Gyllenhammar, 1987; Tosi and Hamedani, 1992). In most of these studies the leukocytes were stimulated and therefore the measured amounts of ROS were not representative of an *in vivo* resting state. The method of adding lucigenin to a whole blood sample and detecting "inherent" superoxide using the luminescent component of a dual channel Lumi-aggregometer as outlined herein, indicates that there is a component of superoxide generation and release in the absence of specific leukocyte stimulation. Moreover, as a baseline LDCL signal was detected within whole blood and PRP sample, spontaneous release of superoxide occurs at a baseline physiological state, in accordance with the observations made by Lu *et al* (1996).

Sources

Baseline increases in LDCL and attainment of a steady state LDCL level implies continuous generation and release of superoxide. While it is possible that this release occurred *ex vivo* as an artifact of blood sampling and/or handling, efforts were made to minimize such a problem.

Within whole blood samples, potential sources of superoxide that may generate superoxide in quantities sufficient for detection within whole blood include the leukocytes. To date a number of studies have demonstrated spontaneous superoxide generation in neutrophil preparations (Tortorella *et al*, 2000; Szuster-Ciesieiska *et al*, 2001). As illustrated in Figure 2.4.13, pure neutrophil preparations released superoxide in a spontaneous fashion similar to that observed within a whole blood preparation.

Within a series of blood element isolation experiments it was also observed that alternative sources of superoxide independent of neutrophils might very well have contributed to the observed baseline LDCL. Addition of lucigenin to a PRP suspension demonstrated an immediate increase in detectable LDCL, an observation that was demonstrated within a PPP preparation too (Figure 2.4.11). Within a PRP sample, potential sources of superoxide that might be responsible for the baseline LDCDL include platelets themselves (Seno *et al*, 2001) despite evidence they need to be activated first (Freedman and Keaney, 1999). However, similar increases in baseline LDCL were observed within PPP samples casting doubt that platelets serve as the primary source of baseline LDCL. Rather, the results suggest a product/s found in plasma contribute significantly to the observed increases in baseline LDCL. Supporting this hypothesis was the observation that no significant baseline LDCL was observed within washed platelet preparations. Although not being assessed in the current study, potential sources of superoxide that may be responsible for the baseline LDCL signals found in plasma, include xanthine/xanthine oxidase (Aslan *et al*, 2001; Desco *et al*, 2002).

Aggregation-associated LDCL

Following the establishment of a plateau in the luminescent signal post administration of lucigenin (Figure 2.4.1), induction of platelet aggregation by ADP was demonstrated to induce a significant increase in the luminescent signal (Figure 2.4.1), an effect that was termed as aggregation-associated LDCL. Like the baseline increase in LDCL, the aggregation-associated luminescent signal was either abolished or significantly inhibited by the administration of SOD, indicating the presence of extra-cellular superoxide (Figure 2.4.2). The aggregation-associated increase in LDCL was also demonstrated to correlate with the extent of platelet aggregation (Figure 2.4.4), the rate of LDCL generation (Figure 2.4.6) and the rate of platelet aggregation (Figure 2.4.7)

Sources/mechanisms

Experiments performed in the current study suggest that an interaction between platelets and neutrophils might be the principal mechanism behind the phenomenon of an aggregation-associated LDCL signal. In sample preparations devoid of leukocytes (i.e. PRP; Figure 2.4.11) no significant aggregation-associated LDCL was associated post induction of platelet aggregation. In samples devoid of platelets (Figure 2.4.12) no significant increase above the baseline LDCL was observed following administration of ADP. As illustrated in Figure 2.4.1, a lag period was observed between the induction of platelet aggregation and the commencement of the aggregation-associated increase in LDCL, the period of which was shown to be a function of the concentration of ADP used. Moreover, the addition of ADP to a pure neutrophil preparation was not associated with any significant increase in LDCL beyond that of the baseline signal (Figure 2.4.13). Therefore, it seems likely that the aggregation-associated LDCL results primarily from an agent released from platelets that interacts with neutrophils to stimulate superoxide production and release. Moreover, aggregation-associated LDCL may result from a direct interaction between platelets and neutrophils.

Agreeing with this hypothesis behind the phenomenon of the aggregation-associated LDCL are the results obtained by Nagata *et al* (1993), who demonstrated that adhesion of activated platelets (thrombin) to monocytes and neutrophils induces release of superoxide in a P-selectin dependent fashion. Membranes prepared from activated platelets induced superoxide production, an effect that was not reproduced when the supernatant of activated platelets was used. Utilizing anti-P-selectin, anti-sialyl-Lewis X antibody or recombinant P-selectin, superoxide generation from the addition of activated platelets to a preparation of neutrophils was significantly inhibited (Nagata *et al.*, 1993). Confirming the observation of Nagata *et al* (1993), Ott *et al* (1996) revealed that binding of purified platelet membranes to neutrophils caused a dose-dependent increase in CD11b surface expression, a decrease in neutrophil L-selectin expression and release of superoxide as detected by cytochrome *c* reduction, all indicative of neutrophil activation.

Evidence from investigations performed by others describing a close relationship between platelets and neutrophils also adds weight to the hypothesis that an interaction between activated platelets and neutrophil/monocytes serve as the primary mechanism of the aggregation-associated LDCL. Throughout the literature there is extensive evidence that

platelets and neutrophils influence the actions of one another (Carulli *et al.*, 1995; Dallegri *et al.*, 1989; McGarrity *et al.*, 1988a; McGarrity *et al.*, 1988b; Moon *et al.*, 1990; Pratico *et al.*, 1993; Spisani *et al.*, 1992; Tsuji *et al.*, 1994; Valles *et al.*, 1993). Moreover, circulating degranulated platelets and monocyte-platelet aggregates have been shown to be more prominent in subjects with SAP than control subjects. Furthermore, they were more sensitive to ADP and more readily formed monocyte-platelet aggregates when stimulated with ADP compared to samples from NVs (Furman *et al.*, 1998a).

Platelets as a superoxide source

Freedman and Keaney demonstrated superoxide generation from aggregating platelets activated by ADP (5μM) (Freedman and Keaney, 1999). In the current study the addition of ADP in a range of concentrations, 1 minute post administration of lucigenin, initiated both platelet aggregation and aggregation-associated LDCL (Figure 2.4.1). However, induction of platelet aggregation by ADP in a PRP preparation, did not produce any increase in the LDCL signal over the baseline LDCL signal (Figure 2.4.11). Reasons for the absence of detectable superoxide within a PRP preparation lie in the differences between the two studies. Freedman and Keaney utilized a concentration of platelets that was significantly higher than that used herein (Freedman and Keaney, 1999). Therefore, the absence of an aggregation-associated LDCL signal post induction of platelet aggregation in a PRP preparation and the requirement of significantly more platelets (and a higher concentration of lucigenin to detect superoxide) implies that the contribution of superoxide generated from aggregating platelets alone, is significantly less than that released in whole blood.

Sources of superoxide within platelets may theoretically result from a dysfunction of the nitric oxide synthase (NOS) enzyme system. Despite extensive evidence demonstrating that platelets contain NOS (Chen and Mehta, 1996; Wallerath *et al.*, 1997), there are no investigations to date demonstrating that a dysfunction of NOS (uncoupling, section A.2.2.1 of chapter 1) described within cell systems that are deficient in NOS cofactors (Vasquez-Vivar *et al.*, 1998), functions as a source of platelet-derived superoxide. However, more recently Seno *et al* (2001), utilizing western blotting techniques demonstrated that platelets possess $p22^{phox}$ and $p67^{phox}$, components of the phagocyte like NAD(P)H oxidase superoxide-generating system (summarized in section A.3.2.1 and Figure 1.1 of chapter 1). The physiological importance of this enzyme in platelets remains uncertain.

[2.4.6] Study Limitations

Direct comparisons between the amounts of superoxide within a whole blood sample and the quantities generated from a PRP sample fall outside the scope of the current study and cannot be made accurately utilizing this method of detection of superoxide. The rates of photon scatter and hence detectable luminescence are different between a whole blood sample and a platelet-rich plasma preparation.

Identification of the true source of superoxide baseline and aggregation-associated LDCL beyond the scope of the current study. It seems possible not only neutrophils (as primary source) and platelets but also compounds residing in plasma may affect the levels of detectable LDCL.

As with many techniques that assess the amount of ROS within a sample remote from its source, reliable estimations of the amount of superoxide detected *ex vivo* may not relate to the amounts of detectable superoxide *in vivo*. Contributions from alternative sources of superoxide such as the vascular endothelium (Griendling *et al.*, 2000), or that results from shear stress (De Keulenaer *et al.*, 1998; Huang *et al.*, 1998), along with various anti-oxidants (nitric oxide derived from the vascular endothelium) compounds and actions that are absent from a donated blood sample, theoretically influence the amounts of detectable superoxide.

Apart from release of superoxide, nitric oxide has also been demonstrated to be generated post induction of platelet aggregation (Freedman and Keaney, 1999; Freedman *et al.*, 1997; Freedman *et al.*, 1998). Despite not assessing nitric oxide generation, it is theoretically possible that the baseline and aggregation-associated increases in LDCL would be influenced by the presence of nitric oxide through its ability to react with superoxide to form peroxynitrite.

[2.4.7] Conclusions

In a simple and sensitive method superoxide can be detected prior to and post induction of platelet aggregation within a whole blood sample. Superoxide generation post induction of platelet aggregation was shown to be platelet aggregation dependent and associated with the extent and rate of aggregation. As this observation was made in whole blood, an experimental model most reflecting *in vivo* conditions, this method of superoxide monitoring may provide

a clearer insight into the physiological and pathological roles played by superoxide. Such pathological phenomena include platelet hyper-aggregability and reduced platelet responsiveness to nitric oxide. Their relationship to baseline and aggregation-associated LDCL forms the basis of the third section of this chapter.

[2.5] Superoxide release as measured by lucigenin-derived chemiluminescence in subjects with coronary artery disease: its relationship to platelet hyper-aggregability and hypo-responsiveness to nitric oxide

[2.5.1] Introduction

Modulation of platelet function by reactive oxygen species

It is now well accepted that ROS play an integral role in the pathogenesis of many cardiovascular disease conditions. Examples include hypercholesterolaemia (Warnholtz *et al.*, 2001), atherosclerosis (Miller *et al.*, 1998; Schachinger and Zeiher, 2000; Singal *et al.*, 1998), hypertension, diabetes and heart failure (Indik *et al.*, 2001).

As discussed in section C.12.1 of chapter 1, platelet function can be modulated by the actions of various ROS. In section I of the current chapter, addition of SOD/catalase inhibited the extent of platelet aggregation in samples from SAP and ACS patients. Despite not demonstrating a significant relationship between the extent of platelet aggregation and change in platelet aggregation post administration of SOD/catalase (Figure 2.3.6), the results suggested that superoxide has an important role in the regulation of platelet function in these subjects.

Influence of reactive oxygen species/oxidative stress on platelet responsiveness to nitric oxide

In a study performed by Haramaki *et al* (2001), platelets from long term smokers (>13 years) were found to be resistant to the anti-aggregatory and cGMP-elevating effects of NTG. Intra-platelet concentrations of reduced glutathione, a known anti-oxidant, were significantly less in smokers compared to non-smokers (Haramaki *et al.*, 2001). Moreover, the ratio of intra-platelet reduced glutathione to oxidized glutathione, was significantly reduced in long-term smokers compared to nonsmokers. Furthermore, administration of NAC potentiated the anti-aggregatory effects of NTG, but only in the non-smoking subjects. No potentiation of the inhibitory effect of NTG by NAC was observed within the nitrate-resistant smoking population (Haramaki *et al.*, 2001). Reasons for this paradoxical finding within the nitrate-resistant smoking cohort remain uncertain.

In section I of the current chapter and utilizing samples from subjects with SAP or an ACS, a significant relationship was observed between the extent of platelet responsiveness to SNP and the degree of change in SNP responsiveness post SOD/catalase administration (Figure 2.3.8), suggesting that superoxide plays a significant role in the phenomenon of reduced platelet responsiveness to nitric oxide.

Experimental study

As described in section 2.2.8.4 of the current chapter, a number of problems are associated with using SOD for the assessment of the role of superoxide within particular phenomena. In section II of this chapter a simple method of assessing the levels of superoxide within a whole blood sample utilizing lucigenin was developed in order to assess further the role of superoxide in the phenomena of platelet hyper-aggregability and platelet hypo-responsiveness to donors of nitric oxide. Therefore, in a series of blood samples obtained from a cohort of NVs, SAP patients and ACS patients, the extent of baseline and aggregation-associated LDCL were examined. Moreover, the relationships between the extent of platelet aggregation/degree of platelet responsiveness to SNP and the baseline and aggregation-associated LDCL were also examined.

[2.5.2] Current study hypothesis

This study was designed to test the following *null* hypotheses in blood samples from a cohort of subjects that included healthy normal volunteers, patients with SAP or an ACS.

Primary:

- *Utilizing two separate concentrations of lucigenin there is no significant difference in the extent of baseline or aggregation-associated LDCL within blood samples obtained from cohorts of NVs, SAP patients and ACS patients.*

Secondary:

- *There is no significant relationship between the extent of platelet aggregation induced by ADP and 1) the extent of baseline LDCL, 2) the extent of aggregation-associated LDCL among NV, SAP and ACS patients.*

- *There is no significant relationship between the degree of platelet responsiveness to SNP and 1) the extent of baseline LDCL 2) the extent of aggregation-associated LDCL among SAP and ACS patients.*

[2.5.3] Methods

[2.5.3.1] Subjects

Studies were performed on blood samples obtained from the following groups:

- Healthy NVs (n = 21; 14 males and 7 females) aged 26 to 67 years; (mean ± S.D), 42 ± 11 years not taking medication that may influence platelet aggregation.

- Patients with SAP (n = 33; 20 males and 13 females aged 49 to 81 years; (mean ± S.D), 67 ± 9 years) undergoing elective diagnostic cardiac catheterization and coronary angiography. In all cases at least one haemodynamically significant (> 50%) stenosis was present in a major coronary artery.

- ACS patients (n = 25; 20 males and 5 females aged 37 to 88 years; (mean ± S.D), 66 ±12 years).

For all patients studied a background medication profile was recorded at the time of recruitment with the clinical characteristics of the study cohort being displayed in Table 2.7. Numbers of subjects used in individual experiments are indicated below (Results: Section 2.5.4). No patient was receiving an ADP or glycoprotein IIb/IIIa receptor antagonist at the time of enrolment. The study was approved by the North Western Adelaide Health Service Ethics of Human Research Committee with informed consent being obtained prior to study entry.

[2.5.3.2] Blood Sampling

Blood samples from the subjects enrolled in this study were collected and prepared according to the method described in Section 2.3.3. All experiments were commenced within 5-10 minutes following blood collection.

[2.5.3.3] Platelet aggregation studies

Whole blood platelet aggregation studies were performed according to the method described in section 2.3.3. Responses to ADP (final concentration 1μM) and SNP (10μM) were assessed for each sample studied. Platelet aggregation was monitored continuously for 7 minutes with the responses being recorded for electrical impedance in Ohms using aggro/Link 4.71 (Chrono-Log, Haverstown, PA, USA).

[2.5.3.4] LDCL parameters

Platelet aggregation and lucigenin derived chemiluminescence (LDCL) was performed according to the method outlined in section 2.4.3 utilizing lucigenin at 12.5 and 125μM. Baseline and aggregation-associated superoxide generation (expressed in mV) was assessed for each sample.

[2.5.3.5] Chemicals

Adenosine 5'-diphosphate (ADP) sodium salt and physiological saline were obtained as indicated in section 2.3.3, lucigenin as indicated in section 2.4.3.

[2.5.3.6] Statistical Analysis

The anti-aggregatory effect of SNP was normalized to the extent of ADP- induced aggregation. Differences between SAP and ACS patients regarding specific risk factors and anti-anginal pharmacotherapy were examined using Fisher's exact test. All data were analyzed for Gaussian distribution utilizing the Kolmogorov-Smirnov test. Log transformations of data were performed on non-Gaussian data or they were assessed using the Kruskal-Wallis test followed by a Dunn's post hoc multiple comparison. Mann Whitney U-test was performed on non-parametric unpaired data. Differences between the standard deviations were assessed utilizing Bartlett's statistic. Differences in the magnitude of the LDCL signals across the groups studied were evaluated utilizing ANOVA followed by Bonferroni's post hoc multiple comparison. Degrees of significance for correlation were determined by linear and non-linear regression analysis. Statistical analysis was performed using the computer programs outlined in section 2.3.3.6 of chapter 2. Statistically significant

differences were limited to $p < 0.05$ with the results being expressed as a mean ± S.E.M unless otherwise indicated.

[2.5.4] Results

[2.5.4.1] Clinical Characteristics

The clinical characteristics of the cohorts of subjects examined in this study are summarized in Table 2.7. The proportions of male subjects, those with hypertension and current smokers, were all well matched between patient cohorts. However, there was significantly more ACS patients with diabetes and significantly fewer subjects with hypercholesterolaemia, compared to the SAP patient cohort. For a further summary of the levels of significance between the two patient cohorts regarding the proportion of subject's with/without coronary risk factors, see Appendix table 4.

Medication profile

All SAP and ACS patients were on multiple anti-anginal pharmacotherapies prior to study enrollment. As summarized in Appendix table 4, there were no significant differences in the numbers of subjects being treated with aspirin, ACE inhibitors, statins, Ca^{2+} antagonists and β-adrenoceptor antagonists for the SAP and ACS cohorts. Not surprisingly, significantly more ACS patients were receiving nitrates compared to the SAP subject cohort. There was also a trend towards more SAP patients being treated with perhexiline (Fishers exact test $p = 0.067$). No patient was receiving GPIIb/IIIa or ADP receptor antagonist treatment.

Table 2.7 Clinical characteristics of the SAP and ACS patients

Clinical Characteristics	Stable Angina Pectoris Patients (n =33)	Acute Coronary Syndrome Patients (n = 25)
Men / Females, n	20/13	20/5
Age (Mean ± S.D), years	67 ± 9	66 ± 12
Diabetes, n (%)	5 (15)	13 (52) *
Hypertension, n (%)	19 (58)	14 (56)
Hypercholesterolaemia, n (%)	29 (88) *	8 (32)
Smokers, n (%)	6 (18)	6 (24)
Medications		
Aspirin, n (%)	28 (84)	22 (88)
Nitrates, n (%)	7 (21)	25 (100) *
ACE Inhibition, n (%)	15 (45)	8 (32)
NAC, n (%)	0 (0)	3 (12)
Perhexiline, n (%)	12 (36)	3 (12)
Statins, n (%)	16 (48)	11 (44)
Ca^{2+} Antagonists, n (%)	22 (66)	16 (64)
β-adrenoceptor antagonists, n (%)	6 (18)	5 (20)

**Significant differences between the subject cohorts. For a further summary on the differences in proportions of risk factors or anti-anginal pharmacotherapies see Appendix table 4.*

[2.5.4.2] Platelet response to ADP

Platelet response to ADP (1μM) for each subject cohort examined was separated according to gender and is summarized in Table 2.8. In section I of chapter 2 the influence of aspirin pharmacotherapy on platelet response to ADP (1μM) was assessed for each gender. However, in the current investigation, there were no female ACS patients not receiving aspirin. The extent of platelet aggregation was accordingly examined across the three disease states and between the genders only.

Within each disease state the extent of platelet aggregation for each gender was found to conform to a Gaussian distribution. Kolmogorov-Smirnov test NV Females KS = 0.29, p = ns, Males KS = 0.17, p = ns; SAP Females KS = 0.1, p = ns, Males KS = 0.11, p = ns; ACS Females KS = 0.29, p = ns, Males KS = 0.17, p = ns. By 2-way ANOVA and confirming the results that were observed in section I of chapter 2, there were significant differences between the disease states and across the genders regarding the extent of platelet aggregation in response to ADP (1μM), with no significant interaction between the two determinants (2-way ANOVA; Disease state $F = 3.8$, $p < 0.05$, Gender $F = 9.4$, $p < 0.01$; Disease state x Gender F

= 0.36, p = 0.69). Utilizing Bonferroni's post hoc multiple comparison test platelet response to ADP (1μM) was significantly less in the male NV subject group compared with the female ACS patients (p < 0.01).

Table 2.8 Platelet aggregation in response to ADP

	Normal Volunteers	*Stable Angina Pectoris Patients*	*Acute Coronary Syndrome Patients*
Males	5.9 ± 0.9 (14)	6.7 ± 0.6 (20)	9.2 ± 1.2 (20)
Females	9.8 ± 2.4 (7)	9.5 ± 1.2 (13)	12.2 ± 3.2 (5)

Blood samples were obtained from NVs, SAP patients and ACS patients. Subject groups were separated according to gender with the numbers of subjects being indicated in parentheses. Data displayed above is the Mean ± S.E.M.

[2.5.4.3] Platelet hypo-responsiveness to sodium nitroprusside

Platelet responsiveness to *in vitro* SNP (10μM) was examined within a series of SAP (n = 29) and ACS (n = 24) patients. Data representing platelet responsiveness to SNP for both cohorts conformed to a Gaussian distribution (Kolmogorov-Smirnov; SAP KS = 0.13, p = ns; ACS KS = 0.11, p = ns). However, significant differences between the standard deviations of each cohort, were observed (F = 3.4, p < 0.01). Therefore, utilizing a Mann-Whitney U-test, the extent of platelet responsiveness to SNP was found to be significantly less in the ACS cohort compared to SAP patients (Mann Whitney U-test; p = 0.021) (Figure 2.51).

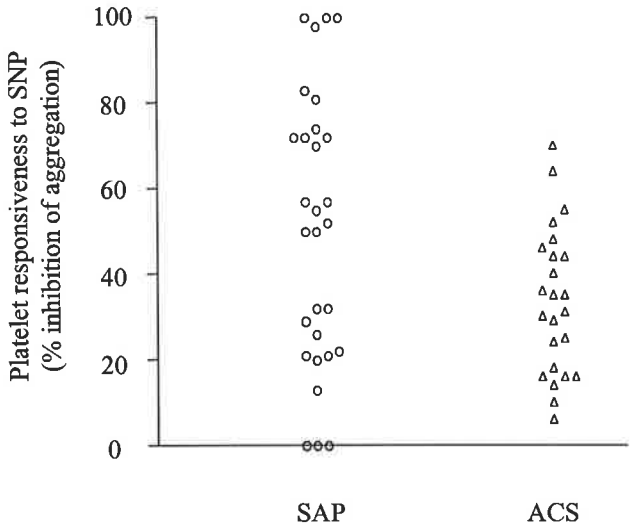


Figure 2.5.1 Platelet responsiveness to SNP

Inhibition of ADP (1μM)-induced platelet aggregation by SNP (10μM) in whole blood samples obtained from SAP and ACS patients. Mann Whitney U-test p = 0.021.

[2.5.4.4] Differences in LDCL across disease states

Having established that lucigenin administration to a whole blood sample is capable of detecting superoxide prior to and post induction of platelet aggregation (section II of this chapter), differences in the luminescent parameters between the cohorts of NVs, and patients with SAP or ACS, were examined. This component of the investigation was performed using two different concentrations of lucigenin (125μM and 12.5μM) as recruitment and examination of subjects utilizing the higher concentration of lucigenin began prior to release of a series of publications casting doubt over the validity of high concentrations of lucigenin to quantify superoxide (Liochev and Fridovich, 1998; Skatchkov *et al.*, 1999; Vasquez-Vivar *et al.*, 1997). Furthermore, recruitment also began prior to the completion of the results that make up section II of this chapter. However, given the assertion that the use of lucigenin is a reliable detector of the presence of superoxide (Afanas'ev, 2001; Afanas'ev *et al.*, 1999; Afanas'ev *et al.*, 2001; Li *et al.*, 1999d) we included the data obtained with lucigenin (125μM).

Baseline LDCL (lucigenin 125μM)

Utilizing lucigenin (125μM), the extent of baseline LDCL was examined in a series of blood samples obtained from NVs (n = 12), patients with SAP (n = 18) and ACS patients (n = 14). All data populations examined conformed to a Gaussian distribution (NVs KS = 0.25, p = ns; SAP KS = 0.23, p = ns; ACS KS = 0.22, p = ns). However, significant differences within the standard deviations between the subject populations were found (Bartlett's statistic = 36.5, $p < 0.01$). Consequently a log transformation of the data was performed (Bartlett's statistic post log transformation = 1.56, p = 0.45, with all subject populations remaining normally distributed).

By 1-way ANOVA a significant difference was found in the log transformed baseline LDCL between the relative subject populations (1-way ANOVA F = 7.1, $p < 0.01$). Utilizing Bonferroni's post hoc multiple comparison test, baseline LDCL from the ACS subject population was significantly greater than that of the SAP ($t = 3.7$, $p < 0.01$), with a trend towards a significant difference between the ACS and NV population ($t = 2.486$, p = ns; $t_{crit} = 2.491$). For a further summary see the upper panel of Figure 2.5.2.

Baseline LDCL (lucigenin 12.5μM)

Similar to the above study, but utilizing lucigenin (12.5μM), baseline LDCL was also examined within a cohort NVs (n = 9), SAP patients (n = 15), and ACS patients (n = 11). As demonstrated above, data from each subject population was shown to conform to a Gaussian distribution but to contain significant differences between the standard deviations of the subject groups (Kolmogorov-Smirnov; NV KS = 0.27, p = ns; SAP KS = 0.12, p = ns; ACS KS p = 0.18, p = ns; Bartlett's statistic = 19.1, $p < 0.01$). Accordingly a log transformation of all the data was performed (Bartlett's statistic post log transformation = 2.9, p = 0.23).

By 1-way ANOVA on the log transformed data, a significant difference in baseline LDCL as detected by lucigenin (12.5μM) was observed across the subject cohorts (1-way ANOVA F = 6.2, $p < 0.01$). LDCL signals for all subject cohorts were significantly lower when utilizing lucigenin (12.5μM) compared to lucigenin (125μM), for the simple reason of a lower concentration of the luminophore. Utilizing Bonferroni's post hoc multiple comparison test, baseline LDCL in the ACS group was significantly greater than both the NV ($t = 3.2$, $p < 0.01$) and SAP ($t = 2.9$, $p < 0.05$) subject groups. There was no significant difference in baseline LDCL between the NV and SAP subject groups ($t = 0.74$, p = ns). For a further summary of the differences between the subject cohorts regarding baseline LDCL as detected by lucigenin (12.5μM) see the lower panel of Figure 2.5.2.

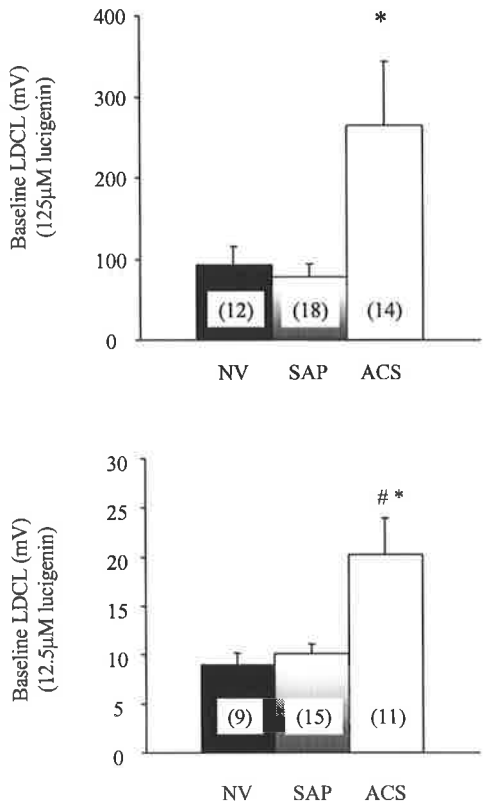


Figure 2.5.2 Baseline LDCL

Baseline LDCL in blood samples obtained from NV, SAP and ACS patients for two separate concentrations of lucigenin (125µM upper panel, and 12.5µM lower panel). ***Upper panel****: 1-way ANOVA on log transformed data F = 7.1, p < 0.01, * p < 0.01 vs SAP.* ***Lower panel****: 1-way ANOVA on log transformed data F = 6.2, p < 0.01;# p < 0.05 vs SAP, *p < 0.01 vs NVs by Bonferroni's post hoc multiple comparison test. Numbers of subjects are indicated within the bars. un-transformed data shown.*

Aggregation-associated LDCL (lucigenin 125µM)

Aggregation-associated LDCL utilizing lucigenin (125µM) was examined in the same subjects as baseline LDCL. Data from all subject cohorts conformed to a Gaussian distribution (NVs KS = 0.27, p = ns; SAP KS = 0.28, p = 0.063, ACS KS = 0.33, p = 0.087). However, significant differences within the standard deviations between the subject populations were found (Bartlett's statistic = 8.0, p = 0.018). Accordingly a log transformation of the data was performed.

By 1-way ANOVA on log transformed data, a slight but significant difference in the extent of aggregation-associated LDCL across the subject populations (1-way ANOVA: F = 3.26, p = 0.047). Utilizing Bonferroni's post hoc multiple comparison test, there were no significant

difference between any combination of subject groups. For a further summary see the upper left panel of Figure 2.5.3.

Aggregation-associated LDCL (lucigenin 12.5μM)

Within the same subject cohorts that were examined for baseline LDCL using lucigenin (12.5μM), differences in aggregation-associated LDCL was also examined. All data within each subject population was shown to be Gaussian (NV KS = 0.12, p = ns; SAP KS = 0.16, p = ns; ACS KS = 0.16, p = ns). However, as demonstrated with the baseline LDCL data, significant differences in the standard deviations between each subject group were observed (Bartlett's statistic = 30.3, $p < 0.01$). Accordingly a log transformation of the data was performed (Bartlett's statistic post log transformation = 2.0, p = 0.36).

By 1-way ANOVA on the log transformed data, significant differences in aggregation-associated LDCL (lucigenin 12.5μM) was observed across the subject cohorts, (1-way ANOVA F = 11.1, $p < 0.01$). Utilizing Bonferroni's post hoc multiple comparison test, aggregation-associated LDCL within the ACS subject group was significantly greater than that of both the NV ($t = 4.7$, $p < 0.01$) and SAP ($t = 2.9$, $p < 0.05$) subject cohorts. There was no significant difference in the degree of aggregation-associated LDCL between the NV and SAP subject groups ($t = 1.2$, p = ns). For a further summary see the lower panel of Figure 2.5.3.

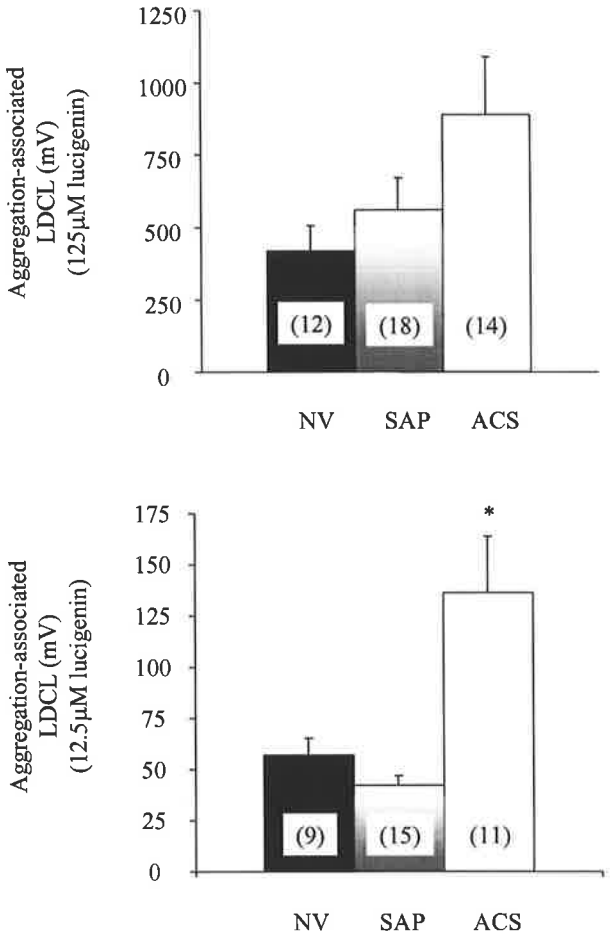


Figure 2.5.3 Aggregation-associated LDCL

Aggregation-associated LDCL within blood samples obtained from NV, SAP and ACS patients for two separate concentrations of lucigenin (125μM upper panel, and 12.5μM lower panel). ***Upper panel****: 1-way ANOVA on log transformed data F = 3.26, p = 0.047.* ***Lower panel****: 1-way ANOVA on log transformed data F = 11.1, p < 0.01 * p < 0.01 vs SAP and NVs utilizing Bonferroni's post hoc multiple comparison test. Numbers of subjects are indicated within the bars. Non-transformed data shown.*

[2.5.4.5] The extent of aggregation and its relationship to baseline LDCL

Having established that there were significant differences between the subject cohorts regarding the extent of baseline and aggregation-associated LDCL, the relationship between the extent of platelet aggregation and the luminescence parameters was examined.

Lucigenin (125μM)

The relationship between the extent of platelet aggregation and baseline LDCL as determined utilizing 125μM lucigenin, was examined in NVs (n = 12), SAP (n = 18) and ACS patients (n = 14). All data populations conformed to a Gaussian distribution (NV aggregation KS = 0.23,

p = ns; SAP aggregation KS = 0.25, p = ns; ACS aggregation KS = 0.18, p = ns; luminescence data shown in 2.5.4.4).

Treating each subject cohort separately, the extent of platelet aggregation in response to ADP (1μM) correlated with baseline LDCL in samples from the NV cohort only (regression analysis: NV r = 0.62, p = 0.03, run test p = 0.65, top left panel of Figure 2.5.4; SAP patients r = 0.01, p = 0.96, run test p = 0.57, middle left panel; ACS patients r = 0.12, p = 0.96, run test p = 0.34, bottom left panel).

Lucigenin (12.5μM)

The relationship between the extent of ADP (1μM)-induced platelet aggregation and baseline LDCL was examined in a cohort of NVs (n = 9), patients with SAP (n = 15) and ACS (n = 11) utilizing lucigenin (12.5μM). All data populations conformed to a Gaussian distribution (Kolmogorov-Smirnov: NV aggregation KS = 0.17, p = ns; LDCL KS = 0.13, p = ns, SAP aggregation KS = 0.14, p = ns, LDCL KS = 0.16, p = ns; ACS aggregation KS = 0.11, p = ns, LDCL KS = 0.16, p = ns).

Treating each of the subject cohorts separately, no significant relationship between the extent of platelet aggregation and baseline LDCL was observed for all subject groups (regression analysis: NV r = -0.26, p = 0.49, run test p = 0.93, top right panel of Figure 2.5.4; SAP r = -0.37, p = 0.17, run test p = 0.29, middle right panel; ACS r = 0.38, p = 0.24, run test p = 0.83, bottom right panel).

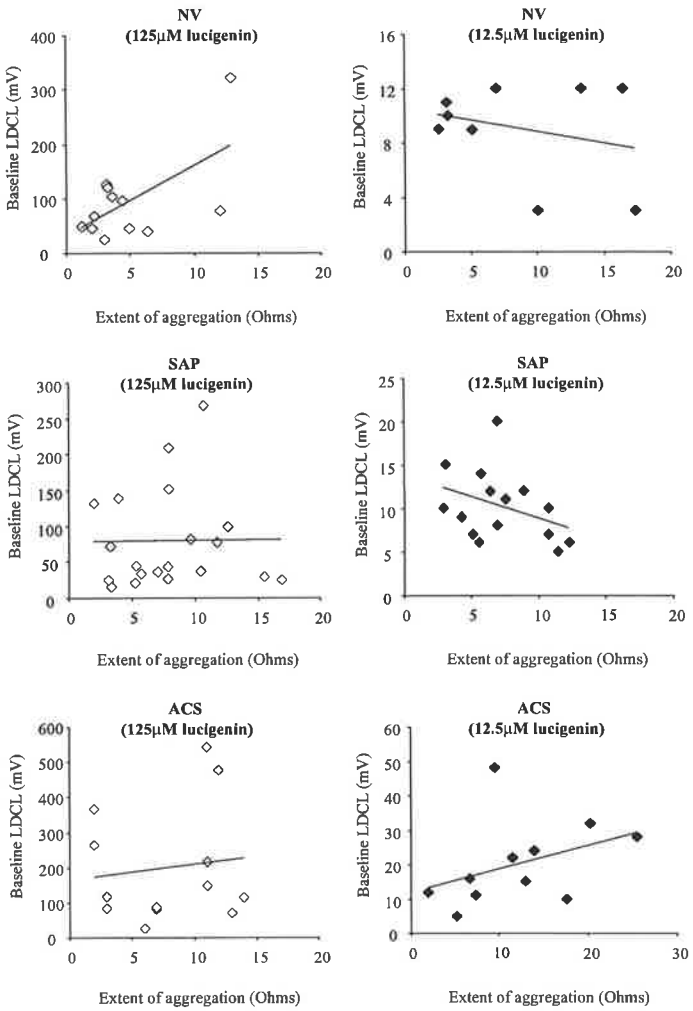


Figure 2.5.4 Platelet aggregability and baseline LDCL

The relationship between the extent of platelet aggregation ADP (1μM) and baseline LDCL was examined in NVs, SAP and ACS patients, utilizing two different concentrations of lucigenin (125μM left panels and 12.5μM right panels). ***Left panels (125μM lucigenin)****: A significant relationship between the extent of platelet aggregation and baseline LDCL was observed within the NV cohort only (regression analysis: NV r = 0.62, p = 0.034, run test p = 0.65, upper left panel; SAP r = 0.01, p = 0.96, run test p = 0.57, middle left panel; ACS r = 0.12, p = 0.69, run test p = 0.34, bottom left panel).* ***Right panels (12.5μM lucigenin)****: No significant relationship was observed between the extent of platelet aggregation and the baseline LDCL for all subject cohorts examined (regression analysis: NV r = -0.26, p = 0.49, run test p = 0.5, upper right panel; SAP r = -0.37, p = 0.17, run test p = 0.29, middle right panel; ACS r = 0.38, p = 0.24, run test p = 0.83, bottom right panel).*

[2.5.4.6] The extent of aggregation and its relationship to the aggregation-associated LDCL

Lucigenin (125μM)

The relationship between the extent of platelet aggregation and aggregation-associated LDCL was also examined. All data populations (aggregability and LDCL) conformed to a Gaussian distribution. Kolmogorov-Smirnov test: NV LDCL KS = 0.25, p = ns; SAP LDCL KS = 0.27, p = 0.029; ACS LDCL KS = 0.3, p = ns). Aggregability data shown in 2.5.4.5.

Treating each subject cohort separately, the extent of platelet aggregation strongly correlated with the degree of aggregation-associated LDCL data for the NV cohort, a relationship that was not observed within the SAP and ACS populations (regression analysis: NV $r = 0.71$, $p < 0.01$, run test $p = 0.73$, upper left panel of Figure 2.5.5; SAP $r = 0.14$, $p = 0.55$, run test $p = 0.027$, middle left panel; ACS $r = -0.11$, $p = 0.69$, run test $p = 0.71$, bottom left panel).

Lucigenin (12.5μM)

The relationship between the extent of platelet aggregation and aggregation-associated LDCL was also examined using lucigenin (12.5μM). All data populations regarding the extent of platelet aggregation and aggregation-associated LDCL, conformed to a Gaussian distribution (Kolmogorov-Smirnov test: NV LDCL KS = 0.27, p = ns; SAP LDCL KS = 0.12, p = ns; ACS LDCL KS = 0.18, p = ns; aggregability data shown in 2.5.4.5).

No significant relationship was observed for any subject cohort for platelet aggregation and the extent of aggregation-associated LDCL (regression analysis: NV $r = -0.074$, $p = 0.85$, run test $p = 0.50$, upper right panel of Figure 2.5.5; SAP $r = -0.28$, $p = 0.29$, run test $p = 0.76$, middle right panel; ACS $r = 0.43$, $p = 0.18$, run test $p = 0.97$, bottom right panel).

Summary: Extent of aggregation versus baseline/aggregation-associated LDCL

In the majority of cases no significant relationship was observed for the comparison between the extent of platelet aggregation and baseline/aggregation-associated LDCL. An exception to this conclusion was the significant relationship between the extent of platelet aggregation and baseline/aggregation-associated LDCL within the NV cohort (lucigenin (125μM)). The relationship between the extent of platelet aggregation and baseline LDCL parallels the relationship between platelet aggregation and aggregation-associated LDCL.

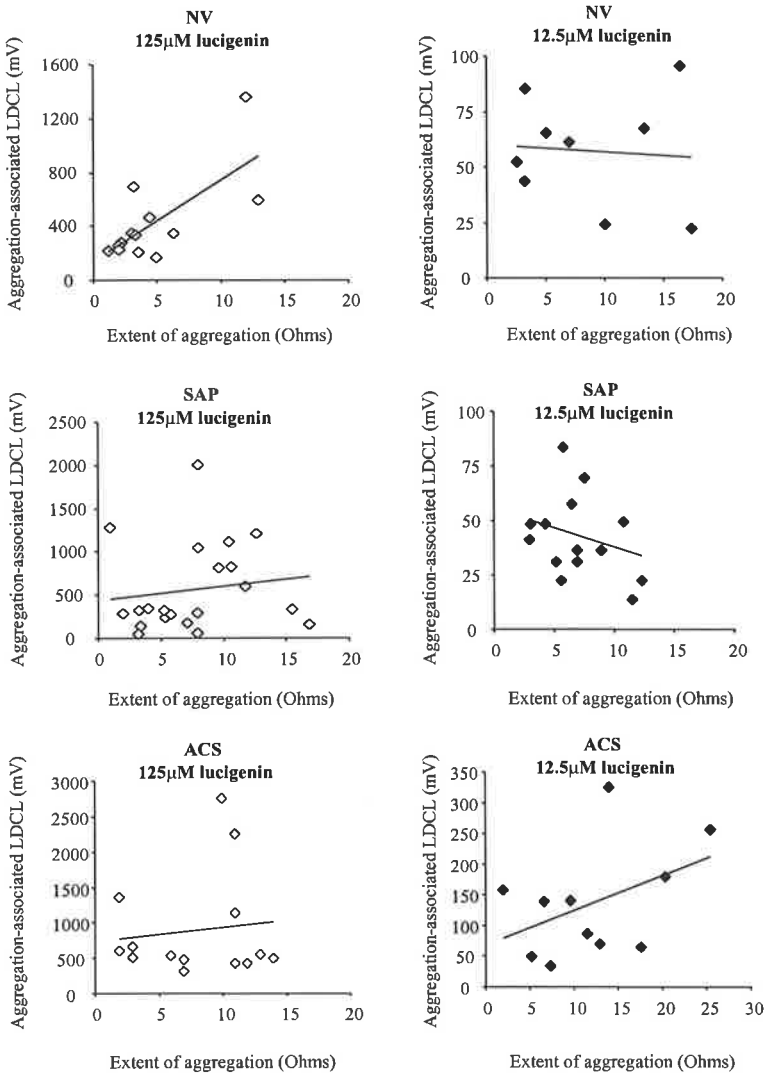


Figure 2.5.5 Aggregability and aggregation-associated LDCL

The relationship between the extent of platelet aggregation ADP (1μM) and aggregation-associated LDCL was examined in NVs, SAP and ACS patients, utilizing two different concentrations of lucigenin (125μM upper panel and 12.5μM lower panel). ***Left panels (125μM lucigenin)****: A significant relationship between the extent of platelet aggregation and aggregation-associated LDCL was observed within the NV cohort only (regression analysis: NV r = 0.71, p < 0.01, run test p = 0.73, upper left panel; SAP r = 0.14, p = 0.55, run test p = 0.027, middle left panel; ACS r = 0.12, p = 0.69, run test p = 0.71, bottom left panel).* ***Right panels (12.5μM lucigenin)****: No significant relationship was observed between the extent of platelet aggregation and the aggregation-associated LDCL for all subject cohorts examined (regression analysis: NV r = -0.074, p = 0.85, run test p = 0.50, upper right panel; SAP r = -0.28, p = 0.29, run test p = 0.76, middle right panel; ACS r = 0.43, p = 0.18, run test p = 0.97, bottom right panel).*

[2.5.4.7] Platelet responsiveness to SNP and its relationship to baseline LDCL

Within section I of the current chapter, a significant relationship was observed between the extent of baseline SNP responsiveness and the degree of change in SNP responsiveness post administration of SOD/catalase. This result suggests that superoxide is involved in the phenomenon of attenuated platelet responsiveness to SNP. Having examined whether the

extent of platelet aggregation was related to the baseline and aggregation-associated LDCL, the relationship between platelet responsiveness to SNP (10μM) and the luminescent parameters was examined in a series of patients with SAP or ACS.

Lucigenin (125μM)

The relationship between SNP responsiveness and the extent of baseline LDCL (lucigenin 125μM) was examined within a series of blood samples obtained from SAP (n = 12) and ACS patients (n = 12). Data from all patient populations (platelet responsiveness to SNP and LDCL) conformed to a Gaussian distribution (Kolmogorov-Smirnov test: SAP SNP KS = 0.20, p = ns; LDCL KS = 0.33, p = ns; ACS SNP KS = 0.12, p = ns, LDCL KS = 0.21, p = ns). Treating each patient group separately and as illustrated in Figure 2.5.6, there was no significant relationship between platelet responsiveness to SNP and the extent of baseline LDCL (regression analysis: SAP r = 0.52, p = 0.084, run test p = 0.42, upper left panel; ACS r = 0.44, p = 0.15, run test p = 0.17, bottom left panel).

Lucigenin (12.5μM)

As with the results described above, the relationship between platelet responsiveness to SNP (10μM) and the extent of baseline LDCL detected using lucigenin (12.5μM), was also examined in a cohort of SAP (n = 15) and ACS patients (n = 11). All data within the two patient cohorts conformed to a Gaussian distribution (SAP SNP KS = 0.14, p = ns, LDCL KS = 0.12, p = ns; ACS SNP KS = 0.18, p = ns, LDCL KS = 0.18, p = ns).

Treating each subject group separately, there was no significant relationship between platelet responsiveness to SNP (10μM) and baseline LDCL (regression analysis SAP r = 0.21, p = 0.45, run test p = 0.76, upper right panel of Figure 2.5.6; ACS r = -0.45, p = 0.12, run test p = 0.52, bottom right panel of Figure 2.5.6).

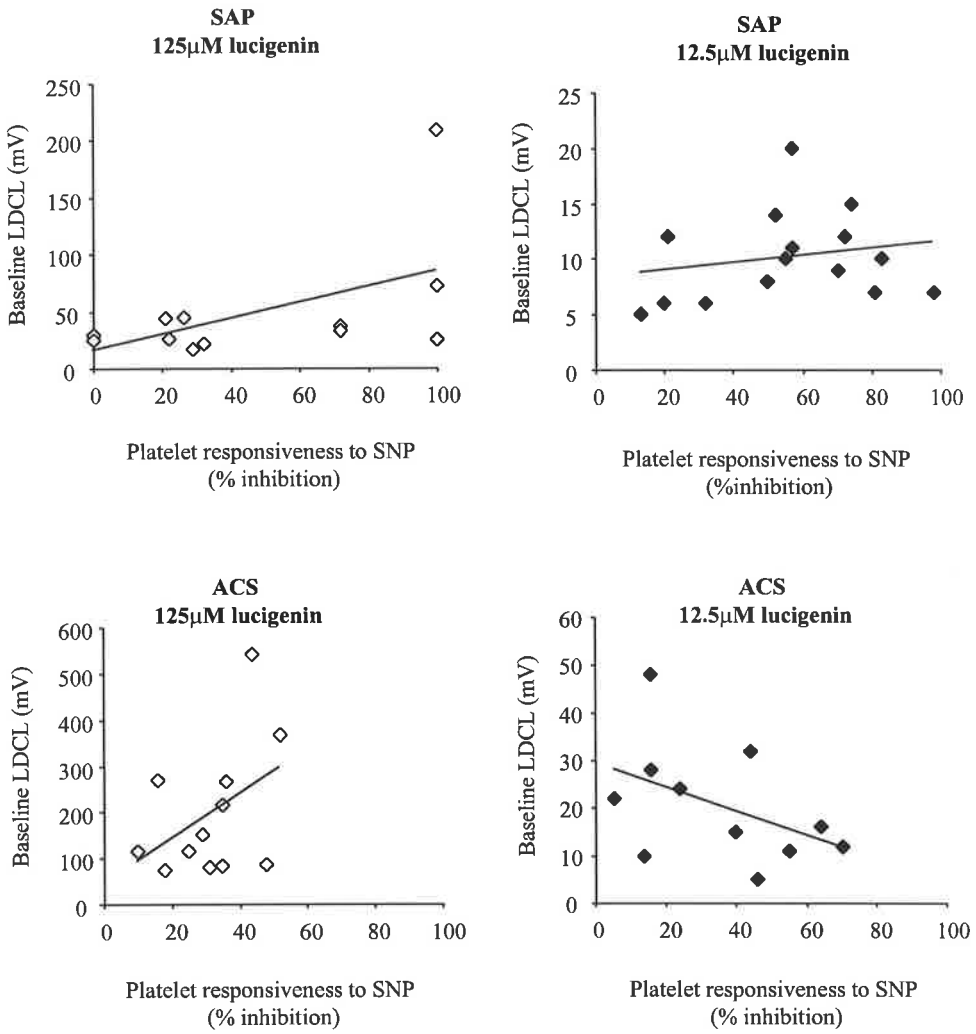


Figure 2.5.6 Platelet responsiveness to SNP and baseline LDCL

The relationship between SNP responsiveness and baseline LDCL was examined in SAP and ACS patients, utilizing two different concentrations of lucigenin (125μM upper panel and 12.5μM lower panel). ***Left panels****: No significant relationship was observed between platelet responsiveness to SNP and baseline LDCL for cohorts of SAP and ACS patients utilizing 125μM lucigenin (regression analysis: SAP r = 0.52, p = 0.084, run test p = 0.42, upper left panel; ACS r = 0.44, p = 0.13, run test p = 0.17, bottom left panel).* ***Right panels****: No significant relationship between platelet responsiveness to SNP and the extent of baseline LDCL was observed for a cohort of SAP and ACS patients utilizing 12.5μM lucigenin (regression analysis SAP r = 0.21, p = 0.45, run test p = 0.76, upper right panel; ACS r = -0.45, p = 0.12, run test p = 0.52, bottom right panel).*

[2.5.4.8] Platelet responsiveness to SNP and its relationship to aggregation-associated LDCL

Lucigenin (125μM)

The relationship between SNP responsiveness and the extent of aggregation-associated LDCL (lucigenin 125μM) was examined within a series of blood samples obtained from SAP (n = 12) and ACS (n = 12) patients. All data populations (platelet responsiveness to SNP and LDCL) conformed to a Gaussian distribution (Kolmogorov-Smirnov test: SAP SNP KS =

0.20, p = ns, LDCL KS = 0.32, p = ns; ACS SNP KS = 0.11, p = ns, LDCL KS = 0.26, p = ns).

Treating both patient cohorts separately, no significant relationship was observed between platelet responsiveness to SNP and the extent of aggregation-associated LDCL (regression analysis: SAP r = -0.058, p = 0.86, run test p = 0.61, upper left panel of Figure 2.5.7; ACS r = 0.24, p = 0.44, p = 0.78, bottom left panel of Figure 2.5.7).

Lucigenin (12.5μM)

Like the examinations performed above, the relationship between SNP responsiveness and the extent of aggregation-associated LDCL as detected utilizing lucigenin (12.5μM), was examined in a cohort of SAP (n = 15) and ACS patients (n = 11). All data within both patient cohorts was shown conform to a Gaussian distribution (Kolmogorov-Smirnov; SAP SNP KS = 0.14, p = ns, LDCL KS = 0.16, p = ns; ACS SNP KS = 0.18, p = ns, LDCL KS = 0.16, p = ns).

No significant relationship was observed between platelet responsiveness to SNP (10μM) and the extent of aggregation-associated LDCL (12.5μM) in either patient population (regression analysis; SAP r = 0.41, p = 0.13, run test p = 0.29, upper right panel of Figure 2.5.7; ACS patients r = -0.18, p = 0.6, run test p = 0.52, bottom right panel of Figure 2.5.7).

Summary: Platelet responsiveness to SNP versus baseline/aggregation-associated LDCL

In samples from SAP or ACS patients no significant relationship was observed between the extent of SNP responsiveness and baseline/aggregation-associated LDCL utilizing either concentration of lucigenin. However, a non-significant trend towards a positive relationship between platelet responsiveness to SNP and baseline LDCL was observed in the SAP patient cohort (125μM lucigenin). This result is at odds with the observations of section I (current chapter) and is thus assumed to have resulted from chance alone given the absence of any other significant relationship between SNP responsiveness and LDCL.

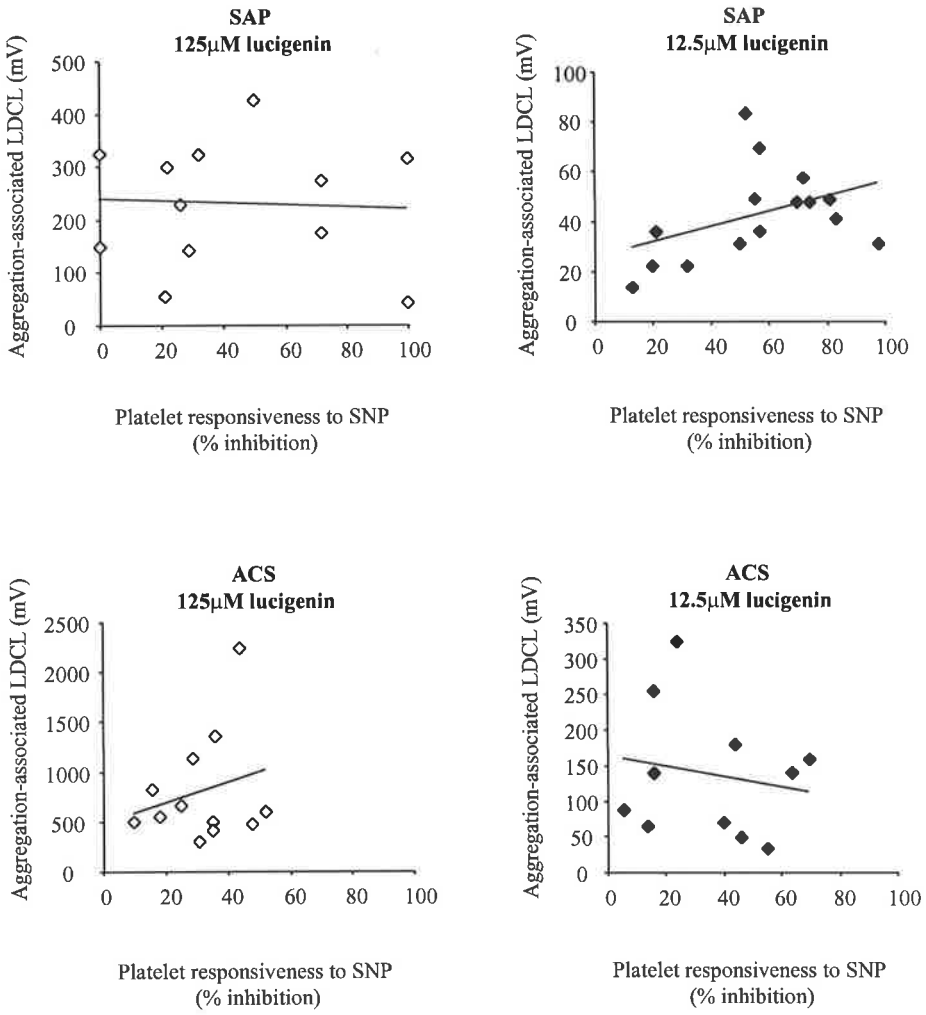


Figure 2.5.7 Platelet responsiveness to SNP and aggregation-associated LDCL

The relationship between SNP responsiveness and aggregation-associated LDCL was examined in SAP and ACS patients, utilizing two different concentrations of lucigenin (125μM left panels and 12.5μM right panels). ***Left panels****: No significant relationship was observed between platelet responsiveness to SNP and aggregation-associated LDCL for cohorts of SAP and ACS patients utilizing 125μM lucigenin (regression analysis: SAP r = -0.058, p = 0.857, run test p = 0.61, upper left panel; ACS r = 0.24, p = 0.44, run test p = 0.78, bottom left panel).* ***Right panels****: No significant relationship between platelet responsiveness to SNP and the extent of aggregation-associated LDCL was observed for a cohort of SAP and ACS patients utilizing 12.5μM lucigenin (regression analysis SAP r = 0.41, p = 0.13, run test p = 0.29, upper right panel; ACS r = -0.18, p = 0.60, run test p = 0.52, bottom right panel).*

LDCL in ACS patients (aggregability)

As illustrated within Figures 2.5.2 and 2.5.3 the extent of either baseline or aggregation-associated LDCL was significantly greater in the ACS patient cohorts compared to the SAP and NV populations. We therefore examined whether ACS patients generated greater amounts of superoxide per unit of aggregation compared to SAP patients. Significance of the regression results was analyzed in sections 2.5.4.5/2.5.4.6. Utilizing ANCOVA and as illustrated in Figure 2.5.8, the extent of baseline and aggregation-associated LDCL, expressed

as a function of the extent of platelet aggregation, was consistently greater in the ACS patient populations compared to the SAP cohorts (Baseline LDCL (125μM lucigenin) SAP vs ACS patients ANCOVA $F = 8.34$, $p < 0.01$, left upper panel of Figure 2.5.8; Baseline LDCL (12.5μM lucigenin) SAP vs ACS patients ANCOVA $F = 4.69$, $p = 0.041$, right upper panel; Aggregation-associated LDCL (12.5μM lucigenin) SAP vs ACS patients ANCOVA $F = 8.56$, $p < 0.01$, bottom right panel). This relationship holds true for all comparisons apart from the relationship between the extent of platelet aggregation and aggregation-associated LDCL (lucigenin 125μM) (Aggregation-associated LDCL (125μM lucigenin) SAP vs ACS patients ANCOVA $F = 2.24$, $p = 0.14$, lower left panel of Figure 2.5.8). Despite the difference between the patient cohorts failing to reach statistical significance the regression line representing the ACS patient cohort (dotted line) remained above that representing the SAP patient population (solid line).

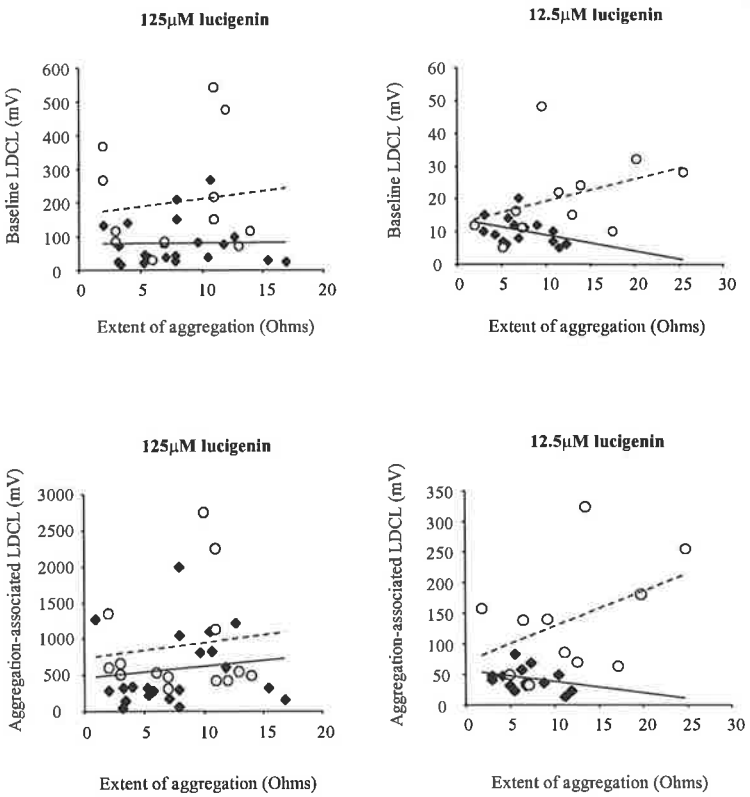


Figure 2.5.8 Platelet aggregability and LDCL (SAP and ACS patients)

ACS patients release superoxide in greater amounts per Ohm of platelet aggregation compared to SAP patients. ***Left panels 125μM lucigenin****: extent of platelet aggregation versus baseline LDCL (SAP vs ACS ANCOVA; $F = 8.34$, $p < 0.01$, upper panel); extent of platelet aggregation versus aggregation-associated LDCL (SAP vs ACS ANCOVA; $F = 2.24$, $p = 0.14$ bottom panel).* ***Right panels 12.5μM lucigenin:*** *extent of platelet aggregation versus baseline LDCL (SAP vs ACS ANCOVA; $F = 4.69$, $p = 0.041$, upper panel); extent of platelet aggregation versus aggregation-associated LDCL (SAP vs ACS ANCOVA; $F = 8.59$, $p < 0.01$, bottom panel). Key:- open circles dotted line = ACS patients; closed diamonds solid line = SAP patients.*

LDCL in ACS patients (SNP responsiveness)

Having demonstrated that ACS patients release greater amounts of superoxide per Ohm compared to SAP patients, the relationship was also examined using the extent of SNP responsiveness.

As indicated in sections 2.5.4.7/2.5.4.8 there was no significant relationship between platelet responsiveness to SNP and baseline/aggregation-associated LDCL as quantified using either 125 or 12.5μM lucigenin. However, like the results demonstrated for platelet aggregability, ACS patients consistently release significantly greater amounts of superoxide compared to SAP patients when the extent of baseline/aggregation-associated LDCL was expressed as a function of SNP responsiveness.

Baseline LDCL (125μM lucigenin) SAP vs ACS patients ANCOVA $F = 13.6$, $p < 0.01$, upper left panel of Figure 2.5.9. Baseline LDCL (12.5μM lucigenin) SAP vs ACS patients ANCOVA $F = 5.65$, $p = 0.026$, upper right panel. Aggregation-associated LDCL (125μM lucigenin) SAP vs ACS patients ANCOVA $F = 11.58$, $p < 0.01$, bottom left panel. Aggregation-associated LDCL (12.5μM lucigenin) SAP vs ACS patients ANCOVA $F = 12.2$, $p < 0.01$).

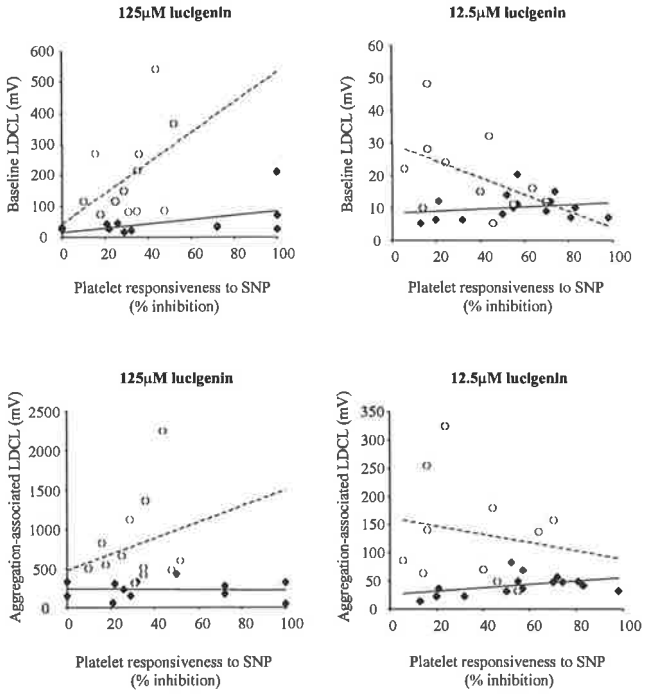


Figure 2.5.9 Platelet responsiveness to SNP and LDCL (SAP and ACS patients)

ACS patients release superoxide in greater amounts compared to SAP patients when either baseline or aggregation-associated LDCL is expressed as a function of responsiveness to SNP. ***Left panels 125μM lucigenin:*** *Platelet responsiveness to SNP versus baseline LDCL (SAP vs ACS ANCOVA $F = 13.6$, $p < 0.01$ upper panel); platelet responsiveness to SNP versus aggregation-associated LDCL (SAP vs ACS ANCOVA $F = 11.6$, $p < 0.01$ bottom panel);* ***Right panels 12.5μM lucigenin:*** *Platelet responsiveness to SNP versus baseline LDCL (SAP vs ACS ANCOVA $F = 5.7$, $p = 0.026$ upper panel). Platelet responsiveness to SNP versus aggregation-associated LDCL (SAP vs ACS ANCOVA $F = 12.2$, $p < 0.01$ bottom panel). Key:- open circles dotted line = ACS patients; closed diamonds solid line = SAP patients.*

[2.5.5] Discussion

The current study is the first investigation examining the relationship between the phenomena of platelet hyper-aggregability and hypo-responsiveness to donors of nitric oxide with the presence of superoxide anion as detected utilizing LDCL. The results can be summarized as follows: -

- Platelets from patients with an ACS were demonstrated to be hyper-aggregable towards ADP (1μM) and hypo-responsive to the anti-aggregatory effects of SNP (10μM), an observation that concurs with previous results.

- The extent of baseline and aggregation-associated LDCL in samples obtained from ACS patients were demonstrated to be significantly greater than that of samples obtained from

SAP patients and NVs, indicating a heightened level of oxidative stress is occurring in these subjects.

- No significant relationship was observed between the extent of platelet aggregation and the baseline/aggregation-associated LDCL for the SAP and ACS patients, nor was there a significant relationship between SNP responsiveness and the baseline LDCL and aggregation-associated LDCL.

- When the extent of platelet aggregation or % inhibition of aggregation by SNP was expressed as a function of baseline or aggregation-associated LDCL, ACS patients were demonstrated to release significantly greater amounts of superoxide per unit aggregation/% inhibition of aggregation, compared with SAP patients.

Lucigenin derived chemiluminescence as a measure of extracellular superoxide; relationship to angina pectoris

As summarized within the introduction to this chapter (section 2.2.8), there are a number of techniques that have been used to quantify the extent of ROS production (Briheim *et al.*, 1984; Smith and Weidemann, 1993). Caldefie-Chezet *et al* (2002) demonstrated that the amount of ROS detected by luminol and 2'-7'-dichlorofluorescin diacetate (DCFH-DA) following neutrophil stimulation with PMA, were significantly correlated. Both luminol and DCFH-DA function as ROS probes that readily permeate cell membranes (Bass *et al.*, 1986; Rest, 1994; Rothe and Valet, 1990). Furthermore, Caldefie-Chezet *et al* (2002) determined that the extent of LDCL and cytochrome *c* reduction (extracellular superoxide probe), were significantly correlated. However, no correlation was observed between results obtained using LDCL and DCFH-DA, indicating that lucigenin does not permeate cells, but rather reflects extracellularly derived superoxide (Caldefie-Chezet *et al.*, 2002). Therefore the results of the current study suggest that differences between patient populations observed herein (baseline and aggregation-associated LDCL (Figure 2.5.2/2.5.3)), reflect the amount of superoxide that is generated and then released extracellularly.

Irrespective of what concentration of lucigenin (125μM/12.5μM) was utilized to examine the levels of whole blood superoxide, blood samples from ACS patients were demonstrated to

contain significantly greater amounts of superoxide at baseline and generated during platelet aggregation, compared to SAP patients and NV (Figures 2.5.2/2.5.3). Moreover, when the extent of platelet aggregation or the degree of SNP responsiveness was expressed as a function of baseline or aggregation-associated LDCL, ACS patients were shown to release significantly greater amounts of superoxide per Ohm/% inhibition of aggregation compared to SAP patients (Figures 2.5.8/2.5.9).

Agreeing with the results observed herein, but utilizing alternative techniques to quantify the extent of oxidative stress, Dubois-Rande *et al* (1994), demonstrated that plasma malondialdehyde levels were significantly higher in those subjects admitted with an ACS compared to those with SAP or NVs. The observation of elevated levels of ROS within subjects with an ACS compared to SAP patients, are also in accordance with observations made by other investigators (McMurray *et al.*, 1992; Kostner *et al.*, 1997).

Throughout the literature a number of investigators have suggested that the use of elevated concentrations of lucigenin is associated with artifactual increases in the concentration of superoxide, due to "redox cycling" (Liochev and Fridovich, 1998; Vasquez-Vivar *et al.*, 1997). As such, lower concentration of lucigenin or alternative detection methods, have been recommended (Skatchkov *et al.*, 1999). In the current study two concentrations of lucigenin were utilized to examine both baseline and aggregation-associated LDCL in whole blood samples obtained from a number of subjects. Across all the cohorts examined a ~10-fold difference in LDCL was observed when either the extent of baseline or aggregation-associated LDCL was compared for lucigenin used at 125 or 12.5μM (Figure 2.5.2/2.5.3). Furthermore, the proportion of LDCL between the subject cohorts, were similar. The extent of LDCL in ACS is ~2-fold greater than SAP or NV irrespective of which concentration of lucigenin was used. Therefore these results suggest that the contribution to an artifactual elevation in the amount of detectable LDCL with elevated concentrations of lucigenin due to "redox cycling", is negligible.

Acute coronary syndromes and inflammation

MI is associated with an inflammatory reaction, a response that is now known to involve the humoral and cell-mediated arms of the immune system (Frangogiannis *et al.*, 2002). A number of markers of systemic inflammation have been demonstrated to correlate with

subsequent risk of MI and may also be predictive of outlook in specific high-risk populations, such as those with diabetes (Buffon *et al.*, 1999; de Beer *et al.*, 1982; Festa *et al.*, 2000; Liuzzo *et al.*, 1999; Rader, 2000; Retterstol *et al.*, 2002; Ridker *et al.*, 1997).

Utilizing neutrophil myeloperoxidase content as an index of neutrophil activation in samples from a series of different patient cohorts Buffon *et al* (2002) recently documented a pronounced neutrophil activation within patients with UAP as neutrophils transversed the coronary vascular bed. However, this level of neutrophil activation was not observed in patients with SAP, patients with acute variant angina or in control subjects (free of significant CAD), a result that concurs with the observations made within the current study. Interestingly and perhaps more importantly, neutrophil activation across the coronary circulation was not confined to the vascular bed in which the culprit lesion was located. This result was interpreted as representing a widespread inflammatory process occurring throughout the coronary vasculature.

Utilizing either concentration of lucigenin, baseline LDCL within the ACS subject cohort was elevated compared to that of the SAP and NVs (Figure 2.5.2). This result agrees with the hypothesis put forward by Buffon *et al* (2002) that a widespread inflammatory process occurs within those patients with an ACS. Like that demonstrated for the baseline LDCL data, the extent of aggregation-associated LDCL for the ACS subject population was significantly greater than that of the SAP and NV cohorts (Figure 2.5.3). Therefore, not only are ACS patients experiencing a widespread inflammatory process represent by an increased baseline LDCL, or a reduced level of myeloperoxidase as described by Buffon *et al* (2002), they also have the ability to release significantly greater amounts of superoxide upon platelet activation, a process that occurs readily in subjects with an ACS.

Potential mechanisms behind an increased ability of ACS patients to produce increased levels of superoxide compared to SAP patients were not explored within the current study. However, it has been reported that an ACS is associated with an enhanced inflammatory response (Liuzzo *et al.*, 1999). Concurring with the observations made herein, Takeshita *et al* (1997), utilizing luminol-dependent chemiluminescence demonstrated that whole blood chemiluminescent counts in ACS patients were twice that of SAP patients. Moreover, there was no significant difference in the numbers of polymorphonuclear leukocytes between the ACS and SAP cohorts (Takeshita *et al.*, 1997). Therefore these results suggest that an

absolute difference in immune effector cells capable of generating superoxide does not pre-determine the ability of ACS subjects to generate more superoxide than that of SAP patients. An examination of the immune effector cell profiles for each subject cohort was not performed in the current study.

Coronary risk factors/disease states and superoxide

Throughout the literature, risk factors for CAD have been associated with elevated levels of ROS. Superoxide production is increased in animal models and human subjects with hypercholesterolaemia (Miller *et al.*, 1998; Mugge *et al.*, 1994; Ohara *et al.*, 1993; Warnholtz *et al.*, 1999; White *et al.*, 1996), hypertension (Fukai *et al.*, 1999; Kerr *et al.*, 1999; Pagano *et al.*, 1997; Rajagopalan *et al.*, 1996) and heart failure (Ellis *et al.*, 2000). Moreover, an altered endothelium-dependent vascular relaxation has also been associated with an enhanced degradation of nitric oxide by superoxide in animal models of hypertension, diabetes, cigarette smoking and heart failure.

Herein the baseline and aggregation-associated LDCL parameters for ACS patients were greater than levels obtained from patients with SAP and NVs (Figures 2.5.2/2.5.3). The proportion of subjects with specific risk factors for CAD between the SAP and ACS were well matched apart from there being significantly more subjects with diabetes in the ACS subject cohort and significantly fewer with hypercholesterolaemia compared to the SAP patient cohort (Table 2.7/Appendix table 4). Given a number of investigations have demonstrated that diabetes is associated with elevated levels of ROS (Hink *et al.*, 2001; Keegan *et al.*, 1995; Mayhan, 1997; Nassar *et al.*, 2002), one can not totally rule out that the observed differences between the ACS and SAP patients cohorts regarding the extent of baseline and aggregation-associated LDCL, results from difference in proportion of subjects with diabetes mellitus.

Countering the argument that the proportion of subjects with diabetes is the sole reason behind the observed differences between the ACS and SAP patients regarding the extent of LDCL, the difference in the proportion of SAP patients with hypercholesterolaemia compared to ACS patients. Throughout the literature hypercholesterolaemia has long been associated with elevated levels of ROS (Cai and Harrison, 2000; Croft *et al.*, 1990; Davi *et al.*, 1997; Miller *et al.*, 1998; White *et al.*, 1996). Within this investigation both the ACS and

SAP patient groups were evenly matched regarding the use of statins. The study was therefore unable to determine the effect of statin use on the luminescent parameters examined.

Anti-anginal pharmacotherapies and superoxide

As summarized in Table 2.7, both the SAP and ACS subject cohorts were well matched regarding the use of various medications. Differences between the subject cohorts were observed only with the use of prophylactic nitrates. Identification of particular pharmacotherapeutic determinants that may influence the extent of LDCL was not possible with the current study design due to insufficient numbers. Therefore, one cannot exclude the possibility that the observed differences between the ACS and SAP subject populations regarding the extent of LDCL resulted from differences in nitrate use. A number of investigators have demonstrated that exposure to NTG and other organic nitrates results in enhanced superoxide formation, a phenomenon that has been proposed as a mechanism of nitrate tolerance (Dikalov *et al.*, 1999; Dikalov *et al.*, 1998a; Munzel *et al.*, 1996a). Moreover, prevention and/or reversal of nitrate tolerance has been demonstrated by some investigators with the use of scavengers or inhibitors of superoxide formation (Berkenboom *et al.*, 1999; Fink *et al.*, 1999).

Countering the argument that differences in LDCL between the ACS and SAP subject groups resulted from differences in the use of nitrates are in the differences in the use of *N*-acetylcysteine (trend towards significantly more ACS patients being treated with NAC compared to SAP patients). Known to potentate the effects of NTG at the platelet (Chirkov and Horowitz, 1996; Loscalzo, 1985) and vascular levels (Horowitz *et al.*, 1983; Mehra *et al.*, 1994; Winniford *et al.*, 1986), *N*-acetyl cysteine has also been suggested to function as an anti-oxidant (Aruoma *et al.*, 1989; Cabassi *et al.*, 2001). Therefore one may assume that the differences between the luminescent parameters across the two subject groups, would be theoretically less significant.

Delineating the exact contribution of organic nitrates and *N*-acetyl cysteine use towards the observed differences in luminescent parameters between the SAP and ACS patients is beyond the scope of the current study. Furthermore, differences between subjects receiving and not receiving ACE-inhibition (proportions of SAP and ACS patients were evenly matched) and

its effect on the luminescent parameters were also unable to be examined because of the current study design.

Platelet aggregability and superoxide

Within section II of this chapter, the extent of whole blood platelet aggregation was demonstrated to be associated with LDCL. Most notably, the extent of platelet aggregation was correlated with the extent of aggregation-associated LDCL. Within the current study this relationship was examined further by determining whether the extent of platelet aggregation was correlated with the baseline and aggregation-associated LDCL in NVs, SAP and ACS patients.

Baseline LDCL

Utilizing lucigenin at either concentration, the extent of platelet aggregation was not significantly correlated to the extent of baseline LDCL, with the exception of the NVs examined using lucigenin (125μM). Within the literature there is evidence demonstrating that platelets primed with sub-threshold concentrations of platelet agonists are activated and undergo aggregation following exposure to superoxide (Pratico *et al.*, 1993) and other reactive species (Ambrosio *et al.*, 1994; Iuliano *et al.*, 1994; Iuliano *et al.*, 1991). Furthermore, platelets that have been exposed to anoxia followed by reperfusion generate superoxide and hydroxyl radical which in turn activate the arachidonic acid pathway of platelet activation (Leo *et al.*, 1997). Given the extent of baseline LDCL was shown to be an inhibitable superoxide signal (section 2.4.4.3) and that lucigenin quantifies the extent of extracellularly released superoxide (Caldefie-Chezet *et al.*, 2002), absence of any relationship between the extent of platelet aggregability and the baseline-LDCL within the patient cohorts for both lucigenin concentrations (NVs also with 12.5μM lucigenin) suggests a minor role for baseline-LDCL and hence extracellularly derived superoxide in the phenomenon of platelet hyper-aggregability. This observation is in accordance with the results of section I of the current chapter in which there was no significant relationship between the extent of platelet aggregation and the degree of change in aggregation post administration of SOD/catalase. Given the differences in mean LDCL between the subject groups, this emphasizes an important role for superoxide in regulation of platelet aggregation in SAP/ACS patients, rather than hyper-aggregability as such.

Aggregation-associated LDCL

Within section II of this chapter, the extent of platelet aggregation was correlated with aggregation-associated LDCL for a small cohort of NVs. The extent of platelet aggregation was also related to the rate of aggregation-associated LDCL. Despite these initial observations, a number of conflicting results were documented when the relationship between aggregability and the extent of aggregation-associated LDCL was examined further in different subject cohorts. In a series of subjects that included NV, SAP and ACS patients no significant relationship was observed between the extent of platelet aggregation and aggregation-associated LDCL (Figure 2.5.5). The only exception to this observation was the NV cohort, where the extent of aggregation-associated LDCL was examined using lucigenin (125μM). As no other significant relationship was observed across the subject cohort using either concentration of lucigenin, it was concluded that the NV (lucigenin 125μM) result occurred through chance alone.

Reasons for observed differences in results between section II and the current study are multiple. One important difference exists between the subjects used to develop the technique (section II) and the subjects examined within the current section. Most if not all SAP and ACS patients examined within this section were receiving multiple anti-anginal medications at the time of study enrollment (Table 2.7). No subjects examined within section II were receiving medications that might influence platelet or superoxide function. It therefore remains a possibility that the anti-anginal medications used by the SAP and ACS patients cloud any potential relationship. A number of anti-anginal medications used by the patients within the current have anti-oxidant properties with examples including ACE-inhibitors and statins (Berkenboom *et al.*, 1999; Hornig *et al.*, 2001; Kalinowski *et al.*, 2002). Complicating the relationship even further, a number of anti-anginal medications and CAD risk factors have shown pro-oxidant properties (Ohara *et al.*, 1993; Dikalov *et al.*, 1999; Guzik *et al.*, 2000; Schoonmaker *et al.*, 2000).

Despite the use of particular anti-anginal pharmacotherapeutics or CAD risk factors, one would then assume that the relationship between the extent of platelet aggregation and the aggregation-associated LDCL would hold true for the NV subject cohort. No such relationship was observed when using lucigenin (12.5μM) as the probe. Reasons for this inconsistency may simply lie in insufficient numbers of NVs with a large spread of platelet

aggregability. Therefore specific studies are required to examine the possible clinical determinants of both the baseline and aggregation-associated LDCL.

Alternative explanations for an absence of any significant relationship between the extent of aggregation and aggregation-associated LDCL may simply lie in the differences between the concentrations of lucigenin used to examine the extent of aggregation-associated LDCL. As discussed before and at the time of commencement of this study, a high concentration of lucigenin was being utilized. This concentration was changed following the publication of a number of studies suggesting that higher concentrations of lucigenin may artificially elevate the level of superoxide (Skatchkov *et al.*, 1999; Vasquez-Vivar *et al.*, 1997) and thus give higher estimations of the level of superoxide generated post platelet aggregation. Given the observations of Afanas'ev *et al* (1999, 2001a, 20001b), it was decided to include the experiments that utilized lucigenin (125μM) in order to obtain the maximum understanding of superoxides role in the phenomenon of platelet hyper-aggregability. As discussed above, the proportion of LDCL (baseline/aggregation-associated) for all subjects were similar when comparing the extent of LDCL detected using either concentration of lucigenin. It therefore seems unlikely that a difference in concentration of lucigenin should serve as a reason for the absence of any significant relationship between platelet aggregability and aggregation-associated LDCL.

Platelet responsiveness towards nitric oxide and its relationship to superoxide

Within section 2.3.4.4, a relationship between baseline SNP responsiveness and the extent of change in SNP responsiveness post SOD/catalase administration was observed. These results imply a role for superoxide in the phenomenon of a reduced responsiveness to nitric oxide. To further investigate this potential mechanism, the relationship between platelet responsiveness to SNP and the extent of baseline/aggregation-associated LDCL was examined in a series of SAP and ACS patients. Utilizing two concentrations of lucigenin, no significant correlation was found between the extent of platelet responsiveness to SNP and baseline LDCL/aggregation-associated LDCL (Figure 2.5.6 and 2.5.7).

Given the results observed in section I (Figure 2.3.8) and III (Figure 2.5.2/2.5.3) of this chapter, it seems <u>unlikely</u> that superoxide has <u>no</u> role in the phenomenon of hypo-responsiveness to nitric oxide. Rather, one of the most likely explanations for an absence of

any significant relationship between the extent of platelet responsiveness to SNP and both LDCL parameters, is heterogeneity of the subject cohorts examined. Patients enrolled within the current study had multiple risk factors for CAD and were receiving multiple anti-anginal medications at the time of blood sampling. Such heterogeneity within the subject population may very well have obscured the relationship between the extent of platelet responsiveness to SNP and both luminescent parameters. The clinical determinants of not only the luminescent parameters but also platelet hypo-responsiveness to donors of nitric oxide are required. The latter forms the basis of the following chapter.

Platelet hypo-responsiveness to nitric oxide: mechanistic implications

Within section I of the current chapter, platelets from patients with ACS were more resistant to the anti-aggregatory effects of SNP than those from SAP and NV subjects. Within section III of this chapter, both baseline and aggregation-associated LDCL levels were elevated within samples from ACS patients compared to SAP and NV. These results therefore suggest that an excess superoxide augments platelet responsiveness to nitric oxide via alternative mechanism/s apart from a direct scavenging of nitric oxide. Such mechanisms may include the progressive oxidation of guanylate cyclase thus limiting the nitric oxide/cGMP dependent pathway of inhibition of aggregation or augmentation of a cGMP independent pathway of inhibition of platelet aggregation (discussed in section 2.3.5 of the current chapter).

[2.5.6] Study Limitations

A number of limitations exist within the current investigation, not least of which is the unmatched subject cohorts enrolled in the study. Differences in coronary risk factors and pharmacotherapies between the ACS and SAP patients reduces the probability that differences between the subject groups regarding the luminescent parameters results from differences in disease state alone. Despite this observation, the study was designed to examine the relationship between the extent of superoxide within a whole blood sample and the phenomena of platelet hyper-aggregability and hypo-responsiveness to donors of nitric oxide, within discreet subject populations. Like the limitations associated with section II of this chapter there was a significant age difference between the NV and the patient cohorts. However, this was inevitable because of the intent to minimize the recruitment of subjects to the NV subject cohort that may have had undiagnosed CAD. The numbers of subjects

recruited to each patient/NV cohort were insufficient to examine potential determinants that may influence the extent of the luminescence parameters examined.

In much the same way as Cu^{2+}/Zn^{2+}superoxide dismutase functions at an extracellular level to scavenge superoxide, it has now been proved that lucigenin functions only at an extracellular level (Caldefie-Chezet *et al.*, 2002). Given it is still implied that superoxide plays a functional role in the regulation of platelet aggregation in SAP and ACS patients along with platelet hypo-responsiveness to donors of nitric oxide, the failure to demonstrate any significant correlation between the luminescent and platelet parameters may suggest that the effects of superoxide largely function at an intra-platelet level.

Another important limitation is the method used herein to assess the level of superoxide. Concentrations of superoxide detected at an *ex vivo* level may be no reflection of the degree of superoxide that is available *in vivo*. Within a whole blood sample contributions of superoxide from various other sources, including the vascular endothelium (Griendling *et al.*, 2000) are unfortunately absent. A true assessment of the involvement of superoxide in the phenomenon of platelet hyper-aggregability and platelet hypo-responsiveness to nitric oxide, taking into consideration vascular sources of superoxide would only be feasible utilizing a "Folts" model of *in vivo* platelet function (Folts, 1991a). To date no investigation has demonstrated a reduced responsiveness to the anti-platelet effects of nitric oxide utilizing this model. However, the importance of endogenously derived nitric oxide (Yao *et al.*, 1992) and superoxide (Yao *et al.*, 1993) in the phenomenon of cyclical flow reductions has been examined.

[2.5.7] Conclusions

The extent of LDCL within a whole blood sample either prior to or post platelet aggregation obtained from either an SAP and ACS patients was found to be greater than that obtained from a NV. Despite this, no significant relationship was observed between the extent of platelet aggregation/degree of platelet responsiveness to SNP and any of the luminescence parameters. The extent of platelet aggregation and the degree of platelet responsiveness to SNP for SAP and ACS patients does not correlate with the extent of detectable LDCL, either prior to or post platelet aggregation.

[2.6] Chapter Summary

Overall the series of experiments that make up this chapter have established a number of firsts.

Section I: Agreeing with the results published by Chirkov *et al* (1999) and extending the observations to include ACS patients, platelets from patients with acute and chronic ischaemic heart disease are hyper-aggregable towards ADP and hypo-responsive to the anti-aggregatory effects of either SNP or NTG. Further to these observations, superoxide plays an important regulatory role in platelet aggregation for SAP and ACS patients along with serving as a potential mechanism for the attenuated platelet responsiveness to SNP commonly observed within these patients.

Section II: Within this section a method of detecting superoxide within a whole blood sample prior to and post platelet aggregation utilizing LDCL is described.

Section III: Within this section the extent of both baseline and aggregation-associated LDCL was examined with ACS patients having elevated LDCL compared to samples from NVs or SAP patients. Despite the clear difference in the luminescence variables across the subject cohorts examined, no significant relationship was observed between the extent of platelet aggregation and either the baseline or aggregation-associated LDCL, a result that was also observed when the degree of platelet responsiveness to SNP was compared with the luminescent parameters for the SAP and ACS patients. These results suggest that the amount of superoxide present within a whole blood sample prior to induction of platelet aggregation, or released following platelet activation plays a minor role in the phenomena of platelet hyper-aggregability and/or reduced responsiveness to the anti-aggregatory effects of nitric oxide.

Results from experiments within this chapter have contributed to a manuscript and a number of abstracts that were presented in a series of national and international meetings.

- Chirkov Y.Y., **A.S. Holmes**, L.P. Chirkov and J.D. Horowitz, 1999, Nitrate resistance in platelets from patients with stable angina pectoris, Circulation, 100, 129-134.

- **Holmes A.S.**, Y.Y. Chirkov and J.D. Horowitz, 1999, Increased superoxide concentration as a possible basis for platelet hyper-aggregability and nitric oxide resistance in patients with ischaemic heart disease. European Heart Journal, 20, 544.

- **Holmes A.S.**, Y.Y. Chirkov, J.D. Horowitz, 1999, Superoxide generation as a basis for platelet hyper-aggregability and nitric oxide resistance in patients with myocardial ischaemia, Australian and New Zealand Journal of Medicine, 29,6.

Chapter 3

Determinants of platelet hypo-responsiveness to nitric oxide (Nitric oxide resistance)

Chapter Overview

This chapter addresses possible determinants of a reduced platelet responsiveness to donors of nitric oxide in patients with acute and chronic ischaemic heart disease, the phenomenon of which was initially described by Chirkov *et al* (1993, 1996, 1999) and examined within section I of chapter 2. Within this context the chapter's purpose, results and conclusions are summarized below.

[3.1] Summary of the study examining the determinants of platelet hypo-responsiveness to donors of nitric oxide.

Objectives: Examination of the determinants of nitric oxide resistance at the platelet level in a number of subject cohorts that include normal volunteers (NV), NIPs, SAP and ACS patients.

Methods: The extent of ADP (1μM) -induced whole blood platelet aggregation and degree of responsiveness to SNP (10μM) and NTG (100μM) was assessed in a series of NVs (n = 43), patients with non-ischaemic chest pain (NIP, n = 35), patients with SAP (n = 82) and ACSs (n = 135). Possible determinants of nitric oxide resistance for SAP and ACS patients were then examined utilizing univariate and multivariate analysis.

Results: Platelets from patients with SAP and ACS were significantly more aggregable than those obtained from a cohort of NV or NIPs (3-way ANOVA $F = 7.53$, $p < 0.01$). Furthermore, platelets from patients with SAP and ACS exhibited a significant attenuation in responsiveness to SNP (2-way ANOVA $F = 19.8$, $p < 0.01$) and NTG (2-way ANOVA $F = 6.5$, $p < 0.01$). Following multivariate analysis on data obtained from patients with SAP and ACS, an attenuated response to SNP (representing nitric oxide resistance at the platelet level) was significantly more frequent in patients with ACS (odds ratio (OR) 2.3:1). Platelet responsiveness to donors of nitric oxide was also closely correlated to the extent of platelet aggregation, but not related with age or number of coronary risk factors. Coronary risk factors (male gender, ≥ 70 years of age, diabetes mellitus, hypertension, hypercholesterolaemia and smoking) were not significantly associated with an abnormal platelet responsiveness to SNP. Pharmacotherapy with various anti-anginal medications (aspirin, nitrates, ACE inhibitors, SH-donors, Ca^{2+} antagonists and β-adrenoreceptor antagonists) were also not significantly associated with variability in platelet responsiveness

to SNP. Platelet hypo-responsiveness to SNP was demonstrated to be significantly less common in subjects that were treated with perhexiline (OR: 0.3:1) or statins (OR: 0.45:1). However, platelets from SAP patients with triple vessel disease were significantly less responsive to SNP than those with stenoses in one or two vessels.

Conclusions: Patients with SAP and ACS exhibited an increase in platelet aggregability in response to ADP and hypo-responsiveness to the anti-aggregatory effects of NTG and SNP. Nitric oxide resistance in platelets was not correlated with any coronary risk factor examined but was strongly associated with ACS and with extensive coronary disease among patients with SAP. Pharmacotherapy with perhexiline and/or statins may improve platelet responsiveness to donors of nitric oxide.

Table 3.1 Abbreviations used in this chapter

Abbreviation	*Definition*	*Abbreviation*	*Definition*
ACE	*Angiotensin converting enzyme*	NAD(P)H	*Nicotinamide adenine dinucleotide phosphate*
ACS	*Acute coronary syndrome*	NIP	*Non-ischaemic patient*
ADP	*Adenosine di-phosphate*	NQAMI	*Non Q-wave acute myocardial infarction*
ANCOVA	*Analysis of covariance*	NTG	*Nitroglycerine*
ANOVA	*Analysis of variance*	NV	*Normal volunteer*
ASA	*Aspirin*	OR	*Odds ratio*
CAD	*Coronary artery disease*	ox-LDL	*Oxidized low density lipoprotein*
cGMP	*Cyclic guanosine monophosphate*	PROVE IT	*Pravastatin or Atorvastatin Evaluation and Infection Therapy*
CPT-1	*Carnitine palmitoyltransferase-1*	PRINCESS	*Prevention of Re-Infarction by Early Treatment with cerivastatin*
CRP	*C-reactive protein*	PRISM	*Platelet Receptor Inhibition in Ischaemic Syndrome Management*
eNOS	*Endothelial nitric oxide synthase*	SAP	*Stable angina pectoris*
KS	*Kolmogorov-Smirnov*	SEM	*Standard error of the mean*
GTP	*Guanosine tri-phosphate*	SNP	*Sodium nitroprusside*
MIRACL	*Myocardial Ischaemia Reduction with Aggressive Cholesterol lowering*	TIMP	*Tissue inhibitor of metalloproteinase*
MMP	*Matrix metalloproteinase*	UAP	*Unstable angina pectoris*

Determinants of nitric oxide resistance

[3.2] Introduction

[3.2.1] Endothelial dysfunction/nitric oxide resistance

The concept of endothelial dysfunction, initially introduced to describe impairment in the vascular responsiveness towards endothelium-dependent vasodilating agents, has been recently extended to encompass a loss, diminution or imbalance in other vascular functions, such as the anti-inflammatory and anti-thrombogenic properties of the vascular endothelium (Anderson, 1999; Raitakari and Celermajer, 2000; Ross, 1993).

Traditionally, subjects with risk factors for and/or (Reddy *et al.*, 1994; Zeiher *et al.*, 1991), established CAD (Ludmer *et al.*, 1986), as well as those with heart failure (Treasure *et al.*, 1990), demonstrate paradoxical vasoconstrictive responses to endothelium dependent vasodilating agents whilst still retaining intact vascular smooth muscle cell responsiveness to other agents.

In 1980, Armstrong *et al* observed that in some patients with chronic heart failure, pulmonary capillary wedge pressure fell by less than 25% despite the infusion of NTG at very high rates. This phenomenon was designated "nitrate resistance" (Armstrong *et al.*, 1980; Packer *et al.*, 1986). In retrospect, part of the basis for this phenomenon may be mechanisical i.e. diastolic ventricular interaction (Atherton *et al.*, 1997). However, numerous investigators since 1980 have provided evidence for the existence of hypo-responsiveness to NTG and other nitric oxide donors at the level of vascular smooth muscle in such patients. "Nitrate resistance", initially poorly understood (Abrams, 1991), has now been demonstrated to occur in both the systemic (Katz *et al.*, 1992) and coronary vascular beds (Schachinger and Zeiher, 1995).

In a study examining the responsiveness of the coronary arteries to exogenously and endogenously donated nitric oxide, Schachinger *et al* (2000) observed that diminished coronary vasodilatation to acetylcholine combined with resistance to the vascular effects of NTG, was associated, on multivariate analysis, with long term adverse cardiac events. Other investigators have also demonstrated that impairment of responses to nitric oxide donors in other vessels may be associated with paradoxical repossess to endothelium-dependent vasodilators, such as acetylcholine (Adams *et al.*, 1998).

In parallel with similar studies demonstrating impaired vascular responsiveness towards NTG, resistance to the anti-aggregatory effects of various nitric oxide donors has also been observed in platelets from subjects with CAD (Chirkov *et al.*, 1993; Chirkov *et al.*, 1996) and in some disease states or conditions that are risk factors for CAD development (Anfossi *et al.*, 1998; Haramaki *et al.*, 2001; Woods *et al.*, 1993).

[3.2.2] Anti-aggregatory properties of nitric oxide

As described within chapter 1 (A.11 and D.1.3.2), the physiological effects of organic nitrates (i.e. nitric oxide) are not only limited to vascular relaxation, but also include the inhibition and reversal of platelet activation/aggregation (Chirkov *et al.*, 1993; Geiger *et al.*, 1992; Gries *et al.*, 1998; Langford *et al.*, 1996; Nong *et al.*, 1997; Radomski *et al.*, 1987c; Sneddon and Vane, 1988; Yoshimoto *et al.*, 1999).

A detailed description of the nitric oxide dependent pathways involved in inhibition of platelet activation/aggregation is described in chapter 1 (section A.11). However, viewed simplistically, nitric oxide binds to the heme moiety of guanylate cyclase which catalyzes the conversion of guanosine tri-phosphate (GTP) to cyclic guanosine mono-phosphate (cGMP) (Anderson *et al.*, 1994b). cGMP then activates cGMP dependent protein kinases, promoting the re-uptake of Ca^{2+} within various intra-platelet stores, resulting in inhibition of platelet activation/aggregation (Body, 1996).

[3.2.3] Platelet hyper-aggregability

Platelet hypo-responsiveness to exogenous nitric oxide donors in patients with angina pectoris reflects an impaired physiological response to endogenous nitric oxide, and as such represents a theoretical mechanism by which these patients suffer from an increased risk of thrombotic events. Platelet hyper-aggregability documented in patients with SAP (Chirkov *et al.*, 1993; Diodati *et al.*, 1994) and UAP (Ault *et al.*, 1999; Grande *et al.*, 1990), or in subjects with risk factors for CAD (de Padua Mansur *et al.*, 1997; Mandal *et al.*, 1993), might theoretically result from attenuation of the anti-aggregatory effects of nitric oxide that function to counter balance pro-aggregatory mediators. Evidence of platelet hyper-aggregability in particular disease states or in subjects with risk factors for CAD and the mechanism/s and implications behind this phenomenon, are summarized within chapter 1 (section C.8).

[3.2.4] Significance

Viewing the phenomena of platelet hyper-aggregability and hypo-responsiveness to nitric oxide together, the impairment in responsiveness towards nitric oxide in patients with CAD implies a theoretical pro-thrombotic disease state. Indeed, those in most need of nitrate therapy may be the least likely to respond adequately, irrespective of prior nitrate exposure (nitrate tolerance). Hence these subjects may be the most likely to develop thrombotic complications associated with the condition.

Within this context the following investigation was designed to examine further the phenomenon of nitric oxide resistance at the platelet level, in a series of NVs and patients with acute and chronic ischaemic heart disease. Identification of the potential clinical correlates of nitric oxide resistance at the platelet level was undertaken. Common coronary risk factors, extent of fixed CAD, intensity of angina, current medication and the presence of an ACS were all examined as possible correlates of poor platelet responsiveness to nitric oxide.

[3.3] Study Hypotheses

This study was designed to test the following *null* hypotheses in 3 cohorts of subjects that included NVs, NIPs, patients with SAP or an ACS.

Primary:

- *Platelet hypo-responsiveness to SNP in blood samples obtained from a cohort of SAP and ACS patients does not vary with symptomatic status, a series of coronary risk factors or commonly used anti-anginal pharmacotherapy.*

Secondary:

- *In blood samples from SAP and ACS patients, platelet responsiveness to SNP is not correlated with the number of coronary risk factors, age or the extent of fixed coronary artery disease.*

- *In blood samples obtained from SAP patients, platelet responsiveness to SNP is not correlated with anginal severity.*

[3.4] Methods

[3.4.1] Subjects

Studies were performed on blood samples obtained from the following groups:

- Healthy normal volunteers (NV) (n = 43; 23 males and 20 females aged 23 to 76 years; mean 46) not taking any medication that may influence platelet function.

- NIPs (n = 35; 17 males and 18 females aged 35 to 77; mean 54) who had presented with chest pain but were found to have normal coronary arteries and no haemodynamically significant valvular heart disease, at cardiac catheterization and coronary angiography.

- Patients with SAP (n = 82; 57 males and 25 females aged 39 to 76; mean 64) undergoing elective diagnostic cardiac catheterization and coronary angiography. In all cases at least one haemodynamically significant (>50%) stenosis was present in a major coronary artery.

- ACS patients (n = 153; 95 males and 58 females aged 37 to 98 years; mean 67) were admitted for treatment of prolonged chest pain occurring at rest and were studied during the first few hours after admission; eventual diagnosis was UAP (n = 98) or NQAMI (n = 55), based on the presence/absence of transient elevation of creatine kinase and/or troponin I levels.

For all patients a background medication profile was recorded at the time of recruitment with the clinical characteristics of the study cohort being displayed in Table 3.2. No patient was receiving ADP- or glycoprotein IIb/IIIa-receptor antagonists at the time of recruitment. The numbers of subjects used in the individual experiments are indicated below (Results: Section 3.5). The study was approved by the North Western Adelaide Health Service Ethics of Human Research Committee, with informed consent being obtained prior to study entry.

[3.4.2] Blood Sampling

Blood samples were collected from both patients and healthy NVs according to the methods outlined in section 2.3.3 of chapter 2.

[3.4.3] Platelet aggregation studies

Platelet aggregation studies in whole blood samples were examined utilizing a dual-channel impedance aggregometer (Model 560, Chrono-Log, Haverstown, PA, USA). Platelet aggregation studies were performed according to the method described in section 2.3.3 of chapter 2.

[3.4.4] Chemicals

Chemicals utilized within this study were the same as described in section 2.3.3 of chapter 2.

[3.4.5] Statistical Analysis

Categorization of coronary risk factors

Male gender was considered as a risk factor and therefore all comparisons of platelet ADP responses were stratified according to gender. Plasma cholesterol level greater than 5 mmol/L was considered a coronary risk factor. Smokers were defined as subjects who were current smokers. Hypertension was defined as systolic blood pressure greater than 140 mm Hg or diastolic blood pressure greater than 90 mm Hg.

Statistical Analysis

Statistical analysis was performed using the computer programs outlined in section 2.3.3.6 of chapter 2. Differences between proportions of subjects with particular risk factors or medications were examined using Fisher's exact test. Gaussian distributions of data were determined by the Kolmogorov-Smirnov test. Homogeneity of variance was examined using Bartlett's test. Comparisons of platelet responses between NVs and patients were made utilizing ANOVA followed by Bonferroni's post hoc multiple comparison test. Differences between non-parametric data populations were assessed using the Kruskal Wallis test followed by Dunn's post hoc multiple comparison tests. Univariate analysis of the effects of each coronary risk factor and therapy on nitric oxide responsiveness was performed with 1-way ANOVA for categorical variables. Possible interactions among risk factors, therapies and platelet responsiveness to SNP were examined by stepwise multiple regression analysis (multivariate analysis). For the purpose of this analysis, nitric oxide resistance was categorized as hypo-responsiveness of platelets to SNP to an extent of 2 standard deviations (SD) below the mean response documented in healthy NVs. Significance of correlation was

determined by regression analysis with linearity determined by a run test. Comparisons between regression curves were made by ANCOVA. Differences between regression curves post ANCOVA was assessed using the Tukey/Kramer post hoc analysis. Non-parametric regression analysis was performed using Spearman rank correlation. Statistically significant differences were limited to $p < 0.05$ or $p < 0.01$ unless otherwise indicated. Results are expressed as mean ± S.E.M unless otherwise stated.

[3.5] Results

[3.5.1] Clinical characteristics

The clinical characteristics of the study cohort of NIPs (n = 35), SAP patients (n = 82) and ACS patients (n = 153) examined in this study are summarized in Table 3.2.

Risk factors

Of the coronary risk factors examined (male gender, age equal to or over 70, diabetes mellitus, systemic hypertension, hypercholesterolaemia and smoking) the median ($25^{th}/75^{th}$ percentiles) number of coronary risk factors was 1 (1/2) in NIPs, 3 (2/3) in patients with SAP and 3 (2/3) in ACS patients. Utilizing a Kruskal Wallis test, significant differences in the numbers of coronary risk factors was observed across the three subject cohorts (Kruskal Wallis: KW = 92.9, $p < 0.01$). Utilizing Dunn's post hoc multiple comparison test there was no significant difference in the number of risk factors between the SAP and ACS patient cohorts but there was between the SAP and NIP and between the ACS and NIP subject groups ($p < 0.01$).

There were no significant differences between SAP and ACS groups regarding the numbers of subjects with particular risk factors. However, the proportion of subjects ≥ 70 years of age and the numbers of current smokers within the ACS cohort tended to be greater compared to the SAP subjects (Fisher's exact test: $p = 0.054$ and $p = 0.079$ respectively) (see Appendix Table 5 for summary). Apart from the numbers of male subjects within the ACS cohort and the numbers of current smokers, both the SAP and ACS patient populations had a significantly greater proportion of subjects with particular coronary risk factors compared to the cohort of NIPs (see Appendix Table 6 for summary).

Medication

Almost all patients with SAP or an ACS were on multiple anti-anginal pharmacotherapy at the time of study enrolment. The numbers of cases in respect to therapy with aspirin, nitrates, ACE inhibitors, sulphydryl donors (SH) (N-acetyl-cysteine or captopril), perhexiline, statins, Ca^{2+} antagonists and β-adrenoceptor antagonists for patients with SAP and ACS patients were sufficient for multivariate analysis.

The proportion of ACS patients being treated with nitrates was significantly greater than that of SAP patients (Fishers exact test: p = 0.019, Appendix Table 5). The proportion of SAP patients being treated with aspirin tended to be greater than ACS patients (Fishers exact test: p = 0.054, Appendix Table 5). Apart from SH donor, ACE inhibitor and perhexiline pharmacotherapy, a significantly greater proportion of subjects in both the SAP and ACS subject cohorts were receiving aspirin, nitrates, statin (ACS only), and β-adrenoceptor antagonist (SAP only) therapy compared to the NIP cohort (results summarized in Appendix Table 6).

Table 3.2 Clinical characteristics of the patient cohorts

Clinical Characteristics	*Non ischaemic Patients (n = 35)*	*Stable Angina Pectoris Patients (n =82)*	*Acute Coronary Syndrome Patients (n = 153)*
Men / Females, n	17/18	57/25	95/58
Age (Mean ± S.D), years	54 ± 12	64 ± 10	67 ± 10
Diabetes, n (%)	1 (3)	25 (30)	40 (26)
Hypertension, n (%)	9 (25)	43 (52)	83 (54)
Hypercholesterolaemia, n (%)	5 (14)	42 (51)	73 (48)
Smoking, n (%)	5 (14)	10 (12)	34 (22)
Medications			
Aspirin, n (%)	10 (29)	69 (84)	111 (73)
Nitrates, n (%)	6 (17)	47 (57)	112 (73)
ACE Inhibition, n (%)	4 (11)	21 (26)	35 (23)
SH-donors (e.g. captopril/NAC, n (%)	0 (0)	10 (12)	30 (20)
Perhexiline, n (%)	1 (3)	12 (15)	19 (12)
Statins, n (%)	5 (14)	29 (35)	41 (27)
Ca^{2+} Antagonists, n (%)	14 (40)	48 (59)	90 (59)
β-adrenoceptor antagonists, n (%)	2 (6)	20 (24)	25 (16)

Statistical comparisons between subject cohorts regarding the clinical determinants and medication profiles are summarized in Appendix Table 5/6. SH-donors within the SAP cohort equals captopril only.

[3.5.2] Platelet aggregability

Platelet response to ADP (1μM) was examined in a series of blood samples obtained from NVs, NIPs with normal coronary arteries at coronary angiography, patients with SAP and ACS patients. As with the platelet aggregation studies performed in chapter 2 sections 2.3 and 2.5 a low concentration of ADP (1μM), which has been shown not to induce the release of thromboxane A_2 (Kinlough-Rathbone *et al.*, 1983), was utilized. A summary of the mean platelet responses for each clinical condition and classified according to gender and aspirin utilization is displayed in Table 3.3.

Table 3.3 Platelet aggregability in response to ADP

	NV	*NIP*		*SAP*		*ACS*	
	-ASA	-ASA	+ASA	-ASA	+ASA	-ASA	+ASA
Males	7.7 ± 0.7 (23)	8.0 ± 1.3 (11)	6.7 ± 1.3 (6)	12.1 ± 0.9 (9)	8.1 ± 0.5 (48)	12.3 ± 1.0 (26)	9.3 ± 0.5 (68)
Females	9.9 ± 0.6 (20)	9.2 ± 1.1 (12)	10.1 ± 1.1 (6)	11.3 ± 2.2 (4)	13 ± 1.0 (21)	12.7 ± 1.1 (16)	12.8 ± 0.7 (41)

Samples were obtained from NVs, NIPs, SAP and ACS patients. All subjects were categorized according to clinical condition, aspirin utilization and gender. Numbers of subjects are indicated in parentheses with the data representing the mean ± S.E.M.

As displayed within Appendix Table 7 and utilizing the Kolmogorov-Smirnov method for the assessment of distribution of data, the extent of platelet response to ADP (1μM) within each subject group (NV, NIP, SAP and ACS) across both genders and between those subjects utilizing aspirin or not, were demonstrated to conform to a Gaussian distribution.

Gender-related differences and variable aspirin pharmacotherapy across the cohorts of subjects examined potentially complicate assessment of platelet responses to ADP (Meade *et al.*, 1985). Therefore platelet responsiveness towards ADP (1μM) was firstly analyzed according to clinical condition and gender. By 2-way ANOVA a significant difference in platelet responsiveness towards ADP (1μM) was observed across the clinical conditions and between genders (Bartlett's statistic = 9.09, p = 0.24). No significant interactions between these two determinants were observed (See Table 3.4 for summary).

Table 3.4 Platelet response to ADP
Two-way ANOVA contingency table

Determinants	*F*	*p*
Disease State	4.52	< 0.01
Gender	21.86	< 0.01
Interaction		
Disease State/Gender	0.57	0.632

Samples for all subjects listed within Table 3.3 were analyzed according to their clinical condition (Disease State) and gender. No significant interaction between these two determinants was observed.

In order to determine the influence of aspirin pharmacotherapy upon platelet responsiveness towards ADP (1μM), aspirin utilization was included as a determinant. Given that no NV was receiving aspirin at the time of study enrolment, this population was omitted from the analyses. Platelet responsiveness towards ADP (1μM) for samples obtained from NIPs, SAP and ACS patients were then analyzed accordingly with levels of significance being displayed within Table 3.5 (Bartlett's statistic = 11.6, p = 0.39). In much the same way as the results shown in Table 3.4, a significant difference in platelet responsiveness towards ADP (1μM) was noted across the clinical conditions (disease state) and between genders. Aspirin utilization per se was not found to significantly influence platelet aggregability towards ADP (1μM) within this cohort of subjects (ANOVA: F = 1.73, p = 0.19). Interestingly a significant interaction between aspirin utilization and gender was noted. Utilizing Bonferroni's post hoc multiple comparison test, a number of significant differences in the extent of platelet aggregability were observed. For a summary see Appendix Table 8.

Table 3.5 Platelet response to ADP
Three-way ANOVA contingency table

Determinants	*F*	*p*
Disease State	7.53	< 0.01
Aspirin Utilization	1.73	0.19
Gender	8.25	< 0.01
Interactions		
Disease State/Aspirin Utilization	0.25	0.78
Disease State/Gender	0.02	0.98
Aspirin Utilization/Gender	6.41	0.01
Disease State/Aspirin Utilization/Gender	0.56	0.57

Samples were obtained from NIPs, SAP patients and ACS patients. Numbers and mean platelet responsiveness towards ADP (1μM) are as shown in Table 3.3. Platelet responsiveness towards ADP (1μM) in NVs was omitted from this analysis as no NV was receiving aspirin at the time of study enrolment.

Concentrating on the phenomenon of differential inhibition of platelet aggregation by aspirin across the genders (Table 3.5) and as displayed in Figure 3.1, aspirin was significantly more effective in males than females. This was only observed within the SAP/ACS populations. NIP male no ASA vs NIP male ASA unpaired *t*-test $t = 0.65$, $p = 0.52$; NIP female no ASA vs NIP female ASA $t = 0.47$, $p = 0.64$; SAP male no ASA vs SAP male ASA $t = 3.44$, $p < 0.01$; SAP female no ASA vs SAP female ASA $t = 0.7$, p 0.49; ACS male no ASA vs ACS male ASA $t = 2.81$, $p < 0.01$; ACS female no ASA vs ACS female ASA $t = 0.1$, $p = 0.92$).

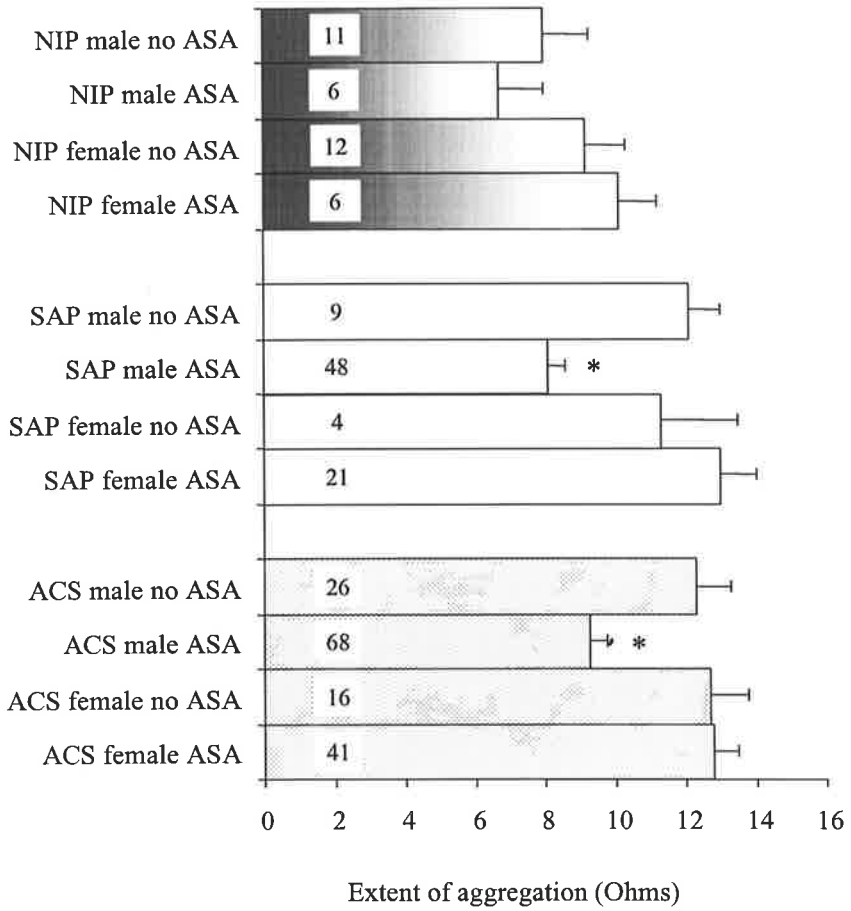


Figure 3.1 Differential inhibition of platelet aggregation by aspirin

*Aspirin use was associated with a significant inhibition ADP-induced platelet aggregation in male SAP/ACS subjects only. No significant inhibition of ADP-induced platelet aggregation was observed within the female SAP/ACS subjects. (numbers of subjects shown in the bars). * $p < 0.01$.*

Platelet aggregability in ACS patients

Platelet response to ADP (1μM) in ACS patients was then analyzed according to the final diagnosis of UAP or NQAMI, gender and aspirin pharmacotherapy. Mean platelet response

to ADP (1μM) for these categories are summarized in Table 3.6. All data populations conformed to a Gaussian distribution (Kolmogorov-Smirnov: UAP; Males -ASA KS = 0.29, p = ns; Males +ASA KS = 0.06, p = ns; Females –ASA KS = 0.18, p = ns; Females +ASA KS = 0.19, p = ns: NQAMI patients Males-ASA KS = 0.25, p = ns; Males +ASA KS = 0.25, p = ns; Males –ASA KS = 0.09, p = ns; Females -ASA KS = 0.21, p = ns; Females +ASA KS = 0.13, p = ns).

Table 3.6 Platelet aggregability in response to ADP(ACS patients)

	Unstable Angina Pectoris Patients		*Non Q-wave Acute Myocardial Infarction*	
	- ASA	+ ASA	- ASA	+ ASA
Men	13.3 ± 1.4 (14)	10.3 ± 0.7 (48)	11.1 ± 1.5 (12)	6.9 ± 0.7 (21)
Females	12.5 ± 1.2 (8)	12.5 ± 0.8 (28)	12.9 ± 1.9 (8)	13.5 ± 1.3 (14)

Samples were obtained from ACS patients with either UAP or a NQAMI and were categorized according to aspirin utilization and gender. Numbers of subjects are indicated in parentheses with the data representing the mean ± S.E.M. See Table 3.5 for 3-way ANOVA summary. -ASA = no aspirin, +ASA = aspirin pharmacotherapy.

In order to determine if the clinical condition (UAP/NQAMI), gender and aspirin utilization, influences platelet responsiveness towards ADP (1μM) in samples from subjects with an ACS, a 3-way ANOVA was performed (Bartlett's statistic = 4.04, p = 0.77). As summarized in Table 3.4 no significant difference in platelet responsiveness towards ADP (1μM) was observed between subjects diagnosed with UAP or a NQAMI. However, there was a significant difference in aggregability between genders and a trend towards a significant reduction in platelet responsiveness towards ADP (1μM) due to aspirin utilization. A number of significant interactions between the determinants were also observed. Both disease state and aspirin utilization significantly interacted with gender. Evaluation of the interactions revealed a significant disease state-gender interaction. Utilizing Bonferroni's post hoc multiple comparison test, male NQAMI patients not receiving aspirin were significantly less aggregable than their UAP counterparts ($p < 0.01$). No other significant differences were observed regarding the disease state-gender interaction. As previously observed the aspirin utilization-gender interaction was significant, with lower ADP responses in males treated with aspirin.

Table 3.7 Platelet response to ADP (ACS patients) three-way ANOVA contingency table

Determinants	*F*	*p*
Disease State	1.56	0.21
Aspirin Utilization	3.65	0.058
Gender	8.0	< 0.01
Interactions		
Disease State/Aspirin Utilization	0.03	0.87
Disease State/Gender	4.37	0.038
Aspirin Utilization/Gender	5.3	0.024
Disease State/Aspirin Utilization/Gender	0.28	0.61

Platelet aggregability in response to ADP (1μM) in samples obtained from ACS patients. Each sample was classified according to the final diagnosis of either UAP or a NQAMI, aspirin utilization and gender. Responsiveness and numbers of subjects used in each category are displayed in Table 3.6.

[3.5.3] Inhibition of platelet aggregation by sodium nitroprusside and nitroglycerine

Platelet responsiveness to sodium nitroprusside

SNP (10μM) significantly inhibited platelet aggregation in samples from all the cohorts of subjects examined, but to varying degrees, consistent with the results displayed in section I of chapter 2. Initially all subjects were analyzed according to their clinical condition and gender and conformed to a Gaussian distribution (Kolmogorov-Smirnov: Male NV KS = 0.19, p = ns; Female NV KS = 0.17, p = ns; Male NIP KS = 0.18, p = ns; Female NIP KS = 0.13, p = ns; Male SAP KS = 0.07, p = ns; Female SAP KS = 0.15, p = ns; Male ACS KS = 0.09, p = ns; Female ACS KS = 0.12, p = ns).

Following a 2-way ANOVA a significant difference in platelet responsiveness to SNP was observed across the cohorts of subjects examined (Bartlett's statistic = 4.94, p = 0.67). There was no significant gender-related difference regarding platelet responsiveness to SNP, confirming the preliminary observations made in chapter 2 section 2.3.4.3. No significant interaction between the two determinants was also observed. Utilizing Bonferroni's post hoc multiple comparison test a number of significant differences were observed (Appendix Table 9). For a summary of the degrees of significance see Table 3.8.

Table 3.8 Platelet responsiveness to SNP two-way ANOVA contingency table

Determinants	*F*	*p*
Disease State	19.88	< 0.01
Gender	0.09	0.76
Interactions		
Disease State/Gender	0.46	0.71

Platelet responsiveness to SNP was assessed according to disease state (NV,NIP, SAP, ACS) and gender. A significant difference in inhibition of aggregation by SNP was apparent between the subject cohorts with no significant role for gender within the populations examined. For a final summary of platelet responsiveness to SNP for all the subject cohorts examined see Figure 3.1.

In order to confirm the results obtained in chapter 2 section 2.3.4.3 where aspirin utilization was also shown not to influence platelet responsiveness to SNP, platelet responsiveness to SNP in samples obtained from NIP, SAP and ACS patients was further analyzed according to aspirin utilization. Platelet responsiveness to SNP from the NV population was omitted from this analysis as no NV was receiving aspirin at the time of study enrolment. All data populations with sufficient numbers to perform the test were demonstrated to conform to a Gaussian distribution (Appendix Table 10).

A summary of the results from the 3-way ANOVA is displayed in Table 3.9 and confirming the results shown in Table 3.8 and chapter 2 section 2.3.4.3 where a significant difference in platelet responsiveness to SNP was found only across the disease states examined and not due to aspirin utilization or gender (Bartlett's statistic = 11.3, $p = 0.42$). The interactions between the examined determinants were also not significant. Utilizing Bonferroni's post hoc multiple comparison test platelet responsiveness to SNP within the NIP subject cohort not receiving aspirin was demonstrated to be significantly greater than that of female SAP patients receiving aspirin ($p < 0.01$), male ACS patients receiving ($p < 0.05$) and not receiving aspirin ($p < 0.01$), and female ACS patients not receiving aspirin ($p < 0.05$).

Table 3.9 Platelet responsiveness to SNP three-way ANOVA contingency table

Determinants	*F*	*p*
Disease State	17.95	< 0.01
Aspirin Utilization	0.93	0.34
Gender	0.61	0.43
Interactions		
Disease State/Aspirin Utilization	0.20	0.82
Disease State/Gender	0.89	0.41
Aspirin Utilization/Gender	1.23	0.27
Disease State/Aspirin Utilization/Gender	1.61	0.20

Platelet responsiveness to SNP in NIP, SAP and ACS patients was analyzed according to disease state, aspirin utilization and gender. Responsiveness to SNP within the cohort of NVs was omitted from this analysis as no NV was receiving aspirin at the time of study enrolment.

Given that there was no significant influence of gender or aspirin utilization on platelet responsiveness to SNP within the cohorts of subjects examined, these results were pooled and analyzed by 1-way ANOVA (Kolmogorov-Smirnov: NV KS = 0.089, p = ns; NIP KS = 0.068, p = ns; SAP KS = 0.12, p = ns; ACS KS = 0.14, p = ns; Bartlett's statistic = 1.17, p = 0.76).

In accordance with the results initially shown in chapter 2 (section 2.3.4.3) platelet responsiveness to SNP was significantly attenuated in samples obtained from patients with SAP and ACS patients compared to the NV and NIP cohorts (1-way ANOVA: F = 16.6, p < 0.01). Utilizing Bonferroni's post hoc multiple comparison test a number of differences between each subject cohort were observed (NIP vs NV t = 0.41, p = ns; SAP vs NV t = 5.0, p < 0.01; SAP vs NIP t = 4.4, p < 0.01; ACS vs NV t = 6.8, p < 0.01; ACS vs NIP t = 5.7, p < 0.01; ACS vs SAP t = 1.7, p = 0.09).

The mean platelet responsiveness to SNP did not differ significantly between the cohort of SAP and ACS patients despite there being a trend towards a significant difference (Unpaired t-test: p = 0.09).

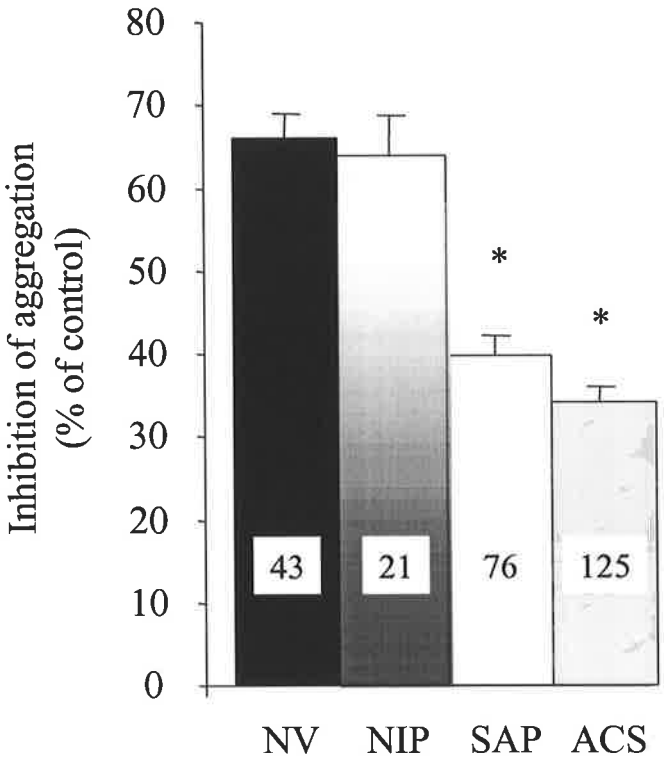


Figure 3.2 Inhibition of platelet aggregation by SNP

*Inhibition of ADP (1μM) induced platelet aggregation by SNP (10μM), in whole blood samples obtained from NVs, NIPs, and patients with SAP and ACS patients. Numbers of subjects indicated within the bars. Overall 1-way ANOVA F = 16.6, p < 0.01; * p < 0.01 vs. N.V and NIP by Bonferroni's post hoc multiple comparison test.*

Platelet responsiveness to nitroglycerine

NTG inhibition of platelet aggregation also varied among the subjects examined. NTG responsiveness for all the cohorts of subjects was analyzed according to disease-state and gender, to see if each sub-group conformed to a Gaussian distribution. (Kolmogorov-Smirnov: Male NV KS = 0.32, p = ns; Female NV KS = 0.16; Male NIP KS = 0.24, p = ns; Female NIP KS = 0.16, p = ns; Male SAP KS = 0.25, p = ns; Female SAP KS = 0.17, p = ns; Male ACS KS = 0.11, p = ns; Female ACS KS = 0.11, p = ns).

By 2-way ANOVA a significant difference across the cohorts of subjects was noted. No gender-related differences in platelet responsiveness to NTG were found. However, a significant interaction between the clinical condition and gender was observed (Bartlett's statistic = 2.17, p = 0.95). Interestingly this disease-state gender interaction was not observed when assessing platelet responsiveness to SNP (Table 3.8). For a summary of the degrees of significance see Table 3.10.

Table 3.10 Platelet responsiveness to NTG two way ANOVA contingency table

Determinants	*F*	*p*
Disease State	6.46	$p < 0.01$
Gender	0.45	0.51
Interactions		
Disease State/Gender	3.78	0.01

Platelet responsiveness towards NTG was analyzed according to disease state (NV, NIP, SAP and ACS) and gender.

In order to determine whether aspirin utilization influences platelet responsiveness to NTG, results from NIP, SAP and ACS patients were analyzed by 3-way ANOVA (Each sub-group with sufficient numbers to perform the test were demonstrated to conform to a Gaussian distribution Appendix Table 11). As with the analysis regarding platelet responsiveness towards ADP and SNP, responsiveness towards NTG within samples obtained from NV was omitted from this analysis as no NV was receiving aspirin at the time of study enrollment.

As summarized in Table 3.11, aspirin utilization did not influence platelet responsiveness towards NTG in NIP, SAP and ACS patients (Bartlett's statistic = 12.2, p = 0.34). Confirming the results observed in Table 3.10, disease-state did influence platelet responsiveness towards NTG, with gender playing no significant role. Platelet responsiveness towards NTG was also found not to be influenced by aspirin utilization. The interaction between aspirin utilization and gender tended towards significance, with a probability of 0.081. However, interpretation of this trend must be done with caution, as there were low numbers of both male and female SAP patients not receiving aspirin. No significant interaction was observed between disease-state and gender, casting doubt on the importance of the significant interaction observed using a 2-way ANOVA and data from all subject cohorts (Table 3.10).

Table 3.11 Platelet responsiveness to NTG three way ANOVA contingency table

Determinants	*F*	*p*
Disease State	4.01	0.021
Aspirin Utilization	1.21	0.27
Gender	0.47	0.49
Interactions		
Disease State/Aspirin Utilization	1.51	0.23
Disease State/Gender	1.51	0.23
Aspirin Utilization/Gender	3.11	0.08
Disease State/Aspirin Utilization/Gender	1.97	0.14

Platelet responsiveness towards NTG was analyzed in samples obtained from NIP, SAP and ACS patients. A significant difference in platelet responsiveness towards NTG was observed across the subjects examined with a trend towards a significant interaction between aspirin utilization and gender.

Given that neither gender nor aspirin was found to significantly influence platelet responsiveness towards NTG within the cohorts of subjects examined, these results were pooled and are displayed in Figure 3.3. (Kolmogorov-Smirnov: pooled data NV KS = 0.16, p = ns; NIP KS = 0.15, p = ns; SAP KS = 0.09, p = ns; ACS KS = 0.079, p = ns; Bartlett's statistic = 2.17, p = 0.54). By 1-way ANOVA platelet responsiveness towards NTG within the NV and NIP cohorts was significantly greater than that of the SAP and ACS patients (1-way ANOVA: $F = 5.85$, $p < 0.01$). Utilizing Bonferroni's post hoc multiple comparison test platelet responsiveness towards NTG within the cohorts of NV and NIP were significantly greater than that of SAP (NV vs SAP $t = 3.4$, $p < 0.01$, NIP vs SAP $t = 3.0$, $p < 0.05$) and ACS patients (NV vs ACS $t = 2.86$, $p < 0.05$, NIP vs ACS $t = 2.5$ p = ns; $t_{crit} = 2.67$). The difference between the NIP and ACS subject cohorts was not significantly different despite a trend towards a significant difference.

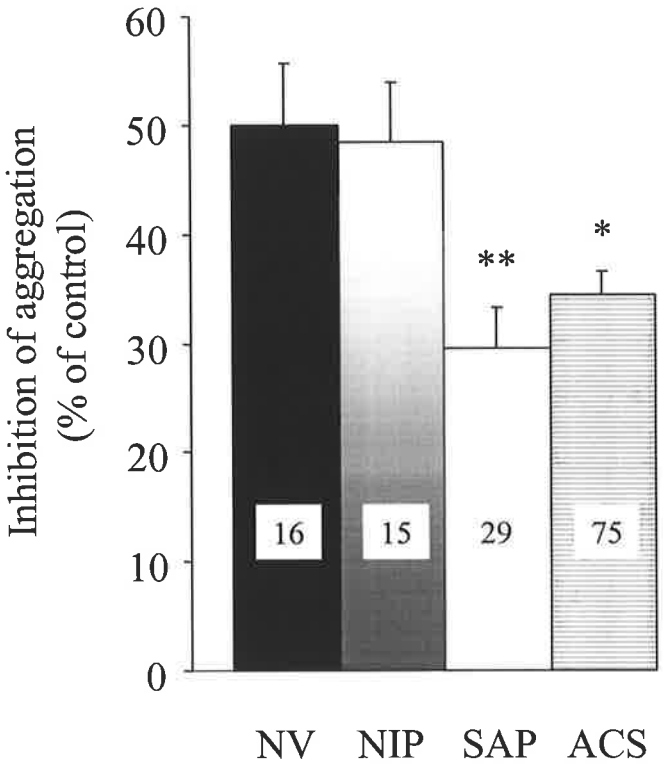


Figure 3.3 Inhibition of platelet aggregation by NTG

*Inhibition of ADP (1μM) induced platelet aggregation by NTG (100μM), in whole blood samples obtained from NVs, NIP, and patients with SAP and patients with an ACS. Numbers of subjects indicated within the bars. Overall by one way ANOVA; $F = 5.85$, $p < 0.01$, * $p < 0.05$, ** $p < 0.01$ vs NV and NIP by Dunnett's multiple comparison test.*

Platelet responsiveness to SNP and NTG in ACS patients

ACS patients can be further classified according to the final diagnosis of either UAP or NQAMI. In order to determine if platelet responsiveness to SNP and NTG varied according to this final diagnosis, the following series of analyses was performed.

Sodium nitroprusside

Platelet responsiveness to SNP within the cohort of ACS patients was classified according to the final diagnosis of UAP or NQAMI, gender and aspirin utility. Data representing each sub-group conformed to a Gaussian distribution (Appendix Table 12). Following a 3-way ANOVA there were no significant differences between the determinants of an SNP response within the ACS (Table 3.12) with no significant interactions between the determinants (Bartlett's statistic = 13.7, p = 0.055). Utilizing a Bonferroni's post hoc multiple comparison test there were no significant differences between any combination of determinants.

Table 3.12 Platelet responsiveness to SNP (ACS patients) three-way ANOVA contingency table

Determinants	*F*	*p*
Disease State	0.009	0.92
Gender	0.81	0.37
Aspirin Utilization	0.05	0.82
Interactions		
Disease State/Gender	0.35	0.55
Disease State/Aspirin Utilization	0.11	0.74
Gender/Aspirin Utilization	0.01	0.92
Disease State/Gender/Aspirin Utilization	0.27	0.60

Platelet responsiveness to SNP within ACS patients that have been analyzed according to the final diagnosis of either UAP/NQAMI, gender and aspirin utilization.

Nitroglycerine

As shown in Tables 3.10/11, a significant interaction (disease state/gender) and a trend towards an interaction (aspirin utilization/gender) were noted regarding platelet responsiveness to NTG in samples from NV, NIP, SAP and ACS patients. Accordingly, platelet responsiveness to NTG within the cohort of ACS patients that had been further classified according to the final diagnosis of UAP or a NQAMI, was also analyzed according to aspirin utilization and gender. Data representing platelet responsiveness to NTG within each of the aforementioned sub-groups followed a Gaussian distribution (Appendix Table 13).

As shown in Table 3.11, a significant difference between subjects diagnosed with UAP or NQAMI was observed (Bartlett's statistic = 10.9, p = 0.14). No significant difference in platelet responsiveness to NTG was observed between those treated or not treated with aspirin, or between genders. Unlike the results displayed in Table 3.11/12 no significant interaction or trend towards an interaction between the disease state and gender, or aspirin utilization was observed. Utilizing Bonferroni's post hoc multiple comparison test male NQAMI patients not receiving aspirin were significantly more responsive to NTG than the male UAP patients receiving aspirin ($p < 0.05$).

Table 3.13 Platelet responsiveness towards NTG (ACS patients) three-way ANOVA contingency table

Determinants	*F*	*p*
Disease State	5.47	0.022
Aspirin Utilization	2.33	0.13
Gender	0.02	0.88
Interactions		
Disease State/Aspirin Utilization	0.76	0.39
Disease State/Gender	0.79	0.37
Aspirin Utilization/Gender	0.07	0.78
Disease State/Aspirin Utilization/Gender	0.42	0.52

Platelet responsiveness towards NTG within the ACS subject cohort was classified according to the final diagnosis of UAP or a NQAMI. Aspirin utilization and gender was also included within the analyses.

Given that no significant difference in platelet responsiveness to SNP and NTG was found as regards to gender or aspirin, these results were pooled. A figure summarizing platelet responsiveness to SNP and NTG within the ACS subject cohorts classified according to the final diagnosis of either UAP or NQAMI is displayed within Figure 3.4. The pooled data populations representing platelet responsiveness to SNP was shown to follow a Gaussian distribution (Kolmogorov-Smirnov: UAP KS = 0.12, p = ns; NQAMI KS = 0.076, p = ns) but to contain significant differences between the standard deviations (F = 1.97, p = 0.0178). Accordingly a log transformation of the data was performed (KS post log transformation UAP KS = 0.09, p = ns; NQAMI KS = 0.16, p = ns) to create equivalent standard deviations (F = 1.29, p = 0.18). By unpaired analysis on log transformed data there was no significant difference in the extent of platelet responsiveness to SNP between those subjects with a final diagnosis of UAP or a NQAMI (unpaired *t*-test t = 0.24, p = 0.81). Within the NTG data populations each sub-group was demonstrated to conform to a Gaussian distribution and to have non-significantly different standard deviations (Kolmogorov-Smirnov UAP KS = 0.11, p = ns; NQAMI KS = 0.12, p = 0.46). Utilizing unpaired analysis platelet responsiveness to NTG was significantly less in the UAP subject cohort compared to those subjects with NQAMI (unpaired *t*-test t = 3.1, $p < 0.01$).

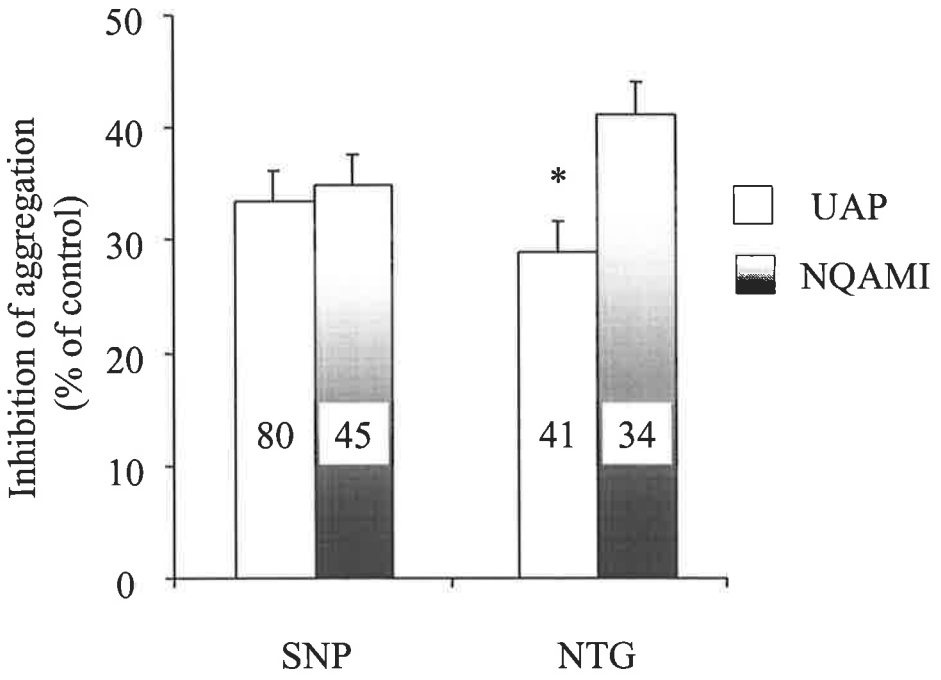


Figure 3.4 Platelet responsiveness to SNP and NTG in ACS patients

*Platelet responsiveness to SNP (10μM) and NTG (100μM) in subjects with an ACS; patients with UAP and patients with a NQAMI. Numbers of subjects studied are indicated within the bars. Unpaired t-test * $p < 0.01$ vs. NQAMI.*

In an attempt to identify the mechanism/s behind a significant difference in NTG responsiveness between UAP and the NQAMI patients, the clinical characteristics of the patient cohort were examined. The proportion of males and patients with diabetes, hypertension, high cholesterol, and current smokers, were well matched across the two patient cohorts. The proportion of patients treated with nitrates, SH-donors (NAC/captopril), perhexiline, statins, Ca^{2+} antagonists and β-blockers were also well matched. However, there were significantly greater UAP patients being treated with aspirin and significantly fewer UAP patients receiving ACE-inhibitors (Fishers exact test: aspirin UAP (68%) vs NQAMI (41%) $p = 0.022$: ACE-inhibition UAP (12%) vs NQAMI (32%) $p = 0.048$). Given aspirin use was shown not to be a significant determinant of NTG-responsiveness within the ANOVA (Table 3.13), it therefore seems unlikely that an absolute difference in aspirin use between the two patient cohorts would serve as the mechanism behind attenuated platelet responsiveness to NTG in UAP patients compared to NQAMI subjects.

[3.5.4] Inter-relationship between aggregability and platelet responsiveness to nitric oxide

Platelet aggregability versus SNP responsiveness

In order to exclude the possibility that the observed reduced degree of platelet responsiveness to SNP within SAP and ACS patients is not an epi-phenomenon of an increase in the extent of platelet aggregability, the relationship between platelet aggregation and platelet responsiveness to SNP was examined across all subject cohorts.

Platelet aggregability versus SNP responsiveness for each clinical condition was assessed according to gender. By ANCOVA, no gender-related differences were observed between platelet aggregability and SNP responsiveness for each of the clinical conditions examined (NV, NIP and SAP patients) (Appendix Table 14). Therefore these results were pooled despite there being a trend towards a significant difference between genders within the SAP cohort (ANCOVA: $F = 3.39$, $p = 0.068$). Data for ACS patients can also be analyzed according to the final diagnosis of either UAP or a NQAMI and then by gender alone. The relationship between platelet aggregability and SNP responsiveness was examined. No gender-related differences regarding the relationship between platelet aggregability and SNP responsiveness were found within the UAP/NQAMI subsets nor within the pooled ACS subject populations (Appendix Table 14).

Given that no gender-related differences were observed regarding the relationship between platelet aggregability and SNP responsiveness, the data were then analyzed according to the final clinical condition of a NV, NIP, SAP or ACS. As displayed within the "normal" (pooled population of NV/NIP) subset of Table 3.14, a trend towards a significant negative correlation between the extent of platelet aggregation and SNP responsiveness was observed for the cohort of NV (regression analysis: $r = -0.32$, $p = 0.09$, run test $p = 0.43$). This result was unlike that observed for the cohort of NIP in which no significant correlation was observed. By ANCOVA these two subject populations were not significantly different from each other and hence were pooled (ANCOVA: $F = 0.87$, $p = 0.35$).

A significant negative correlation between the extent of platelet aggregation and SNP responsiveness was observed for both populations of SAP and ACS patients. These two populations of patients were not significantly different from each other and were also accordingly pooled (ANCOVA: $F = 0.04$, $p = 0.84$). The pooled data populations of NV/NIP

and patients (SAP/ACS) conformed to a Gaussian distribution (Kolmogorov-Smirnov: Patients Aggregation KS = 0.045, p = ns; SNP responsiveness KS = 0.064, p = ns; NV/NIP Aggregation KS = 0.08, p = ns; SNP responsiveness KS = 0.13, p = ns).

As displayed in Table 3.14 and Figure 3.5, a trend towards a significantly negative correlation between the extent of platelet aggregation and SNP responsiveness was observed for the pooled population of NVs (regression analysis: r = -0.26, p = 0.07, run test p = 0.2). For the pooled patient populations a highly significant correlation was noted (regression analysis: r = -0.41, p < 0.01, run test p = 0.4). These two subject cohorts ("normals" NV/NIP and "patients" SAP/ACS) were then found to be significantly different from each other (ANCOVA: F = 50.9, p < 0.01). As displayed in Figure 3.5 the extent of platelet aggregation within patients with acute or chronic IHD predicts platelet responsiveness to SNP, a relationship that is not observed within the pooled normal subject cohort.

Table 3.14 Platelet aggregability versus SNP responsiveness regression and ANCOVA summary table

		Regression analysis			*ANCOVA Summary*		*Outcome*
		r	*F*	*p*	*F*	*p*	
Normals	*NV*	-0.32	3.02	0.09	0.87	0.35	Pooled
	NIPS	-0.13	0.34	0.56			
Patients	*SAP*	-0.44	18.3	< 0.01	0.04	0.84	Pooled
	ACS	-0.38	20.27	< 0.01			
Overall data	*Normals*	-0.26	3.41	0.071	50.94	< 0.01	Figure 3.5
	Patients	-0.41	39.90	< 0.01			

Platelet aggregability versus platelet responsiveness to SNP was initially analyzed according to gender for each clinical condition (Appendix Table 14). By ANCOVA the data populations for NV/NIPs and SAP/ACS did not significantly different and were therefore pooled. By regression analysis a trend towards a significant negative correlation between platelet aggregability and SNP responsiveness exists for the pooled normal subject population. A highly significant negative correlation exists for the pooled patient population exists. By ANCOVA both populations were significantly different from each other. See Figure 3.5 for a further summary.

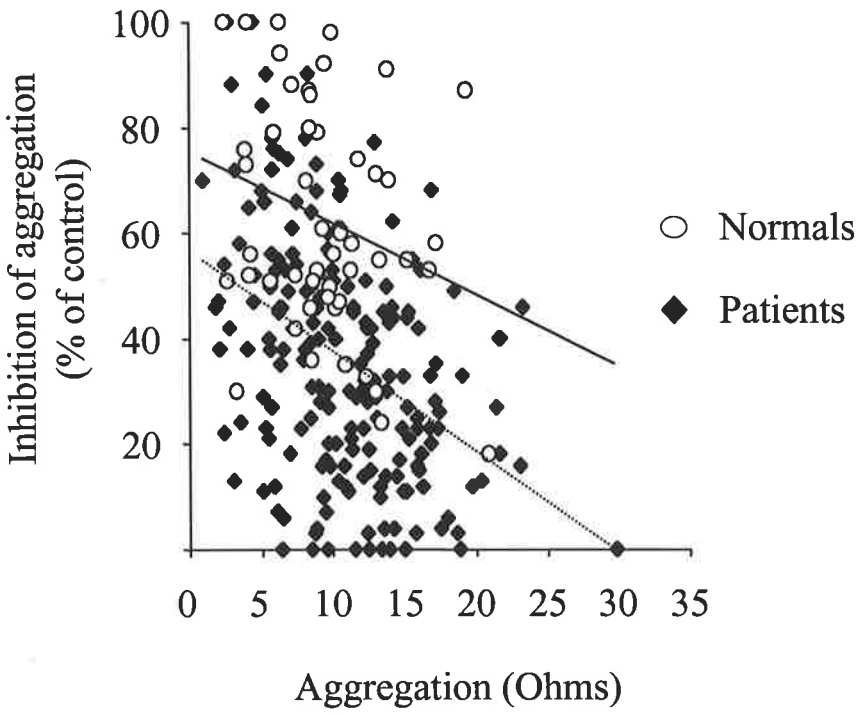


Figure 3.5 Aggregability versus SNP responsiveness: Comparison of normal subjects and patients with angina pectoris

The relationship between the extent of platelet aggregation in response to ADP (1μM) versus responsiveness to SNP (10μM) was assessed in normal subjects (pooled population of NV and NIPs) and patients (pooled population of SAP and ACS patients). By regression analysis $r = -0.26$, $p = 0.071$ for normals (solid line) and $r = -0.41$, $p < 0.01$ for patients (dotted line). ANCOVA revealed the existence of two separate populations $F = 50.9$, $p < 0.01$.

Relationship between platelet aggregability and NTG responsiveness

The platelet aggregability and NTG responsiveness relationship was firstly analyzed according to gender. Within the NV, NIP and female SAP patient cohorts no significant differences between aggregability and NTG responsiveness where noted. (See Appendix Table 15 for the regression and ANCOVA summary). Of the cohorts of subjects examined, male SAP patients were the only subject population that demonstrated a significant negative correlation for platelet aggregability versus responsiveness towards NTG (regression analysis: $r = -0.73$, $p < 0.01$, run test $p = 0.11$).

Within the ACS subject populations categorized according to the final diagnosis of either UAP or NQAMI, no gender-related differences were observed and genders were therefore pooled. However, within this pooled population of ACS patients (UAP/NQAMI patients) a significant difference between the relationships of aggregability and NTG responsiveness was noted for UAP and NQAMI patients (ANCOVA: $F = 7.42$, $p < 0.01$, Appendix Table 15).

Table 3.15 summarizes the regression and ANCOVA analysis for each clinical condition. No significant correlation between platelet aggregability and NTG responsiveness was observed for the data populations of NV and NIPs. By ANCOVA these subject populations were determined not to be significantly different from each other and were accordingly pooled and designated "normals". Unlike the results observed for the normals, patients with SAP showed a significant negative correlation between the extent of platelet aggregation and NTG responsiveness (regression analysis: $r = -0.47$, $p < 0.05$, run test $p = 0.87$). ACS patients diagnosed with NQAMI demonstrated a trend towards a significant negative correlation for the relationship between platelet aggregability and NTG responsiveness (regression analysis: $r = -0.32$, $p = 0.068$, run test 0.32, Appendix Table 15 and Table 3.15). Data from all the pooled subject populations conformed to a Gaussian distribution (Kolmogorov-Smirnov: NV Aggregation KS = 0.09, p = ns, NTG responsiveness KS = 0.09, p = ns; SAP patients Aggregation KS = 0.12, p = ns, NTG responsiveness KS = 0.09, p = ns; UAP patients Aggregation KS = 0.11, p = ns, NTG responsiveness KS = 0.12, p = ns; NQAMI Aggregation KS = 0.13, p = ns, NTG responsiveness KS = 0.08, p = ns).

Table 3.15 Platelet aggregability versus NTG responsiveness regression and ANCOVA summary table

			Regression analysis			*ANCOVA Summary*		*Outcome*
			r	*F*	*p*	*F*	*p*	
Normals	*NV*		-0.10	0.14	0.71	0.04	0.85	Pooled
	NIPS		-0.49	3.1	0.11			
Patients	*SAP*		-0.47	7.54	0.01	4.18	0.02	Separate populations
	ACS	*UAP*	-0.32	3.56	0.07			
		MI	-0.11	0.47	0.50			
Overall data	*Normals*		-0.09	0.26	0.61	6.24	< 0.01	Figure 3.6
	SAP		-0.47	7.54	0.01			
	UAP		-0.32	3.56	0.07			
	MI		-0.11	0.47	0.50			

Platelet aggregability versus platelet responsiveness towards NTG was initially analyzed according to gender for each clinical condition with no gender-related differences being observed (Appendix Table 15). By ANCOVA the data populations for NV/NIPs did not significantly different and were therefore pooled. Within the ACS subject population, the relationship between aggregability and NTG responsiveness for subjects with UAP or NQAMI (MI) were significantly different from each other and therefore were treated independently. Figure 3.6 summaries the analysis further.

By ANCOVA a significant difference between the four subject cohorts (normals, SAP, UAP and NQAMI patients) was noted (ANCOVA: $F = 6.24$, $p < 0.01$) with each population being significantly different from each other (Tukey/Kramer post hoc analysis: $p < 0.01$). For a further summary see Figure 3.6.

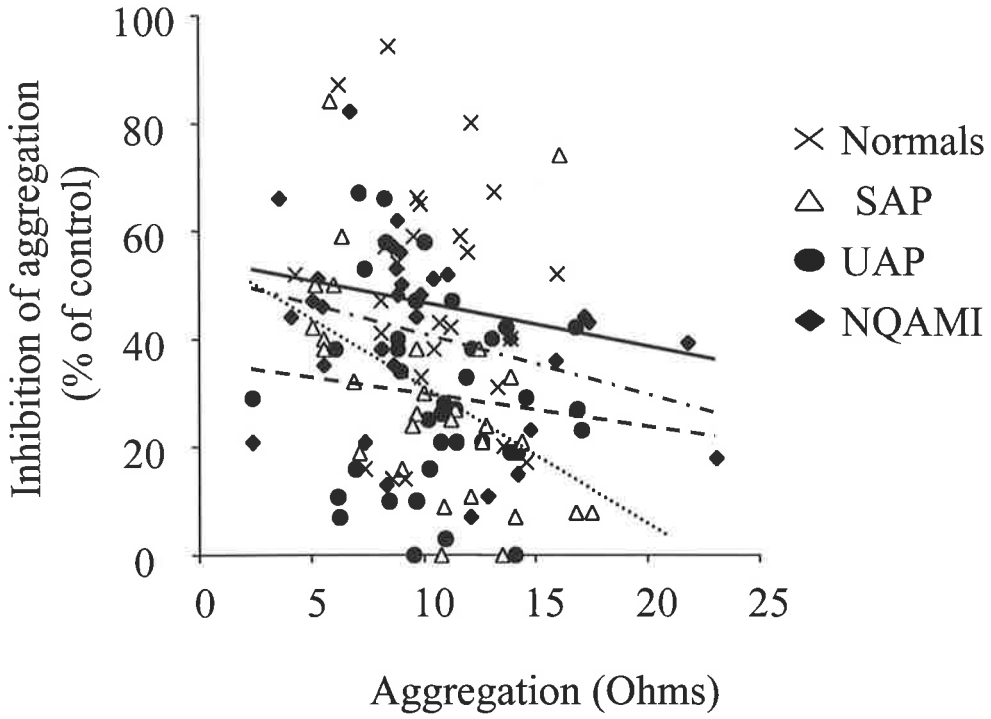


Figure 3.6 Aggregability versus NTG responsiveness: Comparison of normal subjects and patients with angina pectoris

The relationship between the extent of platelet aggregation in response to ADP (1μM) versus responsiveness towards NTG (100μM) was assessed in normal subjects (pooled populations of NV and NIP) solid line, SAP patients (dotted), UAP patients (dashed) and NQAMI patients (dotted and dashed). By regression analysis $r = -0.09$, $p = 0.61$ for normals; $r = -0.47$, $p < 0.05$ for SAP patients; $r = -0.32$, $p = 0.07$ for UAP patients; $r = -0.11$, $p = 0.5$ for NQAMI patients.

[3.5.5] Univariate and multivariate analysis

Inhibition of aggregation by SNP did not significantly differ between the samples obtained from NVs and NIPs. Mean (± S.E.M.) percentage inhibition of aggregation by SNP in platelets from NVs was 66 ± 3 and in NIPs was 64 ± 5 respectively (unpaired *t*-test: p = 0.67).

As the mean platelet response to SNP in the cohort of NVs was 66 ± 19 % (Mean ± SD); responses of < 28% inhibition of aggregation, corresponding to > 2 standard deviation below a "normal" SNP responsiveness, were treated as nitric oxide resistant for the multivariate analysis.

Determinants of inter-individual variability in platelet responsiveness to the anti-aggregatory effects of nitric oxide in the cohort of patients with SAP and ACSs were investigated by both univariate and multivariate analysis. Both univariate and multivariate analyses were performed with platelet responsiveness only to SNP, as SNP functions as a more direct donor of nitric oxide than NTG which requires enzymatic thiol-dependent bioconversion to release nitric oxide (Noack and Feelisch, 1991).

On univariate analysis mean platelet responsiveness to SNP was significantly greater in those subjects who were treated with perhexiline ($p < 0.01$). Hypercholesterolaemia and treatment with statins was also associated with an increased platelet responsiveness to SNP upon univariate analysis ($p = 0.04$ and $p = 0.02$ respectively). The coronary risk factors, including male gender, age ≥ 70 years, diabetes mellitus, hypertension and smoking did not significantly affect the platelet responsiveness to SNP. Anti-anginal pharmacotherapy with aspirin, nitrates, ACE inhibition, SH donors, Ca^{2+} antagonists and β-adrenoceptor antagonists also did not significantly affect platelet responsiveness to the anti-aggregatory effects of SNP. Results assessing the influence of coronary risk factors and anti-anginal pharmacotherapy on platelet responsiveness to SNP by univariate analysis are shown in Figure 3.7.

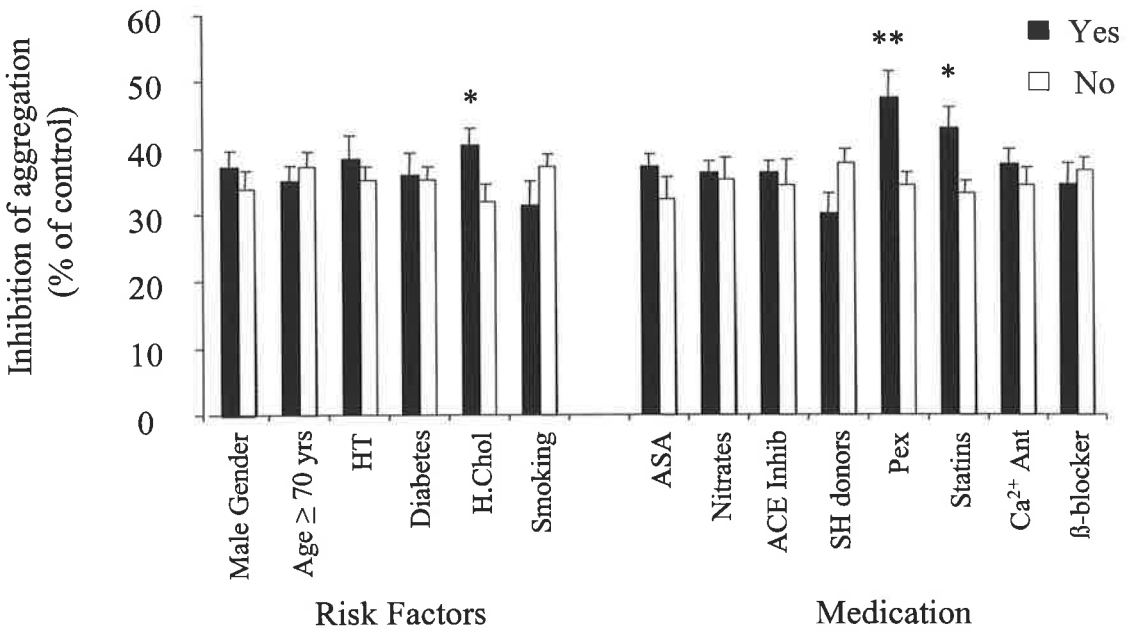


Figure 3.7 Univariate analysis of coronary risk factors and pharmacotherapy with responsiveness to SNP

*Samples were obtained from patients with SAP and ACS patients. Abbreviations used for hypertension (HT), high cholesterol (H.Chol), aspirin (ASA), ACE inhibitors (ACE Inhib), perhexiline (Pex), and Ca^{2+}-antagonists (Ca^{2+}). *p < 0.05, ** p < 0.01.*

Upon multivariate analysis, the presence of ACS (as distinct from SAP; p = 0.012) and the absence of pharmacotherapy with either perhexiline (p = 0.018) or a statin (p = 0.021), were the only significant correlates of nitric oxide resistance at the platelet level (< 28% inhibition of aggregation by SNP). The observation of a significant increase in platelet responsiveness to SNP in hypercholesterolaemic patients upon univariate analysis did not attain significance upon multivariate analysis. Odds ratios, confidence intervals and p values for the significant correlates of nitric oxide resistance are shown in Table 3.16.

Table 3.16 Multivariate analysis of patient characteristics

Determinant	*Odds ratio*	*95% CI*	*p*
ACS	2.29	1.18, 4.46	0.012
Perhexiline therapy	0.31	0.11, 0.88	0.018
Statin therapy	0.45	0.22, 0.90	0.021

Significant correlates of impaired (<28%) platelet responsiveness to SNP in blood samples obtained from a cohort of patients with SAP (n = 82) or ACS (n = 153). CI = confidence interval.

[3.5.6] Relationship between age and platelet responsiveness to SNP and NTG

The possibility of an age-dependent relationship for platelet responsiveness to both SNP and NTG was also examined. This putative relationship was examined in the cohorts of NV, NIP, and patients with SAP and ACS patients.

Age and SNP responsiveness

Initially, an age-dependent relationship for platelet responsiveness to SNP was assessed according to gender for each subject cohort. Following ANCOVA no gender-related differences were observed for the relationship between age and SNP responsiveness and therefore the results were pooled for each clinical condition (Appendix Table 16).

As shown in Table 3.17, no significant correlation between age and SNP responsiveness was observed for NV, NIP, SAP and ACS patients. The relationship between age and SNP responsiveness for NV and NIP and SAP and ACS patients were determined not to be significantly different from each other and were accordingly pooled (Table 3.17 for ANCOVA results). These pooled populations conformed to a Gaussian distribution, apart from the sub-group of data representing patient's age. (Kolmogorov-Smirnov: patients Age KS = 0.115, $p < 0.01$, SNP responsiveness KS = 0.065, p = ns; Normal subjects Age KS = 0.098, p = ns, SNP responsiveness KS = 0.12, p = ns). A log transformation of the data representing the patient's age failed to make the data population Gaussian, therefore a Spearman rank correlation was performed on the original data population. No significant relationship between age and the extent of platelet responsiveness to SNP was observed within the patients examined (Spearman rank correlation: r = -0.048, p = 0.49) corresponding to the result that was observed for NV and NIP (regression analysis: r = -0.07, p = 0.59, run test p = 0.14).

For both the pooled normal and patients populations no significant correlation between age and SNP responsiveness was found. However, both populations was found to be significantly different from each other (ANCOVA: F = 31.2, $p < 0.01$). For a further summary see Figure 3.8.

Table 3.17 Age versus SNP responsiveness
Regression and ANCOVA summary table

		Regression Analysis			ANCOVA Summary		Outcome
		r	*F*	*p*	*F*	*p*	
Normal Subjects	*NV*	-0.16	0.69	0.41	0.52	0.48	Pooled
	NIP	0.24	1.23	0.28			
Patients	*SAP*	0.04	0.14	0.71	2.64	0.11	Pooled
	ACS	-0.05	0.28	0.59			
Overall data	*Normal Subjects*	-0.07	0.29	0.59	31.2	$p < 0.01$	Figure 3.8
	Patients	-0.04	0.26	0.61			

Age versus platelet responsiveness to SNP was initially analyzed according to gender for each clinical condition (see Appendix Table 16 for a summary). By ANCOVA the data populations for NV/NIP and SAP/ACS patients did not significantly different and accordingly these subject populations were pooled. By regression analysis neither normal subjects nor patients showed any significant relationship between age and responsiveness to SNP. However, by ANCOVA these populations were found to be significantly different from each other. See Figure 3.8 for a further summary.

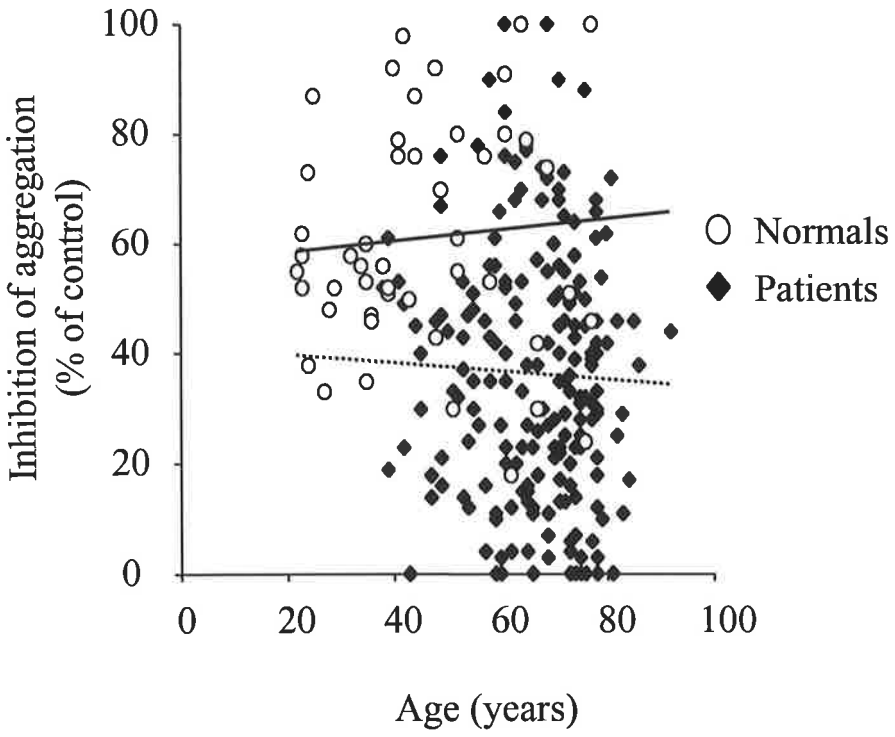


Figure 3.8 Age versus platelet responsiveness to SNP

The relationship between age and platelet responsiveness to SNP (10μM) was assessed in both normal subjects (pooled population of NV and NIP) and in patients (pooled population of SAP and ACS patients). By regression analysis $r = -0.07$, $p = 0.59$ for normal subjects (solid line) and $r = -0.048$, $p = 0.49$ Spearman rank correlation for patients (dotted line). By ANCOVA these two subject populations were significantly different from each other ($F = 31.2$, $p < 0.01$).

Age and NTG responsiveness

As with "age" versus "SNP responsiveness", a possible age-dependent relationship for NTG responsiveness was assessed according to gender for each subject cohort. As shown in Appendix Table 17, there was no significant correlation between age and NTG responsiveness for each gender within the subject cohorts. Therefore the relationship between age and NTG responsiveness was assessed on a pooled population of male and female subjects.

As illustrated in Table 3.18, a significant negative correlation between age and NTG responsiveness was observed for the NIP cohort (regression analysis: $r = -0.61$, $p < 0.05$, run test $p = 0.94$). By ANCOVA this population of NIP and NV along with the patients cohorts were found not to be significantly different from each other and were accordingly pooled. The data from the pooled populations of normals and patients were then shown to conform to a Gaussian distribution (Kolmogorov-Smirnov: NV/NIP Age KS = 0.15, p = ns, NTG responsiveness KS = 0.08, p = ns; Patients KS = 0.11, p = ns, NTG responsiveness KS = 0.07, p = ns).

As shown in Table 3.18, a significant negative correlation between age and NTG responsiveness was found for the pooled "normal" subject cohort (regression analysis: $r = -0.39$, $p = 0.03$, run test $p = 0.72$). In contrast, no age-related relationship to NTG responsiveness was found for the pooled patient population (regression analysis: $r = -0.08$, $p = 0.37$, run test $p = 0.35$). By ANCOVA these two subject populations tended ($p = 0.059$) towards being significantly different from each other.

Given that there was only a trend towards a significant difference between the NV/NIP and SAP/ACS subject groups, all the data were pooled to examine further the relationship between age and platelet responsiveness to NTG (100μM). The pooled populations were found to conform to a Gaussian distribution (Kolmogorov-Smirnov: Age KS = 0.1, p = ns; NTG responsiveness KS = 0.07, p = ns). By regression analysis and as displayed within Figure 3.9 there was a significant correlation between age and platelet responsiveness to NTG (100μM) (regression analysis: $r = -0.3$, $p < 0.01$, run test $p = 0.89$).

Table 3.18 Age versus NTG responsiveness regression and ANCOVA summary table

		Regression Analysis			ANCOVA Summary		Outcome
		r	F	p	F	p	
Normal Subjects	NV	-0.28	0.98	0.39	0.87	0.36	Pooled
	NIP	-0.61	7.9	0.01			
Patients	SAP	-0.27	2.19	0.15	1.73	0.19	Pooled
	ACS	-0.04	0.09	0.76			
Overall data	Normal Subjects	-0.39	5.13	0.03	3.64	0.059	Figure 3.9
	Patients	-0.08	0.80	0.37			

Age versus platelet responsiveness towards NTG was initially analyzed according to gender for each clinical condition (see Appendix Table 17 for a summary). By ANCOVA the data populations were not significantly different from each other and were accordingly were pooled. By regression analysis a significant relationship between age and platelet responsiveness towards NTG was observed for the pooled normal subject population. By ANCOVA both subject populations did not significantly different from each other ($F = 3.64$, $p = 0.059$). For a further summary see Figure 3.9.

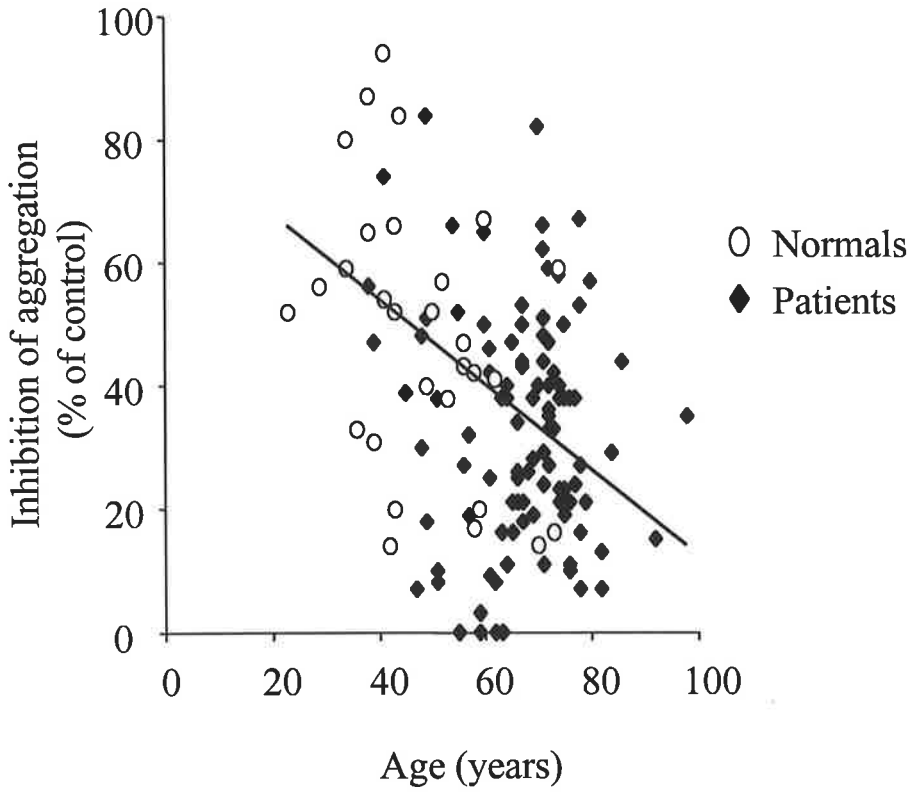


Figure 3.9 Age versus platelet responsiveness to NTG

The relationship between age and platelet responsiveness to NTG (100μM) was assessed in normal subjects (pooled population of NV and NIP) and patients (pooled population of SAP and ACS patients). By regression analysis a significant negative relationship between age and platelet responsiveness towards NTG existed for the pooled population of data ($r = -0.3$ $p < 0.01$, run test $p = 0.89$).

[3.5.7] Total number of coronary risk factors and platelet responsiveness to SNP and NTG

Individual coronary risk factors may synergistically influence platelet responsiveness to SNP and NTG. Therefore the relationship between the total number of coronary risk factors present and platelet responsiveness to SNP and NTG for samples obtained from patients with SAP and ACS patients was evaluated. For both SNP and NTG, the total number of risk factors, clinical condition and gender were included in the analysis.

Sodium nitroprusside

Data populations representing platelet responsiveness to SNP for both the SAP and ACS patients, genders and numbers of risk factors conformed to a Gaussian distribution (Appendix Table 18). By 3-way ANOVA the total number of coronary risk factors was demonstrated not to significantly influence platelet responsiveness to SNP (Bartlett's statistic = 12.7, p = 0.62). No significant interactions between the determinants were observed, further supporting the results shown in Table 3.12. See Table 3.19 for a summary. Subjects with no coronary risk factors (SAP n = 1; ACS n = 2) or subjects with five coronary risk factors (SAP n = 0; ACS n = 2) were omitted from this analysis due to insufficient numbers. Figure 3.10 summarizes the relationship between the number of coronary risk factors and inhibition of platelet aggregation by SNP for both SAP and ACS patients. Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between any combination of points.

Table 3.19 Coronary risk factors influencing platelet responsiveness to SNP three-way ANOVA contingency table

Determinants	*F*	*p*
Risk Factors	0.20	0.89
Disease State	0.80	0.37
Gender	0.11	0.74
Interactions		
Risk Factors/Disease State	0.83	0.48
Risk Factors/Gender	0.24	0.86
Disease State/Gender	0.12	0.73
Risk Factors/Disease State/Gender	0.48	0.69

Platelet responsiveness to SNP in subjects with SAP and ACS patients was assessed according to the number of coronary risk factors, disease state and gender. Subjects with zero (n = 1 SAP; n = 2 ACS) and five (n = 0 SAP; n = 2 ACS) coronary risk factors were omitted from the analysis.

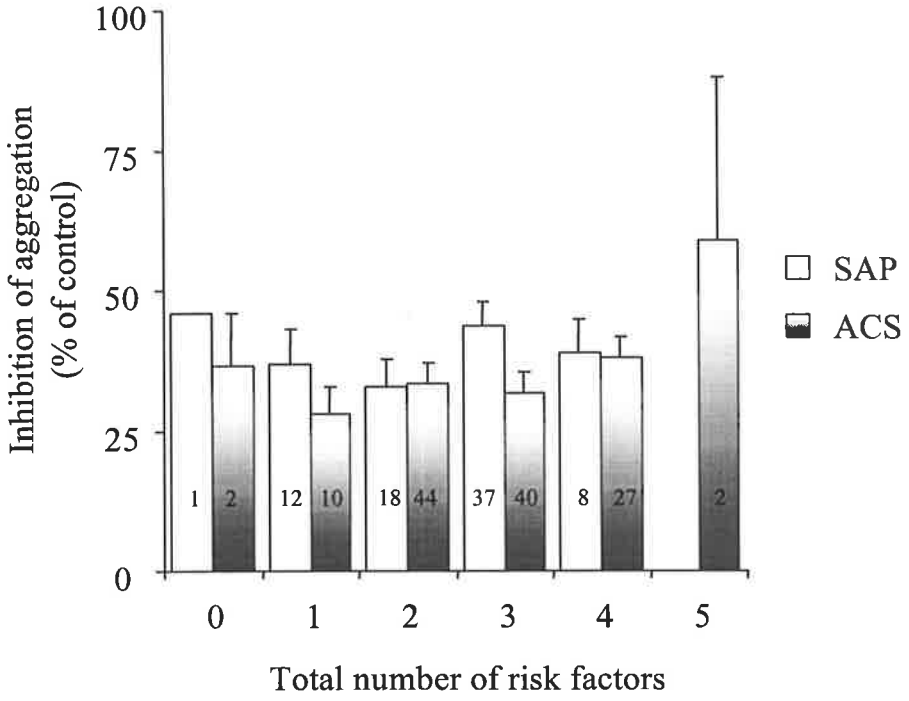


Figure 3.10 Coronary risk factors as a determinant of platelet responsiveness to SNP

Relationship between the number of coronary risk factors and platelet responsiveness to SNP (10μM) in samples from patients with SAP and patients with an ACS. Numbers of subjects are indicated in parentheses. By three-way ANOVA that included the number of coronary risk factors, disease state (SAP/ACS) and gender as potential determinants, no significant difference between the determinants were found. See Table 3.19.

Nitroglycerine

Data populations representing platelet responsiveness to SNP for both the SAP and ACS patients, genders and numbers of risk factors conformed to a Gaussian distribution (Appendix Table 19; Gaussian distributions observed in those data populations with sufficient numbers to perform analysis). In much the same way as the results observed for SNP, no significant difference between the number of coronary risk factors, disease state and gender, was observed regarding NTG responsiveness (Table 3.20) (Bartlett's statistic = 14.6, p = 0.47). No significant interactions were also observed. However, there was a trend towards a significant difference between those with SAP as compared to ACS patients regarding platelet responsiveness to NTG. This analysis, similar to that for SNP responsiveness, was performed on limited data as no subject included in the analysis has zero or five coronary risk factors. Utilizing Bonferroni's post hoc multiple comparison test and much the same as

demonstrated for SNP responsiveness, there were no significant differences between any combination of points. See Figure 3.11 for a further summary.

Table 3.20 Coronary risk factors influencing platelet responsiveness to NTG three-way ANOVA contingency table

Determinants	*F*	*p*
Risk Factors	1.45	0.23
Disease State	2.82	0.09
Gender	0.43	0.52
Interactions		
Risk Factors/Disease State	0.81	0.49
Risk Factors/Gender	0.86	0.46
Disease State/Gender	0.56	0.45
Risk Factors/Disease State/Gender	0.35	0.79

Platelet responsiveness towards NTG in SAP and ACS patients was assessed according to the total number of coronary risk factors, disease state and gender. No SAP or ACS subject had zero or five coronary risk factors. See Figure 3.11 for a further summary.

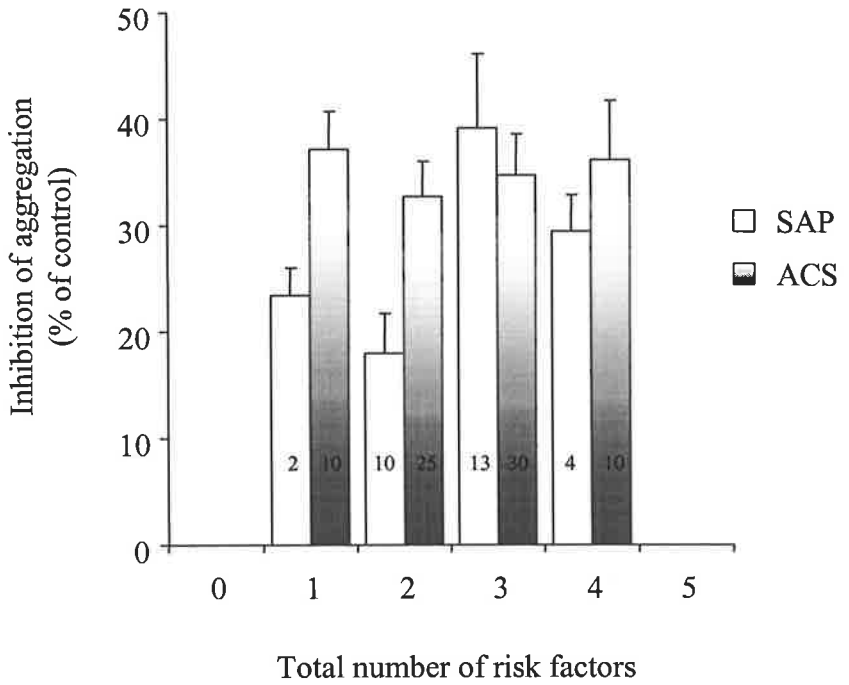


Figure 3.11 Coronary risk factors as a determinant of platelet responsiveness to NTG

Relationship between coronary risk factors and platelet responsiveness to NTG (100μM) and in samples from patients with SAP and patients with an ACS. Numbers of subjects are indicated in parentheses. Gender was also included as a potential determinant with the results of the three-way ANOVA being displayed in Table 3.20. No subject analyzed had zero or five coronary risk factors.

[3.5.8] Extent of coronary artery disease and platelet responsiveness to SNP

An interaction between the extent of coronary disease and platelet responsiveness to SNP was also examined. This interaction was examined in both SAP and ACS patients who were recruited at the time of coronary catheterization allowing reliable determination of the extent of fixed CAD (number of vessels with significant stenosis; > 50% narrowing). Patients at the time of recruitment who were being treated with perhexiline were excluded from this investigation as perhexiline has been demonstrated to improve platelet responsiveness to SNP (Willoughby *et al.*, 2002).

Stable angina pectoris patients

Data representing platelet responsiveness to SNP in subjects with either 1, 2 or 3-vessel disease conformed to a Gaussian distribution (Kolmogorov-Smirnov test: 1-vessel KS = 0.28, p = ns; 2-vessels KS = 0.15, p = ns; 3-vessels KS = 0.13, p = ns). However, the differences between the standard deviations of each population were significant (Bartlett's statistic = 7.2, p = 0.027). A log transformation of the data was performed and also demonstrated significant differences between the groups (Bartlett's statistic post log transformation = 24.5, $p < 0.01$). Therefore utilizing a Kruskal Wallis test for non-parametric data, a significant difference between the groups existed (Kruskal Wallis test: KS = 7.8, p = 0.02). Utilizing Dunn's post hoc multiple comparison test, subjects with significant stenosis in three major vessels were significantly less responsive to SNP than those subjects with signal vessel disease (Dunn's multiple comparison test: 1 vs 2, p = ns; 1 vs 3, $p < 0.05$; 2 vs 3, p = ns). See Figure 3.12 for a further summary.

Acute coronary syndrome subjects

As ACS patients could be categorized according to the final diagnosis of either UAP or NQAMI, this potential determinant was included in the analysis for the ACS subject data. All data populations were demonstrated to conform to a Gaussian distribution (Kolmogorov-Smirnov: MI 1-vessel KS = 0.17, p = ns; 2-vessels KS = 0.16, p = ns; KS = 0.14, p = ns; UAP 1-vessel KS = 0.25, p = ns; 2-vessels KS = 0.24, p = ns; 3-vessels KS = 0.23, p = ns). By two-way ANOVA, the number of major vessels with a significant stenosis and the disease state were not significant determinants of platelet responsiveness to SNP. No significant interaction between these two determinants was observed (Bartlett's statistic = 7.17, p = 0.21). Given this result, the data for both those patients with a final diagnosis of MI or UAP

were pooled. Pooled populations of data also conformed to a Gaussian distribution (Kolmogorov-Smirnov test: 1-vessel KS = 0.17, p = ns; 2-vessels KS = 0.16, p = ns; 3-vessels KS = 0.1, p = ns); there were no significant differences between the standard deviations (Bartlett's statistic = 0.43, p = 0.81). By 1-way ANOVA, there was no significant difference between platelet responsiveness to SNP for ACS patients with 1, 2 or 3-vessel CAD (1-way ANOVA: F = 0.82, p = 0.44). Utilizing Bonferroni's post hoc multiple comparison test, there were no significant differences between any combination of number of vessels (Bonferroni's multiple comparison test 1 vs 2 $t = 0.27$, p = ns; 1 vs 3 $t = 1.05$, p = ns; 2 vs 3 $t = 1.1$, p = ns). For a further summary see Figure 3.12.

Table 3.21 Numbers of vessels with a significant stenosis and its relationship to SNP responsiveness in ACS patients two-way ANOVA contingency table

Determinants	*F*	*p*
Number of vessels involved	0.5	0.61
Disease State	0.3	0.86
Interactions		
Vessel with stenosis/Disease State	1.03	0.36

Platelets responsiveness to SNP in platelets from ACS patients with a final diagnosis of either UAP or a NQAMI obtained prior to catheterization were equated to the extent of CAD.

An examination of a possible relationship between the extent of coronary artery disease and platelet responsiveness to NTG was not performed due to insufficient numbers.

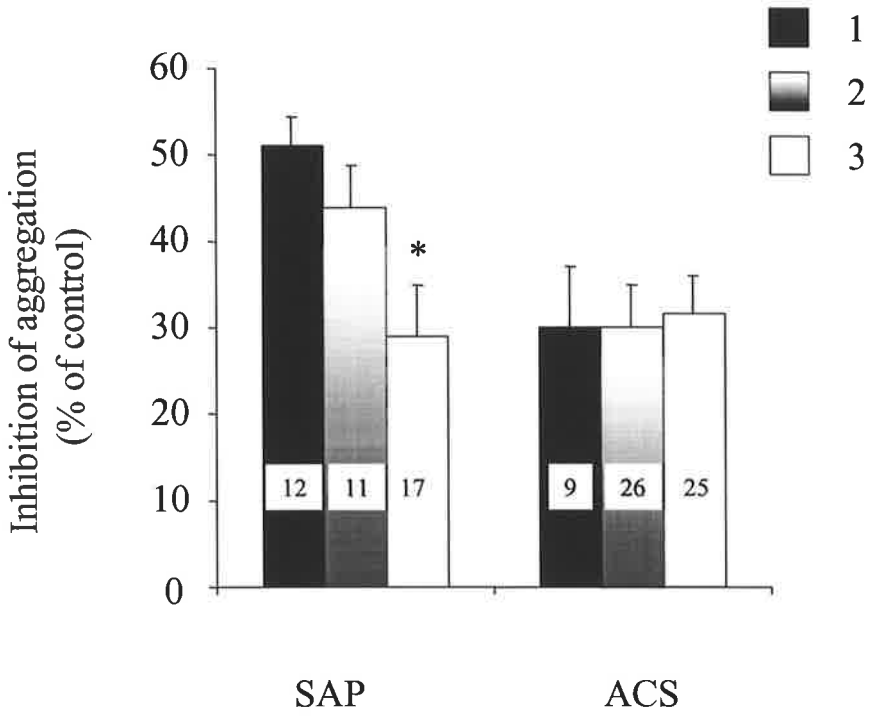


Figure 3.12 Extent of coronary artery disease and platelet responsiveness to SNP

*Extent of CAD and platelet responsiveness to SNP (10μM) in blood samples obtained from patients with SAP and ACS patients. Numbers of subjects studied are indicated within the bars. Subjects being treated with perhexiline at the time of study enrolment were omitted from this analysis. For SAP patients by one way ANOVA F = 4.76 and p < 0.05. *p < 0.01 versus 1 vessel, by Dunnett's multiple comparison test. For ACS patients see Table 3.21.*

[3.5.9] Severity of angina and platelet responsiveness to SNP

Severity of angina, as assessed according to the Canadian Cardiovascular Society classification of angina (Campeau, 1976), and platelet responsiveness to SNP in patients with SAP, was then examined. SAP patients being treated with perhexiline were excluded from this investigation as it has been shown previously that perhexiline potentiates platelet responsiveness to SNP (Willoughby *et al.*, 2002).

Within the cohort of SAP patients that were classified according to angina severity there were insufficient numbers of subjects with a score of 0 and 1 to perform the Kolmogorov-Smirnov test. Accordingly these subgroups were omitted from the analysis. Data within groups 2,3,4 were demonstrated to conform to a Gaussian distribution (Kolmogorov-Smirnov: Cardiovascular classification group 0 KS = N/A; 1 KS = N/A; 2 KS = 0.13, p = ns; 3 KS = 0.17, p = ns; 4 KS = 0.093, p = ns), and shown not to have significant differences between the

standard deviations of each sub-group (Bartlett's statistic = 2.2, p = 0.33). By 1-way ANOVA, there was no association between the severity of angina and platelet responsiveness to SNP (1-way ANOVA: F = 0.69, p = 0.60). Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between each of the three severity of angina classes. See Figure 3.13 for a further summary.

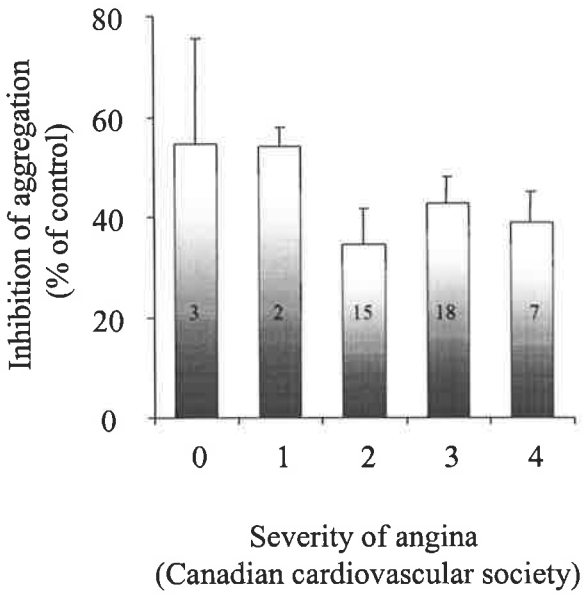


Figure 3.13 Angina severity and platelet responsiveness to SNP

Severity of angina as measured by the Canadian Cardiovascular Society classification of angina and platelet responsiveness to SNP (10μM) in blood samples obtained from patients with SAP. Numbers of subjects studied are indicated within the bars. By 1-way ANOVA, angina severity was not related to SNP responsiveness within the SAP cohort (F = 0.69, p =0.602).

[3.6] Discussion

Results presented in section I of chapter 2 and the previous observations of Chirkov *et al* (1993/6), provided evidence that platelet from patients with SAP differ from those obtained from normal subjects in two separate respects: - 1) they are hyper-responsive to the pro-aggregant ADP and 2) demonstrate attenuated responsiveness to the anti-aggregatory effects of both NTG and SNP. The observed "resistance" to SNP, a more direct donor of nitric oxide than NTG (Ignarro *et al.*, 2002), should thus be regarded as nitric oxide rather than nitrate resistance, implying reduced responsiveness to either endogenously, or exogenously donated sources of nitric oxide.

The current study has extended these initial observations and addressed a number of additional questions. 1) Was the observed platelet hypo-responsiveness towards donors of nitric oxide related in any way to the ischaemic process (stable vs. UAP/ACS) 2) Was it

related to the extent of CAD or severity of angina? 3) Was it ameliorated by treatment of myocardial ischaemia or influenced by the type or number of coronary risk factors?

[3.6.1] Platelet aggregability

Consistent with the results observed within section I of chapter 2 and in keeping with the hypothesis for a major role of thrombus formation in the pathophysiology of an acute ischaemic condition (Davies, 1995), platelets from patients with SAP or an ACS were significantly more aggregable to ADP (1μM) than platelets obtained from a cohort of NVs or NIPs (Table 3.3/4/5). This phenomenon of platelet hyper-aggregability persisted in the combined presence of a number of anti-platelet agents that included organic nitrates, aspirin and others (patients receiving GPIIb/IIIa or ADP receptor antagonists were excluded from the study as ADP was used as an inductor of aggregation). Furthermore, platelets from female subjects were also significantly more aggregable than those of male subjects irrespective of the clinical condition, a result that is consistent with the observations obtained by others (Haque *et al.*, 2001; Meade *et al.*, 1985; Taylor *et al.*, 1987) and may relate to differences in activity of the GPIIb/IIIa receptor (Faraday *et al.*, 1997).

Differential effects of aspirin pharmacotherapy

Our post hoc subgroup analysis of the platelet aggregability data indicated that the effect of aspirin on the extent of inhibition of platelet aggregation differs between males and females across the SAP and ACS populations. Despite not being a randomized or paired comparison, aspirin use was shown to have no significant anti-aggregatory effect in females. A differential effect of aspirin use between genders has been demonstrated in a number of clinical trials. In the "Hypertension Optimal Treatment (HOT)" study, Kjeldsen *et al* (2000) demonstrated that the effect of low dose aspirin (75mg daily) at preventing MI was influenced by gender. Benefits from aspirin use at preventing MI were only observed in males and not in females ($p = 0.001$). It was suggested that absence of a significant effect from aspirin in females was due to a low incidence of MI in the studied females. However, given the results observed within the current study, the failure of aspirin to significantly inhibit platelet aggregation in females only, may serve as a mechanism for the results observed by Kjeldsen *et al* (2000). In the aspirin versus placebo arm of the "Second international study of infarct survival" (ISIS-2) trial, male patients receiving aspirin had a lower odds ratio than female patients, representing fewer vascular deaths. Considerable overlap in the confidence intervals between both male

and female patients was demonstrated, thus making any difference between genders non-significant. However, the direction of benefit from aspirin use was clearly associated with male rather than female patients (ISIS-2 collaborative group, 1988).

[3.6.2] Platelet responsiveness towards nitric oxide

As demonstrated in Figures 3.1/3.2, platelets from patients with either SAP or an ACS manifest a significantly reduced responsiveness to the anti-aggregatory effects of nitric oxide. This confirms and extends the initial observations made by Chirkov *et al* (1993,1996), to include those subjects with acute ischaemic heart disease, a result that agrees with the observations made in section I of chapter 2. In contrast to the results obtained from these acute and chronic IHD patients are the results from patients admitted for evaluation of chest pain but then discovered to be free of fixed CAD. Termed NIPs, they demonstrated a platelet responsiveness towards nitric oxide that was similar to that observed from a cohort of NVs.

This series of results provides the first evidence of a link between the phenomenon of nitric oxide resistance at the platelet level and the presence of symptomatic myocardial ischaemia. Consistent with this hypothesis is the observation that SAP patients with a significant lesion in three major coronary arteries are significantly less responsive to SNP compared to those with a major stenosis in only one vessel (Figure 3.12). Moreover, the number of major vessels that contained a significant stenosis was found not to be a significant determinant for platelet responsiveness to SNP within the ACS patient population. Platelet responsiveness to SNP within these subjects was at a level consistent with a response observed for SAP patients who have significant stenoses in three major vessels, implying a link between ischaemia and responsiveness towards nitric oxide at the platelet level. This relationship was not examined for responsiveness to NTG due to insufficient numbers.

As illustrated in Figure 3.4, platelets from patients with UAP were significantly less responsive to the anti-aggregatory effects of NTG (100μM) compared to samples from patients with a NQAMI. An examination of the clinical characteristics for both patient cohorts demonstrated that both patient populations were well matched, apart from a disparity in the use of aspirin and ACE-inhibitors. Significantly greater numbers of UAP patients were undergoing aspirin pharmacotherapy and not receiving ACE-inhibitors compared to the NQAMI cohort.

As illustrated in Table 3.13, aspirin utilization was not a significant determinant for platelet responsiveness to NTG. It therefore is unlikely that a significant difference in the frequency of aspirin use would serve as a mechanism for attenuated platelet responsiveness to NTG within the UAP cohort.

Evidence demonstrating a potentiation of responsiveness to organic nitrates by ACE-inhibitors is more extensive than aspirin, but no less convincing due to a number of controversies. As discussed in section D.4.2.3 of chapter 1, high dose ACE-inhibitor pharmacotherapy has been used to limit nitrate tolerance (pseudo-tolerance) induction, by several investigators, with varying degrees of success (Dakak *et al.*, 1990; Pizzullo *et al.*, 1996). Debate as to what role the sulfhydryl moiety has within captopril and the ability of ACE-inhibitors/AT_I receptor antagonists to reduce superoxide via direct/indirect inhibition of NAD(P)H oxidase, remains undefined. Doubt over the clinical significance of this difference in the frequency of ACE-inhibitor use serving as a mechanism behind a differential response to NTG between the two cohorts occurs when the extent of platelet responsiveness to NTG was examined according to ACE-inhibitor use. ACE-inhibitor use was found not to be a significant determinant of platelet responsiveness to NTG when examined using ANOVA ($F = 21.7$, $p = 0.15$). Differences between the disease states were still apparent, with no difference between the genders or any significant interactions between the determinants, being observed.

Despite no significant difference between the groups regarding the proportion of subjects treated with organic nitrates, differences in the dosing regimens or preparations, may serve as a simple explanation for the observed difference in the extent of platelet responsiveness to NTG across the cohorts examined. The UAP patient cohort may simply be more tolerant to the anti-aggregatory effects of NTG than the NQAMI patient cohort. A record of the dosing regimen for each patient was not recorded.

Decreased platelet responsiveness to exogenously donated nitric oxide implies reduced platelet sensitivity to endogenous sources of nitric oxide *in vivo*. Given that endogenously derived nitric oxide is critical to the *in vivo* regulation of platelet aggregability (Moncada and Higgs, 1991), then coupling the phenomena of platelet hyper-aggregability and hypo-responsiveness to donors of nitric oxide may contribute to an increased risk of platelet aggregation and intravascular thrombus formation observed in subjects with acute and

chronic IHD (Diodati *et al.*, 1990). A significant negative relationship between the extent of platelet aggregation and platelet responsiveness to SNP (Figure 3.5) was observed. Given platelet responsiveness to donors of nitric oxide was found to be independent of age (Figures 3.7/3.8), the significant negative relationship between platelet aggregability and responsiveness to nitric oxide suggests either a common mechanism influencing both phenomena and/or that the two phenomena are linked via "physiological antagonism". The issue of "one phenomenon or two" has recently been explored via ANCOVA in patients with aortic stenosis (Chirkov *et al.*, 2002). Potential mechanisms describing the phenomena of platelet hyper-aggregability and a reduced responsiveness to nitric oxide were examined within chapter 2.

[3.6.3] Clinical determinants

Evaluation of the clinical determinants of a poor platelet responsiveness towards nitric oxide yielded evidence on both univariate and multi-variate analyses (see Figure 3.7 and Table 3.16), that conventional risk factors for CAD and a number of commonly utilized anti-anginal pharmacotherapies (apart from hypercholesterolaemia and treatment with perhexiline and statins), did not markedly modulate this phenomenon.

Platelet level

Apart from the observations made by Chirkov (Chirkov *et al.*, 1993; Chirkov *et al.*, 1995; Chirkov *et al.*, 1996; Chirkov *et al.*, 1999; Ellis *et al.*, 2001), there are only a few studies that have demonstrated a reduced platelet responsiveness to donors of nitric oxide at a platelet level in samples from subjects with risk factors for CAD. In platelets from patients with hypertension, Woods *et al* (1993) demonstrated that the concentration of SNP required to inhibit Ca^{2+} mobilization induced by U46619 was significantly greater in platelets from hypertensive patients than in those from normotensive controls. In a study examining the anti-platelet properties of NTG in diabetic subjects, Anfossi *et al* (1998) demonstrated that the IC_{50} NTG concentrations were significantly higher in insulin-resistant patients compared to normal subjects. This relationship was established when platelet responsiveness to NTG was examined in lean NIDDM patients compared to obese NIDDM patients. More recently Haramaki *et al* (2001), demonstrated that platelets from chronic smokers were significantly less responsive to the anti-aggregatory effects of NTG compared to platelets from non-

smokers. Moreover, it was demonstrated that reduced platelet responsiveness to NTG was a function of the level of intra-platelet glutathione (Haramaki *et al.*, 2001).

Vascular level

This is the first study to examine the clinical determinants of an attenuated responsiveness towards donors of nitric oxide at the platelet level. However, determinants of the apparently analogous phenomenon within the vasculature, have also been described. In terms of classical risk factors, Schachinger *et al* (2000) demonstrated that the predictive value of coronary endothelial vasodilator dysfunction (as distinct from classical endothelial dysfunction) was independent of the classical risk factors apart from hypertension. In a study examining potential mechanisms behind the higher prevalence of hypertension in African Americans compared to caucasians, Cardillo *et al* (1998) examining healthy subjects demonstrated a significant attenuation in the forearm vasodilator responsiveness post SNP administration within the African American subject cohort. In another vascular investigation, forearm blood flow responses to SNP were demonstrated to be profoundly impaired in hypertensive patients compared to normotensive controls, with the maximal flow responses inversely correlating with mean arterial blood pressure (Preik *et al.*, 1996), results that were also observed in hypertensive rats (Huang and Koller, 1996). In a more recent investigation examining the potential mechanisms behind pre-eclampsia, Suzuki *et al* (2000) demonstrated a marked reduction in the extent of SNP induced relaxation of the human omental resistance arteries of females with pre-eclampsia compared to normotensive pregnant females.

Relationship to ischaemic heart disease

As shown in Table 3.16, the diagnosis of an ACS, as distinct from SAP, was found to be a significant determinant of attenuated platelet responsiveness to SNP on multivariate analysis, with an odds ratio of 2.3:1. Although worsening severity of angina amongst the patients with SAP was associated with diminished platelet responsiveness to SNP, platelets from SAP patients with triple vessel disease were significantly less responsive to SNP than those with stenosese involving only one or two vessels, implying that extent of ischaemia (and potentially of redox stress) may contribute to the phenomenon.

[3.6.4] Pharmacotherapy

Pharmacotherapy with the prophylactic anti-anginal agent perhexiline was associated with augmented platelet responsiveness to nitric oxide on both univariate (Figure 3.7) and multivariate analyses, with an odds ratio for the elimination of nitric oxide resistance of 3.2:1 (Table 3.16).

[3.6.4.1] Perhexiline maleate

Clinical efficacy

Ever since the early 1970's a number of investigators have demonstrated that perhexiline is efficacious in reducing the degree/severity of anginal episodes in symptomatic patients. Horowitz and Mashford demonstrated a reduction in the frequency of attacks in patients suffering from severe angina pectoris (Horowitz and Mashford, 1979), confirming the results obtained from a series of other investigators (Armstrong *et al.*, 1974; Brown *et al.*, 1976; Wallace, 1978). More recently, Cole *et al* (1990), in a randomized double blind placebo controlled trial, found that 65% of subjects randomized to perhexiline showed an improvement in measures of anginal frequency and severity. Stewart *et al* (1996) demonstrated that perhexiline exerts an incremental anti-anginal effect over those of other anti-anginal agents in patients with a UAP or a NQAMI. Perhexiline pharmacotherapy in patients with aortic stenosis improved symptomatic status over three months of therapy (Unger *et al.*, 1997).

Mechanism of action

Calcium antagonist

At one stage perhexiline was proposed to function as an L-type Ca^{2+} antagonist (Fleckenstein-Grun *et al.*, 1978). A study performed by Barry *et al* (1985), comparing the effectiveness of a number of calcium channel antagonists at inhibiting myocyte contractility and calcium influx in cultured chick embryo ventricular myocytes, demonstrated that perhexiline was significantly less effective at inhibiting myocyte contractility (i.e. was a weaker L-channel calcium antagonist) than a range of commonly utilized calcium channel antagonists that included verapamil, diltiazem and nifedipine.

Carnitine palmitoyltransferase-1 (CPT-1) inhibition

Whilst testing the hypothesis that perhexiline promotes a shift in cardiac metabolism from fatty acid utilization towards increased utilization of carbohydrates and thus effectively sparing oxygen, Kennedy *et al* (1996) demonstrated that perhexiline dose dependently inhibits the enzyme carnitine palmitoyltransferase-1 (CPT-1). CPT-1 is a mitochondrial enzyme responsible for long chain acyl carnitine formation, which provides access of long chain fatty acyl-CoA to the site of β-oxidation in the mitochondrial matrix (Murthy and Pande, 1987). In both rat heart and liver specimens perhexiline inhibited CTP-1 more effectively than amiodarone, an agent that also inhibits mitochondrial β-oxidation of fatty acids (Fromenty *et al.*, 1990).

Effects on myocardium and coronary vasculature

In a more recent study, Kennedy *et al* (2000) demonstrated that perhexiline (2μM) improved the diastolic function during ischaemia in a Langendorff-perfused rat heart (60 minutes of low-flow (95% flow-reduction) followed by 30 minutes of reperfusion). Moreover, Kennedy *et al* (1999), whilst examining the coronary vasodilator properties of perhexiline, demonstrated that it has endothelium dependent vasodilator properties that are independent of nitric oxide, prostacyclin and endothelial derived hyperpolarizing factor generated by the actions of bradykinin. To date identification of the mechanism by which perhexiline functions as a coronary small vessel vasodilator in this setting has not been elucidated. There is no evidence that perhexiline exerts any significant vasomotor effects in humans.

Anti-platelet properties

Studies demonstrating a direct anti-platelet effect of perhexiline are limited with only a number of investigations addressing this question specifically. Perhexiline has previously been shown to inhibit aggregation induced by a range of platelet agonists, an effect not correlated with its Ca^{2+} antagonist properties (Ono and Kimura, 1981). Utilizing platelets obtained from both normal subjects and patients with SAP, Willoughby *et al* (1998) examined the platelet CPT-1 and anti-aggregatory effects of perhexiline, amiodarone and trimetazidine in comparison with etomoxir and hydroxyphenylglyoxylate, both specific CPT-1 inhibitors. All compounds were demonstrated to inhibit platelet derived CPT-1. However, only perhexiline, amiodarone and trimetazidine demonstrated a significant *in vitro* anti-aggregatory effect with perhexiline being the most potent, suggesting they exert this effect

independent of their CPT-1 inhibitory functions (Willoughby *et al.*, 1998). However, the clinical significance of this anti-aggregatory effect is unclear. Specifically, it has recently been demonstrated that perhexiline in both SAP and ACS patients, in doses that have no detectable anti-aggregatory effect, improved *ex vivo* platelet responsiveness to SNP to that reflecting responses observed in normal subjects. Despite not being conducted under randomized or blinded conditions, ACS patients not being treated with perhexiline demonstrated no improvement in platelet responsiveness to SNP (Willoughby *et al.*, 2002).

Perhexiline summary

As outlined above, perhexiline utilization has demonstrated many clinical benefits via mechanisms that remain to be fully elucidated. It is possible that perhexiline may interact directly with the determinants of a poor platelet responsiveness to nitric oxide, namely via an interaction with platelet soluble guanylate cyclase and/or various nitric oxide clearance mechanisms. The exact mechanisms by which perhexiline augments platelet responsiveness to nitric oxide falls outside the scope of the current study, but has been evaluated by Willoughby (1999).

[3.6.4.2] Statin utilization and hypercholesterolaemia

The interaction between hypercholesterolaemia, statin pharmacotherapy and platelet responsiveness to SNP is complex. Upon univariate analysis (Figure 3.7), hypercholesterolaemia and statin pharmacotherapy were associated with significantly augmented platelet responsiveness to SNP. Following multivariate analysis (Table 3.16), statin utilization was found to be a significant determinant of improved platelet responsiveness to SNP, with an odds ratio of 0.45:1. An improvement in platelet responsiveness towards nitric oxide following statin utilization, in much the same way as perhexiline pharmacotherapy, has not been previously documented.

Throughout the literature there is now considerable evidence demonstrating that the use of statins is associated with an increased benefit at a secondary (and possibly primary) prevention level (Scandinavian Simvastatin Survival Study, 1994; Downs *et al.*, 1998; Kjekshus and Pedersen, 1995; Morris *et al.*, 1994; Sacks *et al.*, 1996; Shepherd *et al.*, 1995), the potential mechanism/s of which are discussed below.

Anti-platelet/thrombotic actions of statins

One of the suggested mechanisms behind the observed benefit of statin utilization has been the direct and indirect effect of statin use on platelet function. As discussed within chapter 1 (section C.10.2), hypercholesterolaemia, and more importantly ox-LDL, augments platelet activation/aggregation (Carvalho *et al.*, 1974; Davi *et al.*, 1992). Utilizing an annular perfusion chamber and de-endothelialized abdominal rabbit aortae, Badimon *et al* (1991), demonstrated a significantly greater platelet deposition in samples obtained from hypercholesterolaemic rabbits. With platelets from patients with SAP, platelet thrombus deposition on strips of porcine aortic media were demonstrated to be significantly higher in subjects with hypercholesterolaemia compared to normocholesterolemic subjects (Lacoste *et al.*, 1995). Following 10 weeks of pravastatin pharmacotherapy within the hypercholesterolaemic subjects, platelet deposition, along with serum cholesterol concentrations, were reduced significantly. In a more recent study and with the use of a tubular perfusion chamber developed by Badimon *et al* (1987), that assesses the *ex vivo* platelet mediated thrombus formation on porcine aortic media, Thompson *et al* (2002), demonstrated a significant attenuation in platelet mediated thrombus formation in samples from subjects that had undergone treatment with either pravastatin or simvastatin (compared to placebo treatment in which platelet mediated thrombus formation was significantly potentiated).

Plaque stability

The modification of the contents of an atherosclerotic plaque leading to plaque stabilization has also been suggested as another potential mechanism by which statins function (Koh, 2000; Waters, 2001; Waters and Hsue, 2001). In a comprehensive histochemical investigation, Crisby *et al* (2001), demonstrated that the atherosclerotic lesions in subjects treated with pravastatin (40mg/d) compared to no statin pharmacotherapy, contained significantly less lipid and oxidized LDL, fewer macrophages and T-lymphocytes, less matrix metalloproteinase 2 (MMP-2) immunoreactivity, greater tissue inhibitor of metalloproteinase 1 (TIMP-1) immunoreactivity and a higher collagen content, results that agree with the observations of others (Bellosta *et al.*, 1998; Fukumoto *et al.*, 2001; Kurata *et al.*, 2001; Shiomi *et al.*, 2001).

Statin use and endothelial dysfunction

As discussed within section C.6.1.2 of chapter 1, statin use in subjects with hypercholesterolaemia and/or multiple coronary risk factors in most, but not all studies, show an improvement in endothelial function (Anderson *et al.*, 1995a; Egashira *et al.*, 1994; O'Driscoll *et al.*, 1997b; Treasure *et al.*, 1995). The mechanism/s of this effect of statin use on endothelial function remains unresolved, but may include reductions in the direct and indirect effects of superoxide and/or ROS, along with stimulation of nitric oxide production (see below).

Reactive oxygen species

In cultured vascular smooth muscle cells and in spontaneously hypertensive rats the influence of atorvastatin on the generation of ROS and the expression of the NAD(P)H oxidase sub-units was recently examined (Wassmann *et al.*, 2002). *In vitro*, atorvastatin significantly reduced ROS production, down-regulated nox-1 expression and rac1 GTPase translocation required for NAD(P)H oxidase activation. *In vivo,* in spontaneously hypertensive rats, treatment with atorvastatin reduced the vascular mRNA expression of $p22^{phox}$, nox-1 and catalase. Expression of SOD, gluthathione peroxidase, $gp91^{phox}$, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ remained unchanged (Wassmann *et al.*, 2002), suggesting that the vasoprotective effects of statins are related to their cellular anti-oxidant properties.

Nitric oxide stimulation

Laufs *et al* (1998), utilizing human saphenous vein endothelial cells treated with ox-LDL, demonstrated a significant decrease in eNOS mRNA expression and protein formation post ox-LDL exposure. Both simvastatin and lovastatin upregulated eNOS mRNA expression by >3-fold and completely prevented its down-regulation by ox-LDL. Utilizing an experimental protocol similar to the one outlined above, Wassmann *et al* (2001) demonstrated in spontaneously hypertensive rats, that 30 days of treatment with atorvastatin caused a significant reduction in systolic blood pressure, an upregulation of vascular eNOS expression and activity and a reduction of vascular ROS, an observation that concurs with the results of others (Amin-Hanjani *et al.*, 2001; Laufs *et al.*, 2000; Mital *et al.*, 2000; Mueck *et al.*, 2001).

Reduction of inflammation at the sites of an atherosclerotic lesion

Described within section C.7 of chapter 1 and as reviewed by a number of authors, inflammation plays a pivotal role in not only atherosclerotic lesion initiation/development, but also in lesion rupture (Berliner *et al.*, 1995; Libby *et al.*, 2002; Maseri, 1997; Plutzky, 2001; Ross, 1999; Schonbeck and Libby, 2001). Throughout the literature there is considerable evidence demonstrating an increased cardiovascular risk associated with increased basal levels of cytokines, adhesion molecules and acute-phase inflammatory markers that include C-reactive protein or serum amyloid A (Danesh *et al.*, 2000; Haverkate *et al.*, 1997; Hwang *et al.*, 1997; Koenig *et al.*, 1999; Ridker *et al.*, 2001a; Ridker *et al.*, 1998; Ridker *et al.*, 2000a; Ridker *et al.*, 2000b). Data from the Cholesterol and Recurrent Events (CARE) trial demonstrated a significant increase in median CRP levels over the 5 year follow up period in subjects randomized to placebo, directly contrasting to the pravastatin cohort in which a significant reduction in CRP was observed (Ridker *et al.*, 1999). Following on from this initial retrospective observation, a number of other investigators have now prospectively demonstrated a reduction in CRP due to statin utilization (Albert *et al.*, 2001; Ridker *et al.*, 2001b)

[3.7] Limitations of the Study

Despite the observed correlation between the level of extent of fixed CAD and platelet responsiveness to SNP, the Canadian Cardiovascular Society Classification class of angina within the population of SAP patients enrolled within this study did not correlate with platelet responsiveness to SNP. Given that the majority of subjects that were enrolled within this study were moderately or severely symptomatic, the recruitment of SAP patients with a Canadian Cardiovascular Society class of angina score of zero or one, would have been largely fortuitous. Therefore, the comparison between the Canadian Cardiovascular Society class of angina and platelet responsiveness to SNP was largely under-powered to demonstrate a relationship between ischaemia and platelet responsiveness to nitric oxide. Further to this and for the same reasons as outlined above, the lack of recruited patients with either zero or five coronary risk factors, made the comparison between the number of risk factors and platelet responsiveness to SNP and NTG under-powered. Given that impetus for both of these comparisons stemmed from the univariate and multivariate findings, future experiments addressing these particular questions might be useful.

Other forms of pharmacotherapy, which might modulate platelet responsiveness to nitric oxide, have not been fully explored. For example, ACE inhibitors may inhibit expression of a number of components of the NAD(P)H oxidase enzyme system in phagocytes and the endothelium, and thus may potentate nitric oxide responses. Our failure to observe this in a non-randomized non-standardized study design does not exclude the potential existence of such an interaction.

Recent investigations emphasized the likelihood that inflammatory changes within the vasculature underlie plaque destabilization (Buffon *et al.*, 2002). Furthermore, elevation of CRP protein levels may contribute directly to endothelial dysfunction. In retrospect, it is regrettable that within the current study no routine measures of CRP levels were made, as this would have permitted evaluation of a possible relationship between inflammation and platelet dysfunction.

As ADP-induced whole blood platelet aggregation was utilized as the model for the assessment of platelet responsiveness to nitric oxide, subjects treated with either GPIIb/IIIa or ADP receptor antagonists could not be assessed within this investigation.

Finally, it must be mentioned that the finding that the impact of aspirin pharmacotherapy on ADP responses appears to be diminished in females, a potentially very important observation, was not pursued in this series of experiments. The failure to evaluate this further results from the incidental nature of the observation: it remains of great priority to test this issue in a prospective design.

[3.8] Conclusions

The results of the current study further emphasize the importance of variable platelet responsiveness to nitric oxide in patients with angina pectoris. It appears that platelet resistance to the anti-aggregatory effects of nitric oxide is a characteristic of patients with severe SAP or an ACS and is therefore likely to contribute to the poor responsiveness to organic nitrates and hence the adverse clinical course of ischaemia observed in these subjects. Moreover, attenuated platelet responsiveness to nitric oxide may also contribute to platelet hyper-aggregability in patients with angina pectoris. Herein a diagnosis of ACS was found to be a significant determinant of reduced platelet responsiveness to SNP. Given that platelets

from patients with ACS have a markedly reduced degree of nitric oxide synthesis/release during aggregation that serves as a predictor of an adverse outcome (Freedman *et al.*, 1998), then further elucidation of the mechanisms behind nitric oxide resistance and development of therapeutic measures for this anomaly is critical.

The finding in the current study that pharmacotherapy with perhexiline and with statins was associated with a lower incidence of an attenuated platelet resistance to nitric oxide, provides additional impetus towards investigations of both of these agents in the prophylaxis and treatment of chronic and acute ischaemic syndromes.

[3.9] Chapter Summary

The results of the current study highlight further the importance of impaired platelet responsiveness to nitric oxide in subjects with both chronic and acute ischaemic syndromes. Platelets from patients with SAP and ACS exhibit increased platelet aggregability and significantly decreased platelet responsiveness to both SNP and NTG. The extent of this reduction in platelet responsiveness to nitric oxide was not correlated with any risk factors for CAD or age, but rather was partly related to ischaemia within the SAP cohort but also may be related to the mechanism of ACS (plaque rupture) with reduced platelet responsiveness to nitric oxide being particularly prominent in ACS patients. For patients in general, the reduced responsiveness to the anti-aggregatory effects of nitric oxide may also be in part a function of platelet aggregability further suggesting a close interplay between these two phenomena.

By both univariate and multivariate analyses, the current study at the very least established that pharmacotherapy with statins and with perhexiline are associated with a lower incidence of platelet resistance to nitric oxide. An examination of the mechanism/s behind perhexiline and statins action in attenuating nitric oxide resistance falls outside the current scope of this thesis. However, the influence of organic nitrate pharmacotherapy (commonly utilized to alleviate symptoms of myocardial ischaemia) on *ex vivo* platelet responsiveness towards NTG and SNP was assessed in subjects with mild to moderate SAP (Chapter 4).

Results from experiments within this chapter have contributed to a manuscript and a number of abstracts that were presented in a series of national and international meetings.

- Chirkov Y.Y., **A.S. Holmes**, S.R. Willoughby, S. Stewart, R.D. Wuttke, P.R. Sage and J.D. Horowitz, 2001, Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets, Journal of the American College of Cardiology, 1851-7.

- **Holmes AS.**, Y.Y. Chirkov, R.D. Wuttke, S.R. Willoughby, S. Stewart, P.R. Sage and J.D. Horowitz, Determinants of platelet responsiveness to nitric oxide donors in the presence and absence of ischaemic heart disease. 47th Annual Scientific Meeting of the Australian and New Zealand Cardiac Society, Wellington, 1999.

- Chirkov Y.Y., **A.S. Holmes**, R.D. Wuttke, S.R. Willoughby, S. Stewart, P.R. Sage and J.D. Horowitz, Determinants of platelet responsiveness to nitric oxide donors in the presence and absence of ischaemic heart disease. XXIst Congress of the European Society of Cardiology, Barcelona, 1999.

Chapter 4

Prophylactic nitrate pharmacotherapy: evaluation of anti-aggregatory and arterial vasomotor effects

Chapter Overview

This chapter addresses the interaction between acute and chronic nitrate pharmacotherapy on platelet aggregation and platelet responsiveness to SNP and NTG, in patients with SAP. Furthermore, this chapter will address the effect of acute and chronic nitrate therapy on the haemodynamic changes that result from its use and the interrelationship between the above mentioned platelet parameters. Within this context the chapters' purpose, results and conclusions are summarized below.

[4.1] Summary of the study evaluating the anti-aggregatory and arterial vasomotor effects of prophylactic nitrate pharmacotherapy

Background: Organic nitrates and nitric oxide exert potent anti-aggregatory effects *in vivo*, effects that have also been demonstrated *ex vivo*, generally via the utilization of high dose nitrate regimens.

Objectives: (1) To examine the *ex vivo* anti-aggregatory effects, effects on platelet responsiveness to nitric oxide donors and the effects on platelet aggregability utilizing SR isosorbide 5-mono-nitrate and transdermal-nitroglycerine during both acute and chronic pharmacotherapy, in patients with SAP. (2) To correlate the nitrate effects on platelet function and arterial wave reflection.

Methods: Patients with chronic SAP (n = 34) were enrolled in a blinded randomized crossover study of isosorbide 5-mononitrate (ISMN: 120mg) versus intermittent transdermal-NTG (TD-NTG: 15mg/24-hrs). Platelet response to ADP (1μM), inhibition of aggregation by both NTG (100μM) and SNP (10μM) and whole blood superoxide content, as detected using LDCL, were measured at 0, 4,8 and 24-hrs at the end of the "run in" phase (no prophylactic nitrate pharmacotherapy), after initial dosing for 7 days and following 14 days of therapy with each nitrate (0,4,8-hrs only). The effects of each nitrate regimen on pulse wave reflection were also assessed in 12 of the 34 subjects using applanation tonometry techniques, with derivation of a rate-corrected augmentation index AI(x).

Results: Neither ISMN nor TD-NTG was shown to induce any significant changes in platelet response to ADP (1μM), NTG (100μM) or SNP (10μM), or in the levels of superoxide

during any time period. Furthermore, there was no evidence of platelet hyper-aggregability during the "nitrate-free" periods. Conversely, both nitrate preparations were demonstrated to markedly reduce AI(x) at 4 and 8-hrs after acute dosing (ANOVA $F = 29.2$, $p < 0.01$). Persistence of effects during chronic nitrate therapy was also observed (ANOVA $F = 5.9$, $p < 0.01$), but with evidence of an attenuation of effectiveness (ANOVA $F = 5.6$, $p = 0.018$), implying tolerance development at the vascular level. There was no significant difference between the nitrate regimens regarding either initial extent of change in AI(x) or extent of attenuation of response.

Conclusions: (1) Neither nitrate regimen induces any detectable change in platelet aggregation as assessed by the current *ex vivo* technology used herein. This probably reflects a lack of sensitivity of this technology, rather than a lack of actual effects of each nitrate *in vivo*. (2) Neither nitrate regimen alters ADP-induced aggregation, or platelet responsiveness to nitric oxide, during chronic therapy or during nitrate-free periods. This provides evidence for safety of both regimens as regards potential precipitation of ACSs, and argues against "feed-back inhibition" of the nitric oxide/guanylate cyclase cascade by administered nitric oxide donors. (3) Both nitrate regimens induce similar extents of long-acting deceleration of wave reflection, a process which is subject to attenuation during chronic administration, but which does not exhibit the "zero-hour" phenomenon.

Table 4.1 Abbreviations used in this chapter

Abbreviation	Definition	Abbreviation	Definition
ACE	*Angiotensin converting enzyme*	**LDCL**	*Lucigenin derived chemiluminescence*
ADP	*Adenosine di-phosphate*	**MI**	*Myocardial infarction*
AI(x)	*Augmentation index*	**NIDDM**	*Non-insulin dependent diabetes mellitus*
ANCOVA	*Analysis of covariance*	**NTG**	*Nitroglycerine*
ANOVA	*Analysis of variance*	**PVD**	*Peripheral vascular disease*
BPM	*Beats per minute*	**SAP**	*Stable angina pectoris*
CCF	*Chronic cardiac failure*	**SD**	*Standard deviation*
cGMP	*Cyclic guanosine mono-phosphate*	**SEM**	*Standard error of the mean*
Hrs	*Hours*	**SNP**	*Sodium nitroprusside*
IDDM	*Insulin dependent diabetes mellitus*	**SR-ISMN**	*Slow/sustained release isosorbide mono-nitrate*
ISDN	*Isosorbide dinitrate*	**TD-NTG**	*Transdermal nitroglycerine*
ISMN	*Isosorbide mono-nitrate*	**UAP**	*Unstable angina pectoris*
KS	*Kolmogorov-Smirnov*		

Prophylactic nitrate pharmacotherapy: Evaluation of the anti-aggregatory and arterial vasomotor effects

[4.2] Introduction

[4.2.1] Biological effects of organic nitrates

Vasodilatation-peripheral and coronary

The clinical effectiveness of organic nitrates in the management of patients with angina pectoris and/or acute and chronic heart failure has long been established (reviewed by (Darius, 1999; Horowitz, 2000)) and has often been primarily attributed, at least in the context of SAP (Bassenge and Stewart, 1986), to their vasodilator properties on the peripheral veins and arteries. Vasodilatation of the peripheral veins and arteries results in a reduction in ventricular preload and afterload causing a reduction in cardiac work and myocardial oxygen demand, thereby alleviating myocardial ischaemia (Abrams, 1995).

Within the coronary vascular bed, NTG causes coronary vasodilatation mainly of large and intermediate sized vessels (>100μ in diameter), but with little effect on vessels < 100μ in diameter (Sellke *et al.*, 1990). Organic nitrates cause vasodilatation of coronary stenoses although to a lesser extent than native vessels, further contributing to the anti-ischaemic properties of these agents (Brown *et al.*, 1981; Gage *et al.*, 1986; Rafflenbeul and Lichtlen, 1983; Sievert *et al.*, 1989). Nitrates also dilate collateral vessels (Abrams, 1992; Ohno *et al.*, 1991), and exhibit increased vasodilator responsiveness in subendocardial versus epicardial vessels (Gross *et al.*, 1985), thus reducing the probability of diverting coronary flow from ischaemic areas (i.e. inducing coronary "steal"). Moreover, NTG reverses myocardial "hibernation" (Jugdutt *et al.*, 1997), thus increasing myocardial work and oxygen demand. Finally, the arterial vasomotor effects of organic nitrates were also originally thought to be clinically trivial. However, recent studies have shown that relatively low doses of NTG reduced arterial wave reflection, and hence left ventricular afterload (O'Rourke *et al.*, 2001).

Anti-aggregatory

In addition to their vasodilator effects, the inhibitory function of organic nitrates on platelet activation/aggregation may also contribute to the anti-ischaemic efficacy of these agents. To date a number of investigators have demonstrated that organic nitrates exert potent anti-aggregatory effects. Inhibition of platelet adhesion/aggregation has been demonstrated *in vitro* (Schafer *et al.*, 1980), *in vivo* in a number of animal models (Hebert *et al.*, 1997; Lam *et al.*, 1988) and *ex vivo* in man (Chirkov *et al.*, 1993; Diodati *et al.*, 1990; Lacoste *et al.*, 1994), utilizing intravenous, sublingual and TD-NTG, ISMN and ISDN (Bult *et al.*, 1995; Chirkov *et al.*, 1993; De Caterina *et al.*, 1988; Diodati *et al.*, 1990; Drummer *et al.*, 1991; Ivanova *et al.*, 1993; Lacoste *et al.*, 1994; Sinzinger *et al.*, 1992; Yoshimoto *et al.*, 1999).

Despite evidence of significant anti-aggregatory effects, some studies have demonstrated no significant anti-aggregatory effects in humans, despite the administration of haemodynamically effective doses of nitrates (Muikku *et al.*, 1995; Wallen *et al.*, 1994). Further to this, the available data on the anti-aggregatory effects of nitrates are limited regarding direct comparisons between the extents of the anti-aggregatory effects for various nitrate preparations. Furthermore, there is little information as to whether the anti-aggregatory effects of long-acting nitrate preparations are maintained during chronic

administration. That is, whether they are subject to the phenomenon of nitrate tolerance or a "rebound" in platelet aggregability upon nitrate withdrawal.

As outlined in sections D.2-D.4 of chapter 1, there are several potential mechanisms whereby haemodynamic and clinical responses to nitrates during chronic therapy may be less marked than initial responses ("nitrate tolerance/pseudotolerance"). This phenomenon has led to abandonment of continuous nitrate administration as a therapeutic modality in SAP. However, many studies have revealed residual clinical efficacy during chronic therapy of regimens designed to include a "nitrate-free period" (section D.4.1.1 of chapter 1).

[4.2.2] Chronic anti-anginal pharmacotherapy with organic nitrates

Clinical efficacy in stable angina

Within subjects with SAP, an intermittent application of TD-NTG is capable of improving exercise tolerance (DeMots and Glasser, 1989; Parker *et al.*, 1995). Chrysant *et al* (1993), examining the efficacy of sustained release-ISMN in a large cohort of SAP patients demonstrated a significant increase in mean total exercise time of approximately 30 to 50 seconds, 4 and 12-hrs post drug administration, an effect that was sustained over 42 days for the higher ISMN investigated (120 and 240mg).

Evidence regarding the therapeutic efficacy of ISMN and TD-NTG (intermittent use) as prophylactic anti-anginal agents

ISMN

As described above, Chrysant *et al* (1993) demonstrated a significant increase in the mean total exercise time to angina compared to placebo at 4 and 12-hrs following drug administration during chronic therapy. The benefits in prolonging exercise time with either 120mg or 240mg/day chronic dosing regimens were continued with the development of only limited nitrate tolerance. Furthermore, the higher daily doses of SR-ISMN (120mg and 240mg/day) were found to be superior to the lower doses (30mg or 60mg/day) regarding an increase in treadmill walking time to moderate angina (Chrysant *et al.*, 1993). Wisenberg *et al* (1989), utilizing a lower ISMN dosing regimen (60mg/day) also demonstrated a prolongation in exercise time, an effect that was sustained following chronic exposure without significant tolerance induction.

TD-NTG

Soon after the introduction of TD-NTG for the prophylactic management of angina pectoris, a number of investigators demonstrated a rapid and significant loss of efficacy during continuous use (Parker and Fung, 1984; Reichek *et al.*, 1984), despite increases in dosing regimens (Steering Committee, Transdermal Nitroglycerine Cooperative Study, 1991). Overcoming tolerance development by suggesting an intermittent dosing regimen (12-hr nitrate-free period) Cowan *et al* (1987), demonstrated a significant ST-segment reduction at maximum workload with the TD-NTG used in an intermittent fashion compared to continuous use. In a more comprehensive examination of the efficacy of intermittent TD-NTG use, deMotts and Glasser (1989) demonstrated that following chronic administration (2 to 4 weeks), treadmill walking time to an anginal episode was significantly prolonged with the use of 15 or 20mg/24-hrs (12-hr application) rather than 5 or 10mg/24-hrs ISMN or placebo.

Within the aforementioned studies, higher doses of ISMN (e.g. 240mg/24-hrs) and TD-NTG (e.g. 20mg/24-hrs (12-hr application)) were both associated with a higher incidence of nitrate related side effects, without any detectable improvement in efficacy beyond that of the mid range doses of ISMN (120mg/24-hrs) and TD-NTG (15mg/24-hrs (12-hr application)) (DeMots and Glasser, 1989; Parker *et al.*, 1995). The latter "medium" doses of nitrates were therefore used within the current study.

Evidence for anti-aggregatory effects

Despite initial investigations suggesting that the concentrations of organic nitrates required to produce a clinically significant anti-aggregatory effect were well beyond pharmacologically achievable concentrations *in vivo* (Loscalzo, 1985; Mehta and Mehta, 1980), studies have now demonstrated that organic nitrates indeed produce significant anti-platelet effects at therapeutically achievable doses (Diodati *et al.*, 1990; Karlberg *et al.*, 1992). However, much of the evidence for significant *ex vivo* anti-platelet effects of organic nitrates has been derived only from acute studies.

De Caterina *et al* (1984), whilst studying the effects of intravenous infusion of ISDN (1.25 – 125μg/ml for 10 minutes) in a cohort of SAP patients (n = 11), reported a significant decrease in ADP and adrenaline-induced platelet aggregation. Similarly Wolfram *et al* (1996),

demonstrated potent anti-aggregatory effects of TD-NTG and ISDN following exposure for 90 and 150 minutes.

At a more chronic level of exposure Hebert *et al* (1997), demonstrated that the amount of platelet deposition on porcine aortic media following 48-hrs of TD-NTG pharmacotherapy (0.8mg/h) remained significantly inhibited despite the induction of haemodynamic tolerance. Utilizing NTG in a dosing regimen known to induce haemodynamic tolerance in rats (10μg/min for 8-hrs) Booth *et al* (1996), demonstrated a prolongation in bleeding time implying the anti-aggregatory effects of NTG are not diminished during nitrate tolerance induction at a vascular level.

[4.2.3] Pulse wave analysis and the vasomotor effects of organic nitrates

As summarized in section B.2 of chapter 1, established methods for assessment of "endothelial function" rely on measuring endothelium-dependent (acetylcholine, reactive hyperemia) and endothelium-independent responses (NTG, SNP), of vessels *in vivo*.

Analysis of the aortic pressure waveform allows the assessment of the systolic pressure augmentation that results from a reflection of pressure from the periphery of the circulation to the aortic root (Asmar, 1999). The magnitude of this reflection can be quantified as an AI(x), which is defined as the increment expressed as a percentage, in pressure from the first systolic shoulder to the peak pressure of the aortic pressure waveform (Kelly *et al.*, 1989), the magnitude of which at least in part is determined by the pulse wave velocity (Kelly *et al.*, 2001). Given the pulse wave velocity is inversely related to arterial distensibility (Bramwell and Hill, 1922), the AI(x) has been proposed as a means of quantifying aortic distensibility or "arterial stiffness" (O'Rourke and Mancia, 1999; Wilkinson *et al.*, 1998a).

A reduction in aortic distensibility as measured by changes in AI(x), occurs with increasing age (Cameron *et al.*, 1998; Murgo *et al.*, 1980), in patients with CAD (Cameron *et al.*, 1996; Dart *et al.*, 1991; Gatzka *et al.*, 1998; Hirai *et al.*, 1989; Safar, 1999; Stefanadis *et al.*, 1990) and can be reduced by exercise training (Cameron and Dart, 1994). Pulse wave analysis has also been a useful tool in the management/diagnosis of predominately systolic hypertension and heart failure, but not as a diagnostic tool for atherosclerosis or severity of diabetes (O'Rourke *et al.*, 2001; Safar, 2000).

Recently Wilkinson *et al* (2002), in a comprehensive study examining the usefulness of radial artery pulse wave analysis as a means of assessing vasomotor endothelial function, demonstrated a marked reduction in the AI(x) with albuterol or NTG (sublingual 500μg) administration in a cohort of NVs. It was postulated, but not demonstrated, that the effects of albuterol were mainly endothelium-dependent. Importantly, a significant linear relationship was demonstrated between the extent of vascular responsiveness to albuterol (as measured by applanation tonometry) and the change in forearm blood flow during acetylcholine infusion (as measured by venous occlusion plethysmography of the brachial artery), suggesting that pulse-wave analysis provides a non-invasive means of assessing endothelial function *in vivo* (Wilkinson *et al.*, 2002).

[4.2.4] "Rebound" and the "zero hour" phenomenon

NTG and other organic nitrates are effective anti-anginal agents, but their clinical effectiveness is potentially limited both by the development of tolerance (Abrams, 1986; Thadani *et al.*, 1986) and of the "rebound" phenomenon upon nitrate withdrawal (Ferratini *et al.*, 1989; Figueras *et al.*, 1991; Frishman, 1992). A number of studies have demonstrated that intermittent nitrate pharmacotherapy is superior to continuous therapy at limiting tolerance development (Cowan *et al.*, 1987). However, intermittent therapy may be associated with "rebound" myocardial ischaemia during the nitrate free period, usually manifesting as an increase in angina at rest or even MI (Ferratini *et al.*, 1989; Lange *et al.*, 1972; Waters *et al.*, 1989).

Similar to the phenomenon of "rebound", myocardial ischaemia during a nitrate free period is the phenomenon of a decreased exercise capacity prior to nitrate administration, compared to subjects treated with placebo (Parker and Parker, 1998). Known as the "zero-hour" phenomenon, it was originally described in a study on SAP patients, in which the exercise capacity of subjects just prior to TD-NTG application failed to increase (DeMots and Glasser, 1989). This result contrasts to the placebo treatment arm where the time spent walking on a treadmill was significantly increased just prior to placebo administration (DeMots and Glasser, 1989). Despite not demonstrating nitrate tolerance or "rebound" myocardial ischaemia during a 10-12-hr nitrate free period, SAP patients treated with TD-NTG demonstrated a reduced exercise capacity (treadmill walling time) prior to nitrate application, compared to control (Parker *et al.*, 1995). It has been postulated that this phenomenon and

that of "rebound" are representative of pseudotolerance: increased vasoconstrictor tone mediated by increased secretion of endothelin-1, catecholamines and angiotensin II, which is normally algebraically opposed by nitric oxide, but which may persist briefly after nitric oxide donors are withdrawn. Perhaps the most spectacular examples of this phenomenon have been documented in association with munitions industry exposure to NTG (Ebright, 1914).

In contrast to the results observed with the use of NTG or TD-NTG, clear cut "rebound" in myocardial ischaemia has not been demonstrated with the use of ISMN (Chrysant *et al.*, 1993; Cleophas *et al.*, 2000; Olsson *et al.*, 1992; Parker, 1993; Thadani *et al.*, 1994).

[4.2.5] Summary

In the current study; (a) the *ex vivo* anti-aggregatory effects of SR isosorbide 5-mono-nitrate and TD-NTG during both acute and chronic pharmacotherapy in blood samples from patients with SAP (b) effects on platelet responsiveness to nitric oxide donors (c) effects on platelet aggregation (d) and the relationship of the nitrate effects on platelet function and arterial wave reflection, were examined.

[4.3] Current study hypothesis

This study was designed to test the following hypotheses in a cohort of mild to moderate SAP patients.

Primary:

- *That S/R isosorbide-5 mono-nitrate and intermittent transdermal-NTG (120mg and 15mg/24hours respectively) have identical effects in inhibiting ex vivo platelet aggregation both acutely and chronically in patients with SAP.*

Secondary:

- *That neither nitrate preparation affects platelet aggregation response to ADP, nor platelet responsiveness to nitric oxide, as measured utilizing the "direct" nitric oxide donor, sodium nitroprusside.*

- *That the effects of ISMN and TD-NTG on arterial wave reflection, as measured by changes in augmentation index, are identical over a 24-hr dosing period.*

- *That individual platelet and vasomotor responsiveness to organic nitrates are correlated acutely and chronically*

- *That the "zero-hour" phenomenon does not vary significantly between ISMN and TD-NTG.*

- *That whole blood superoxide generation does not vary during chronic therapy with either ISMN or TD-NTG.*

[4.4] Methods

[4.4.1] Subjects

Studies were performed in 34 patients with mild to moderate SAP (Canadian cardiovascular class II-III), (n = 34: 19 males and 15 females aged 52 to 80; mean ± S.D. 67.8 ± 7.5), with severity on assessment unlikely to preclude 1 week without prophylactic nitrate pharmacotherapy and a worsening of anginal symptoms to Canadian cardiovascular class IV (Clinical determinants are summarized in Table 4.2).

For all patients a background medication profile was recorded at the time of recruitment with the clinical characteristics of the study cohort being displayed in Table 4.3. No patient received an ADP or glycoprotein IIb/IIIa receptor antagonist at any time throughout the study. The study was approved by the North Western Adelaide Health Service Ethics of Human Research Committee, with informed consent being obtained prior to study entry.

[4.4.2] Patient exclusion criteria

Clinical parameters

Patients were excluded from the trial if they had previously not tolerated long-acting nitrates. Patients being treated with sulfhydryl-containing agents such as captopril, gold salts, penicillamine, were also excluded. Patients that had an acute MI within the 3 months prior to study involvement were also excluded. Current participation in any other study involving

investigational pharmacotherapy and a known bleeding diathesis were also grounds for trial exclusion.

Experimental parameters

During the "no nitrate" assessment phase of the trial, an aggregation response of < 3.0 ohms with ADP (1.5μM) was also grounds for trial exclusion as the responsiveness to the anti-aggregatory effects of nitric oxide donors would be difficult to quantitate.

Criteria for withdrawal from the trial

During trial involvement a number of criteria were set out for possible withdrawal from the investigation. These criteria included an intolerance of the trial medication, a requirement for intravenous nitrate infusion or addition of agents with known anti-platelet effects (ADP and GPIIb/IIIa receptor antagonists) and also a cardiac emergency requiring adjustment of nitrate dose.

[4.4.3] Nitrate pharmacotherapy protocol

Run-in phase

The trial structure is summarized in Figure 4.2. Seven days prior to the initial active treatment phase patients enrolled within the study stopped all prophylactic nitrate use but were allowed to continue using all other non-nitrate prophylactic anti-anginal medication. A diary documenting angina episodes and NTG use was also kept. On day 7 of the trial, patients were admitted for the initial "no nitrate" phase (approximately 0750-hrs). Blood samples for assessment of platelet function were then obtained throughout the day. Having met all minimal platelet aggregability criteria (see above), patients progressed to the active treatment phases of the trial.

Active treatment phases

Following the initial "no nitrate" assessment phase, patients were randomized to receive either TD-NTG 7.5mg/24-hrs for the 1st week (acute phase) and then 15mg/24-hrs for the 2nd and 3rd weeks (12-hr application) of the trial (chronic phase), or SR isosorbide mono-nitrate 60mgs mane for the 1st week followed by 120mg mane for the 2nd and 3rd weeks. At the end of each chronic phase, patients crossed over to receive the other nitrate preparation.

Patient assessment periods

Each patient at the end of the acute phases of the trial was assessed following a 24-hr nitrate free period (day 15 and day 36). Following blood sampling at 0-hrs (0800-hrs) each patient received the first high dose nitrate preparation. If well tolerated, this therapeutic regimen was then continued for 2 weeks, whereupon each subject returned for further assessment on the final day of the chronic phase of each nitrate preparation (day 28 and day 49). Diaries documenting anginal episodes and additional nitrate use were also kept. For a flow diagram depicting the phases of the trial with assessment periods see Figure 4.2.

[4.4.4] Blood sampling

All blood samples were obtained from the peripheral venous system following 10 minutes of physical inactivity. Blood samples were taken prior to administration of the trial medication (0-hrs) and were repeated 4, 8 and 24-hrs (acute phase only) which corresponded to approximately 0800, 1200, 1600 and 0800-hrs.

As displayed within the Figure 4.2, blood samples were taken at the end of the nitrate free "run in" phase, corresponding to day 7, at the commencement of the maximally tolerated trial treatment with a nitrate free period of 24-hrs prior to evaluation (day 15 drug #1, day 36 drug #2), and during the final day of the active treatment (day 28 drug #1, day 49 drug #2).

[4.4.5] Investigations

All platelet aggregation and whole blood superoxide estimations were performed by laboratory personal blinded to the active treatment phases.

Aggregability

Platelet aggregation studies in whole blood samples from the trial patients were examined utilizing a dual-channel impedance aggregometer (Model 560, Chrono-Log, Haverstown, PA, USA). Platelet aggregation studies were performed according to the method described in section 2.3.3 of chapter 2, with platelet aggregation being induced with ADP (1-1.5μM).

Superoxide

Whole blood superoxide was detected by LDCL and quantified using the method outlined in section 2 of chapter 2. Initially, a high concentration of lucigenin (125μM) was used to estimate superoxide generation in the whole blood samples prior to aggregation (baseline LDCL) and aggregation-associated LDCL (n = 10). However, as described previously, doubts were raised over the suitability of high lucigenin concentrations for the detection of superoxide (Liochev and Fridovich, 1998; Skatchkov *et al.*, 1999; Vasquez-Vivar *et al.*, 1997). Therefore, in 16 subjects, whole blood superoxide was monitored using 12.5μM lucigenin. Throughout the current chapter baseline LDCL is denoted "pre-aggregation LDCL" in order to minimize confusion with the "baseline" (no-nitrate) phase of the study.

[4.4.6] Haemodynamics

All haemodynamic variables were performed and recorded by a technician blinded to the platelet and luminescent results. Subjects (n = 12) were studied in a quiet, temperature controlled room at all time points throughout the trial. Radial artery waveforms were recorded using a Millar micromanometer tipped pressure transducer (Millar SPT 301B, Millar instruments) coupled to a Sphygmocor pulse wave velocity system (Model SCOR-Vx; PWV Medical) from the wrist of the dominant arm. A series of radial artery pressure waves were recorded over a period of eight seconds. A central waveform was generated using a convolutional algorithm and generalized transfer function as previously described (Karamanoglu *et al.*, 1993; O'Rourke *et al.*, 2001; Wilkinson *et al.*, 1998b). From the generated central waveform, an AI(x) corrected for a heart rate of 75bpm was calculated as the difference between the second and first systolic peaks (Wilkinson *et al.*, 2002). Systolic blood pressure was recorded in duplicate in the dominant arm using an automatic digital blood pressure monitor (HEM-705CP; Omron, Japan). A computerized report on an analysis of a radial artery and the synthesized aortic pressure wave is depicted in Figure 4.1.

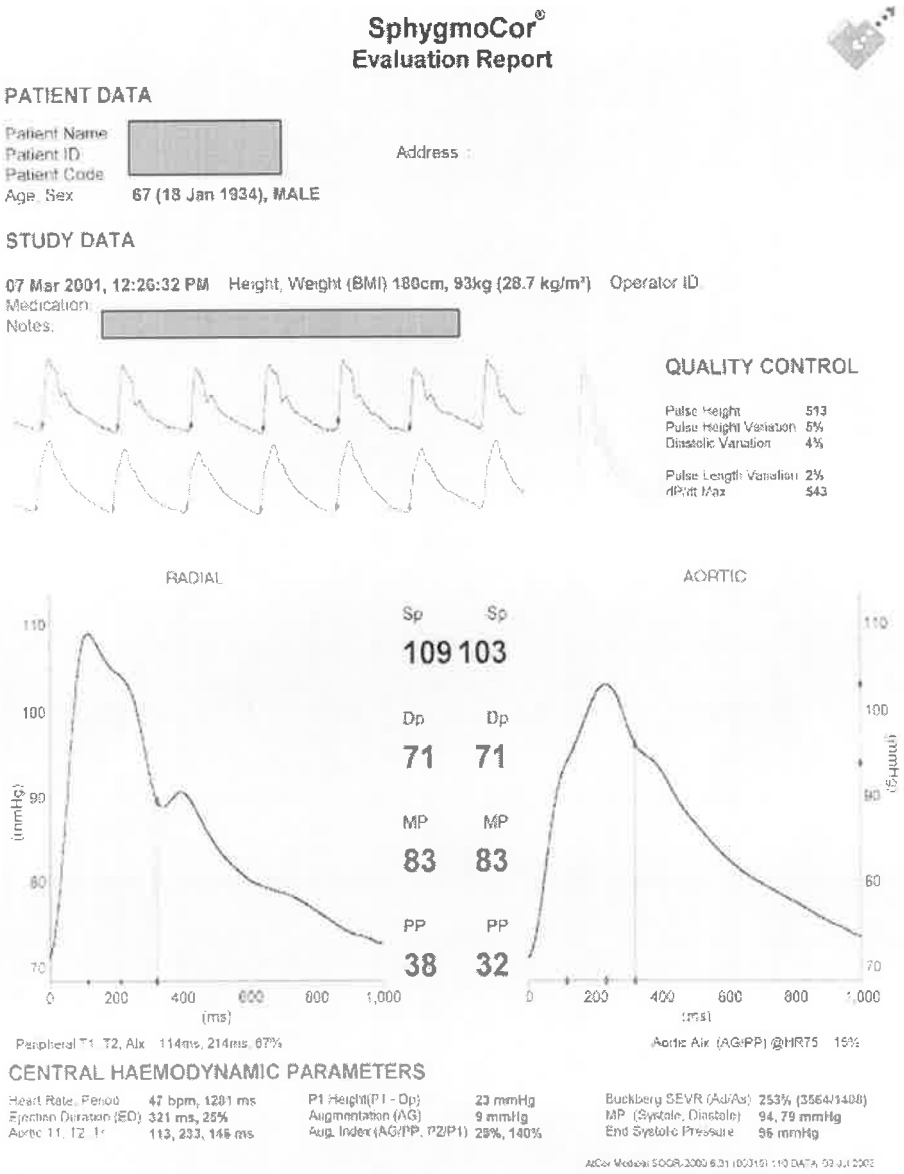


Figure 4.1 A representative sphygmocardiogram

A sphygmocardiogram obtained from a male patient during the "no nitrates" period of the trial. An average radial artery pressure wave (lower left panel) is used to generate an ascending aortic pressure wave (lower right panel), through the use of a convolutional algorithm and a generalized transfer function.

[4.4.7] Chemicals

Chemicals utilized within this study are described in section 2.3.3 of chapter 2.

[4.4.8] Statistical Analysis

Differences between proportions of subjects with particular risk factors or medications were examined using Fishers exact test. Differences between proportions across treatment phases was examined using Friedman's ANOVA for matched non-parametric data followed by Wilcoxon matched paired signed rank test. Gaussian distributions of data were determined by the Kolmogorov-Smirnov test. Homogeneity of variance was assessed using Bartlett's or Levene's F-test. Differences between standard deviations were examined by Bartlett's test. Log transformations were performed on data that failed ANOVA assumptions. Comparisons between variables were made utilizing ANOVA (repeated measures where appropriate) followed by Bonferroni's post hoc multiple comparison test. Significance of correlation was examined by regression analysis with linearity determined by a run test. Comparisons between regression curves were made by ANCOVA. Statistical analysis was performed using the computer programs outlined in section 2.3.3.6 of chapter 2. Statistically significant differences were limited to $p < 0.01$ unless otherwise indicated. Results are expressed as mean ± S.E.M unless otherwise stated.

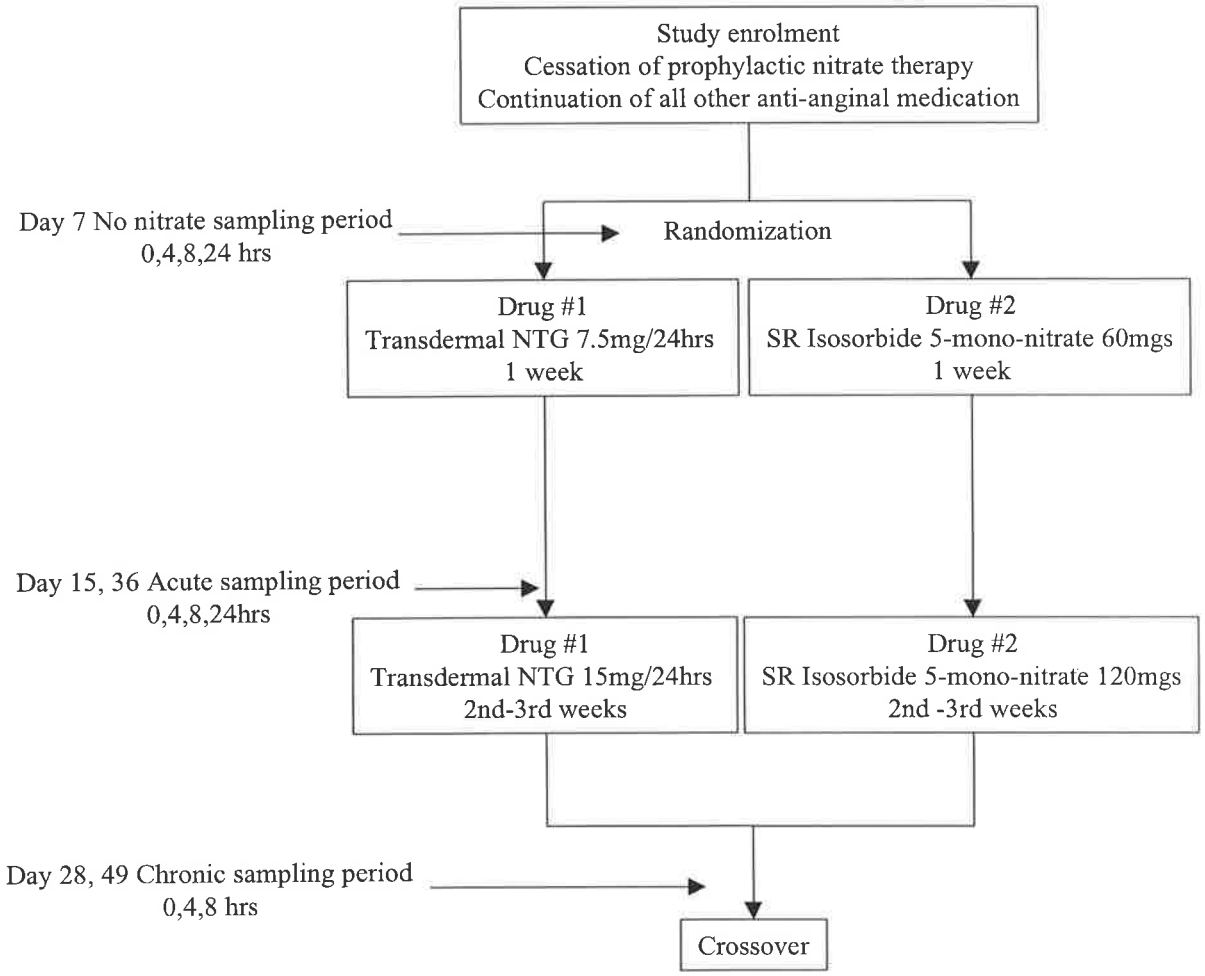


Figure 4.2 Schematic representation of the nitrate treatment regimen

Following 1 week cessation of prophylactic nitrate pharmacotherapy, the cohort of SAP patients underwent randomized crossover trial investigating the platelet anti-aggregatory properties of S/R isosorbide 5'mono-nitrate and intermittent transdermal NTG as outlined in the above schema.

[4.5] Results

[4.5.1] Clinical characteristics

Thirty-four patients, with mild to moderate SAP completed the 7-week protocol. One subject voluntarily withdrew from the investigation on personal grounds and in 2 subjects platelet aggregation was below 3 ohms (see section 4.4.2; patient exclusion criteria). The clinical characteristics of those subjects who completed the study are shown in Table 4.2.

Table 4.2 Clinical determinants of the study cohort

Clinical Determinants	n (%)
Age (Mean ± S.D), years	67.8 ± 7.5
Males, n (%)	19 (56)
Current Smokers, n (%)	7 (21)
Ex-smokers, n (%)	12 (35)
Hypercholesterolaemia, n (%)	32 (94)
Hypertension, n (%)	23 (68)
Previous MI, n (%)	16 (47)
Previous UAP, n (%)	18 (53)
Positive family history, n (%)	14 (41)
NIDDM, n (%)	8 (23)
IDDM, n (%)	1 (3)
Stroke, n (%)	2 (6)
PVD, n (%)	5 (15)
CCF, n (%)	3 (9)

S.D.= standard deviation; MI = myocardial infarction; UAP = unstable angina pectoris; NIDDM = non-insulin dependent diabetes mellitus; IDDM = insulin-dependent diabetes mellitus; PVD = peripheral vascular disease; CCF = congestive cardiac failure.

Medications

As shown in Table 4.3, most subjects prior to study enrolment were receiving a number of non-nitrate prophylactic anti-anginal medications. All subjects were required to continue all non-nitrate prophylactic anti-anginal medication, unchanged throughout the duration of the trial period. Thirty of the thirty-four patients were receiving low dose aspirin on a regular basis.

Table 4.3 Medication profile of the study cohort

Medications	n (%)
Aspirin, n (%)	30 (88)
Ca^{2+} antagonists, n (%)	23 (68)
Perhexiline, n (%)	13 (38)
ACE inhibitors, n (%)	20 (59)
β-adrenoceptor antagonists, n (%)	7 (21)
Statins, n (%)	21 (62)
Digoxin, n (%)	3 (9)

[4.5.2] Adverse effects of the nitrate preparations

Both nitrate preparations were well tolerated with no major adverse events during the investigation period and no acute events requiring hospitalization). Ten subjects experienced

mild headaches that were relieved by paracetamol. One subject experienced daily exertional dyspnea when on either ISMN or TD-NTG throughout both the acute and chronic phases of the trial.

[4.5.2.1] Anginal frequency

Throughout the trial a number of subjects experienced anginal episodes requiring relief through the use of either additional NTG spray or sublingual NTG. A depiction of the raw and median data with the $25^{th}/75^{th}$ quartile values for angina frequency and additional nitrate consumption, is shown in Figure 4.3.

Utilizing Friedman's ANOVA for matched non-parametric data there was no significant difference in angina frequency or additional nitrate consumption across the phases of the trial ("no nitrates"/acute/chronic) for each nitrate preparation examined (ISMN/TD-NTG) (Friedman ANOVA: Angina frequency ISMN; Chi-square = 0.74, p = 0.68, TD-NTG; Chi-square = 0.015, p = 0.99: Nitrate use ISMN; Chi-square = 0.19, p = 0.91, TD-NTG; Chi-square = 0.37, p = 0.83). Utilizing Wilcoxon matched-paired signed-rank test, differences between treatment with either ISMN/TD-NTG regarding angina frequency/nitrate use was also found not to be significant (Wilcoxon matched paired signed rank test: Angina frequency: Acute ISMN vs Acute TD-NTG z = -0.48, p= 0.63; Chronic ISMN vs Chronic TD-NTG z = -0.48, p = 0.68. Nitrate consumption: Acute ISMN vs Acute TD-NTG z = -0.15, p = 0.87; Chronic ISMN vs Chronic TD-NTG z = -0.35, p = 0.72).

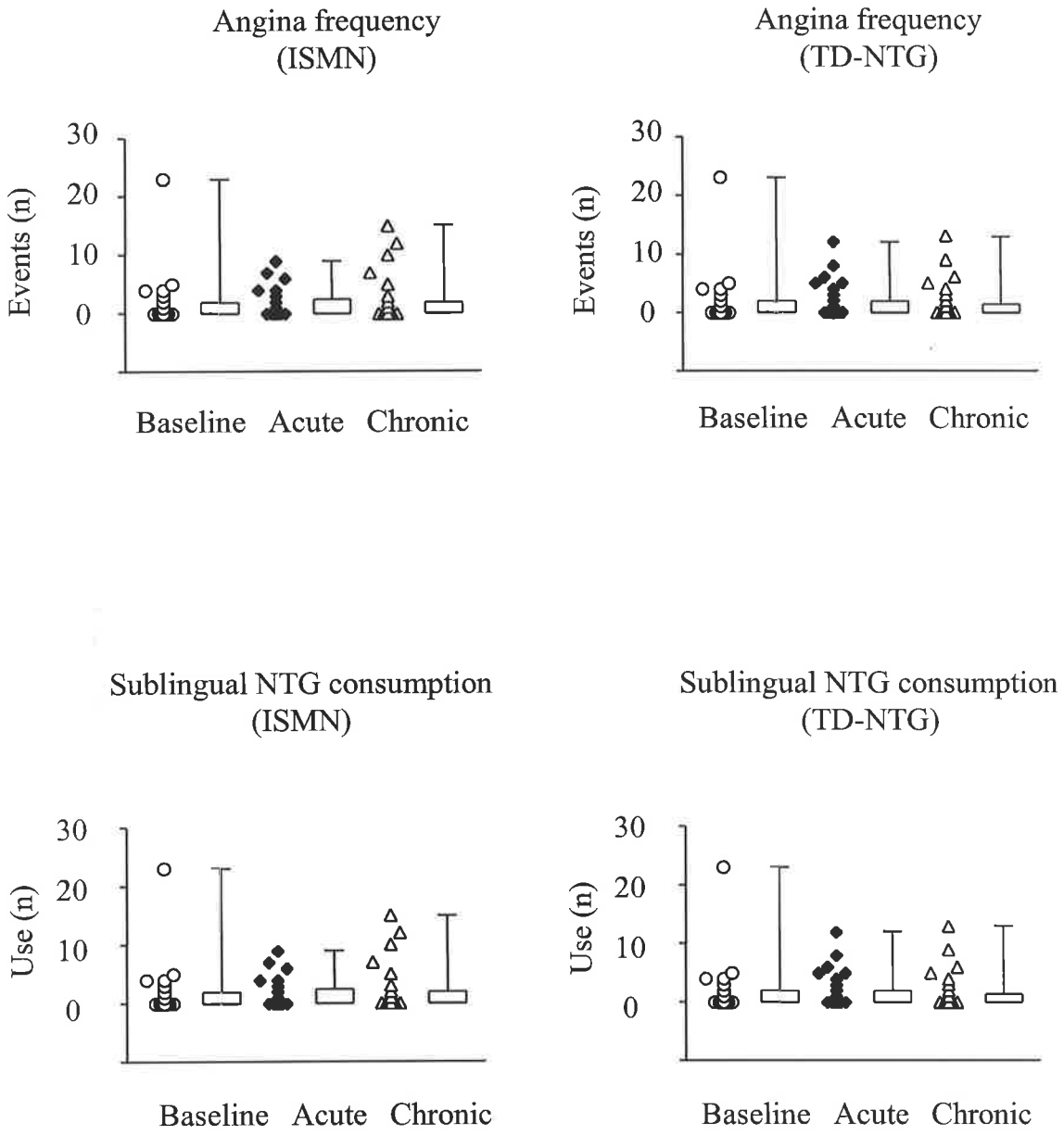


Figure 4.3 Angina pectoris episodes and additional nitrate consumption during trial

The frequency of anginal episodes and nitrate consumption used to relieve angina was recorded throughout all phases of the trial ("no nitrates"/acute/chronic). Raw data with box plots and whiskers (median with 25th/75th quartiles and range) open circles ("no nitrates"), closed diamonds (Acute nitrates), open triangles (chronic nitrates). ***Upper panels****: No significant difference in anginal episodes during all phases of the trial for both ISMN treatment (left: Friedman ANOVA; Chi-square = 0.74, p = 0.68) or TD-NTG (right: Friedman ANOVA; Chi-square = 0.74, p = 0.9).* ***Lower panels****: No significant difference in additional nitrate use during all phase of the trial for both ISMN (left: Friedman ANOVA; Chi-square = 0.37, p = 0.83) or TD-NTG (right: Friedman ANOVA; Chi-square = 0.19, p = 0.9).*

[4.5.3] Platelet and luminescent investigations

[4.5.3.1] Diurnal variability

Platelet response to ADP

Of all the SAP patients enrolled within this investigation (n = 34), blood samples from 2 patients required the addition of ADP (1.5μM) rather than 1.0μM, in order to reach the minimum degree of platelet aggregability defined in section 4.4.2 for successful study participation.

The majority of patients were already undergoing aspirin pharmacotherapy at the time of enrollment (88%). Platelet response to ADP at the zero hour "no nitrate" collection in those subjects not treated with aspirin (n = 4) was not significantly different from that in subjects undergoing aspirin pharmacotherapy (n = 30), and therefore data were pooled.

As well as a variable aspirin intake, gender-related differences within the subject cohort can potentially complicate the assessment of platelet aggregability (Faraday *et al.*, 1997; Meade *et al.*, 1985). Therefore platelet response to ADP was assessed according to gender across the 24-hr period. Firstly, both male and female subject populations were demonstrated to conform to a Gaussian distribution (Appendix Table 20). Therefore by 2-way repeated measures ANOVA (Figure 4.4), there was no significant diurnal variability in platelet response to ADP, despite there being a significant difference between male and female subjects, with the latter being significantly more aggregable than former (2-way repeated measures ANOVA; Time $F = 0.55$, $p = 0.65$; Gender $F = 50.7$, $p < 0.001$, time x gender $F = 0.73$, $p = 0.54$; Bartlett's statistic = 5.29, $p = 0.62$). Utilizing Bonferroni's post hoc multiple comparison test and apart from the obvious gender differences that were all highly significant, there were no significant differences between the time points within the gender populations regarding platelet aggregation. Interestingly platelet aggregability at the zero hour time point for both genders were not significant different from each other, whereas they were at the eight-hr collection point.

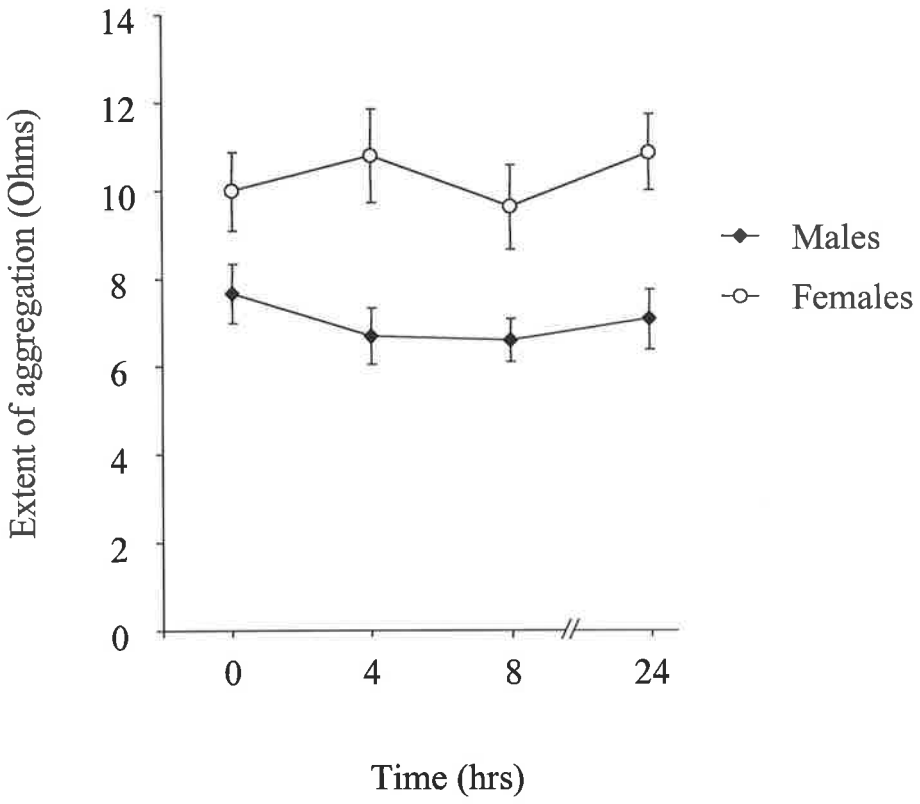


Figure 4.4 Diurnal variability in platelet response to ADP

Variability in platelet response to ADP during the "no nitrate" phase was analyzed according to time and gender. By two-way repeated measures ANOVA no significant diurnal variability in platelet response to ADP was observed despite an obvious and significant gender difference in the extent of platelet aggregation. (2-way repeated measures ANOVA, Time $F = 0.55$, $p = 0.65$; Gender $F = 50.7$, $p < 0.001$, Time x Gender $F = 0.73$, $p = 0.54$).

Diurnal variability in platelet responsiveness to NTG

At the end of the "run in" phase (7 day of a nitrate free period), platelet responsiveness to NTG (100μM) was examined to discern any significant diurnal variability. Given the extent of whole blood platelet responsiveness to NTG (100μM) was demonstrated to be independent of gender (section 3.5.3 of chapter 3) data herein were analyzed on the pooled results from both male and female subjects. Platelet responsiveness to NTG (100μM) for each time point conformed to a Gaussian distribution (0-hrs KS = 0.08, p = ns; 4-hrs KS = 0.1, p = ns; 8-hrs KS = 0.11, p = ns; 24-hrs KS = 0.09, p = ns) with homogeneity of variances across the 24-hr time period (Levene's test: F = 1.72, p = 0.16). In much the same way as demonstrated for platelet response to ADP and as shown in Figure 4.5, no significant diurnal variability in platelet NTG responsiveness was observed (1-way repeated measures ANOVA; Time, F = 2.23, p = 0.088).

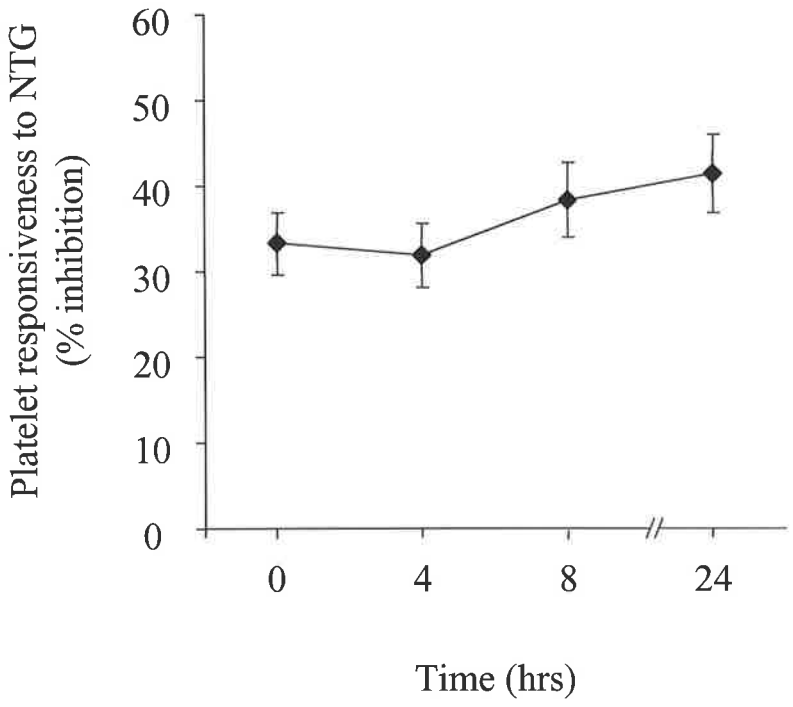


Figure 4.5 Diurnal variability in platelet responsiveness to NTG

Variability in platelet responsiveness to NTG (100μM) (expressed as a % inhibition of ADP-induced aggregation) during the "no nitrate" collection phase was analyzed across 24-hrs with no significant diurnal variability being observed. (1-way repeated measures ANOVA; Time, F = 2.23, p = 0.088).

Diurnal variability in platelet responsiveness to SNP

Platelet responsiveness to SNP (10μM) during the initial "no nitrate" examination phase of the trial was also examined. Platelet responsiveness to SNP (10μM) for each time point conformed to a Gaussian distribution (0-hrs KS = 0.12, p = ns; 4-hrs KS = 0.1, p = ns; 8-hrs KS = 0.16, p = ns; 24-hrs KS = 0.13, p = ns) with homogeneity of variance across the 24-hr time period (Levene's test: F = 0.089, p = 0.96). By 1-way repeated measure ANOVA (Time: F = 2.64, p = 0.053) there was a trend towards, diurnal fluctuation in platelet responsiveness to SNP across the "no nitrate" collection phase of the study. This trend is clearly illustrated in Figure 4.6 and reflects a significant difference in platelet responsiveness to SNP (10μM) between the 8-hr and 24-hr collection time point (Bonferroni's post hoc multiple comparison test t = 2.7, p < 0.05). However, there was no significant difference between the zero and eight-hr data, suggesting that this difference may be spurious.

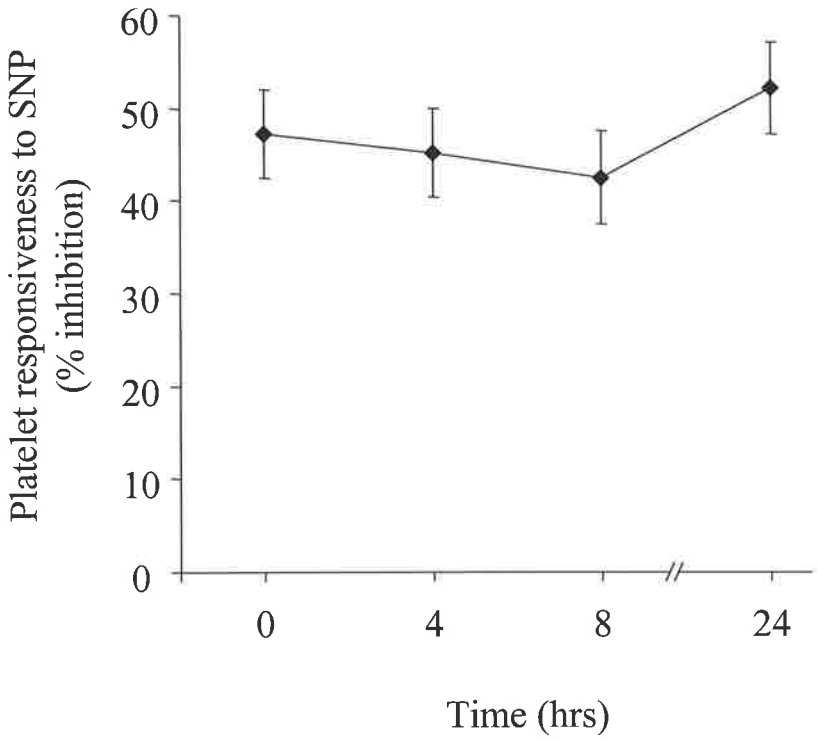


Figure 4.6 Diurnal variability in platelet responsiveness to SNP

Variability in platelet responsiveness to SNP (10μM) (expressed as a % inhibition of ADP-induced aggregation) during the "no nitrate" collection phase was analyzed across the 0,4,8 and 24-hr time period with a trend towards, diurnal variability being observed within the patient cohort (1-way repeated measures ANOVA F = 2.64, p = 0.053).

Diurnal variability in whole blood superoxide

A number of investigators have examined the role of superoxide generation post organic nitrate administration and have suggested that it plays a significant role in the phenomenon of nitrate tolerance (Munzel *et al.*, 1995; Dikalov *et al.*, 1998; Dikalov *et al.*, 1999). Moreover, as indicated in section I of Chapter 2, there may also be a role for superoxide in the phenomenon of platelet hyper-aggregability and hypo-responsiveness to nitric oxide. Therefore superoxide was examined in order to determine the influence of both acute and chronic nitrate pharmacotherapies on superoxide generation in whole blood. As described with the methods section "baseline LDCL" is denoted as "pre-aggregation LDCL" for the current investigation in order to prevent confusion between the luminescence variable and the "baseline" treatment phase of the trial.

Pre-aggregation LDCL

Data obtained from the 16 subjects over the initial "no nitrate" period of the trial for the pre-aggregation LDCL assumed a Gaussian distribution (0hrs KS = 0.12, p = ns; 4hrs KS = 0.14, p = ns; 8hrs KS = 0.18, p = ns; 24hrs KS = 0.19, p = ns). However, heterogeneity of variance was observed (Bartlett's statistic = 20.2, $p < 0.01$) and a log transformation of the data was performed (Bartlett's statistic post log transformation = 6.9, p = 0.074). By 1-way ANOVA no significant diurnal variability in the pre-aggregation LDCL was found as detected by 12.5μM lucigenin (F = 1.75, p = 0.17). A summary of the pre-aggregation LDCL is illustrated in the left panel of Figure 4.7.

Aggregation-associated LDCL

Aggregation-associated LDCL (12.5μM lucigenin) for all data populations over the 24-hour "run in" "no nitrate" period of the trial was also demonstrated to conform to a Gaussian distribution (0hrs KS = 0.16 p = ns; 4hrs KS = 0.22 p = ns; 8hrs KS = 0.23 p = ns; 24hrs KS = 0.16 p = ns). Like the other luminescence variables heterogeneity of variance was observed (Bartlett's statistic = 11.9, p = 0.0078) and a log transformation of the data was performed (Bartlett's statistic post log transformation = 3.6, p = 0.31). By 1-way ANOVA there was a non-significant trend towards a significant diurnal variability in aggregation-associated LDCL (F = 2.3, p = 0.088). A summary of the aggregation-associated LDCL detected by 12.5μM lucigenin is illustrated in the lower right panel of Figure 4.7. Utilizing Bonferroni's multiple comparison test, the trend towards a significant diurnal variability comes from a

slight difference between the 4-hr and 24-hr sampling time points (Bonferroni's multiple comparison test $t = 2.58$, $t_{crit} = 2.74$).

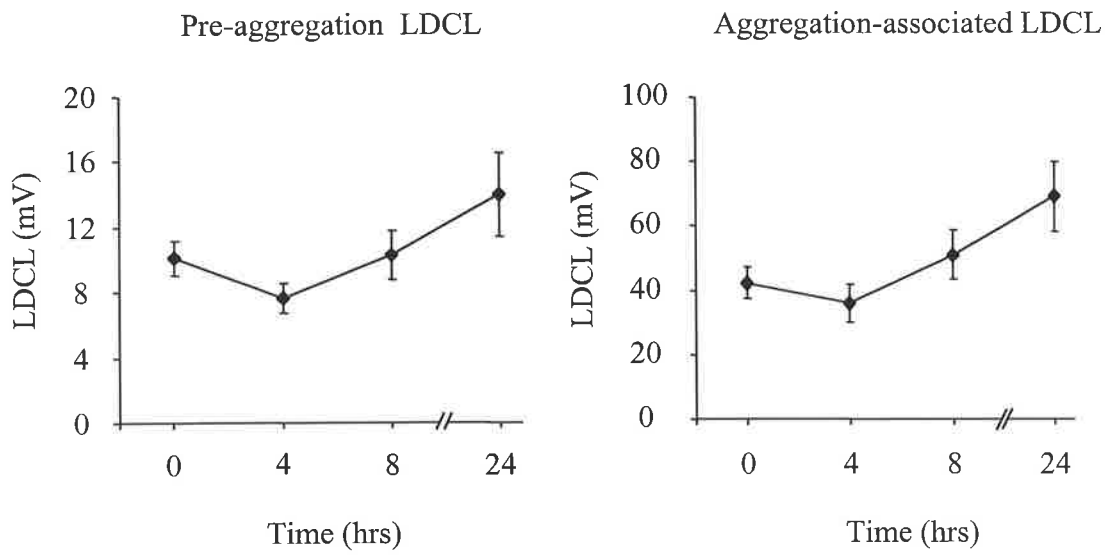


Figure 4.7 Diurnal variability in whole blood superoxide

Diurnal variability in whole blood superoxide level (pre-aggregation LDCL (left) and aggregation-associated LDCL (right)) during the "no nitrate" phase of the study, was examined in 16 subjects utilizing lucigenin (12.5μM). No significant diurnal variability in the pre-aggregation LDCL was observed (log transformed data; 1-way ANOVA F = 1.75, p = 0.17). No significant diurnal variability in (12.5μM lucigenin) aggregation-associated LDCL was also observed (log transformed data; 1-way ANOVA F = 2.3, p = 0.088) though a trend towards a significant variability was observed.

[4.5.3.2] Acute nitrate pharmacotherapy

Acute anti-aggregatory effects of ISMN and TD-NTG

Following a 24-hr nitrate free period and having already been treated for 7 days with either 60mg ISMN or 7.5mg/24-hrs (12-hr application) TD-NTG, each patient returned for the next series of investigations designed to examine the acute anti-aggregatory effects of both nitrate preparations. Immediately following the zero hour blood collection (approximately 8am), each subject received their first dose of either ISMN (120mgs) or TD-NTG (15mg/12-hr application) with further blood samples being taken at 4, 8 and 24-hrs.

Firstly, normality in the data populations for each gender and nitrate preparation during the acute phase of the trial was examined using the Kolmogorov-Smirnov test. All subject populations for platelet aggregability were found to conform to a Gaussian distribution and

contain homogeneous variances (Bartlett's statistic = 15.9, p = 0.38; Appendix Table 21). A repeated measures ANOVA was not performed as data from two subjects on separate occasions were not collected.

ISMN

Utilizing 3-way ANOVA (Figure 4.8 (upper panel) and Table 4.4), there was no significant anti-aggregatory effect following the acute administration of ISMN (120mg) compared to the "no nitrate" platelet response to ADP across the genders over the 24-hr time period. Platelets from female patients were significantly more aggregable than their male counterparts (3-way ANOVA $F = 82.7$, $p < 0.01$). There was no significant change in platelet aggregability across the time period, along with no significant interaction between the determinants. Utilizing Bonferroni's post hoc multiple comparison test and apart from the obvious gender differences, there were no significant differences in platelet aggregability across the 24-hrs within each gender sub-group.

TD-NTG

The absence of any significant anti-aggregatory effect was also observed with acute administration of TD-NTG (15mg/12-hr application). By 3-way ANOVA and as illustrated in the lower panel of Figure 4.8 (Table 4.4), there was no significant difference in aggregability between the treatment phase ("no nitrates"/TD-NTG), or across the time period (Bartlett' statistic = 19.9, p = 0.17). Gender was the only determinant that remained significant, with female patients remaining more aggregable than their male counterparts ($F = 73.6$, $p < 0.01$). Utilizing Bonferroni's post hoc multiple comparison test, there were no significant differences in platelet aggregability across the 24-hrs within either gender.

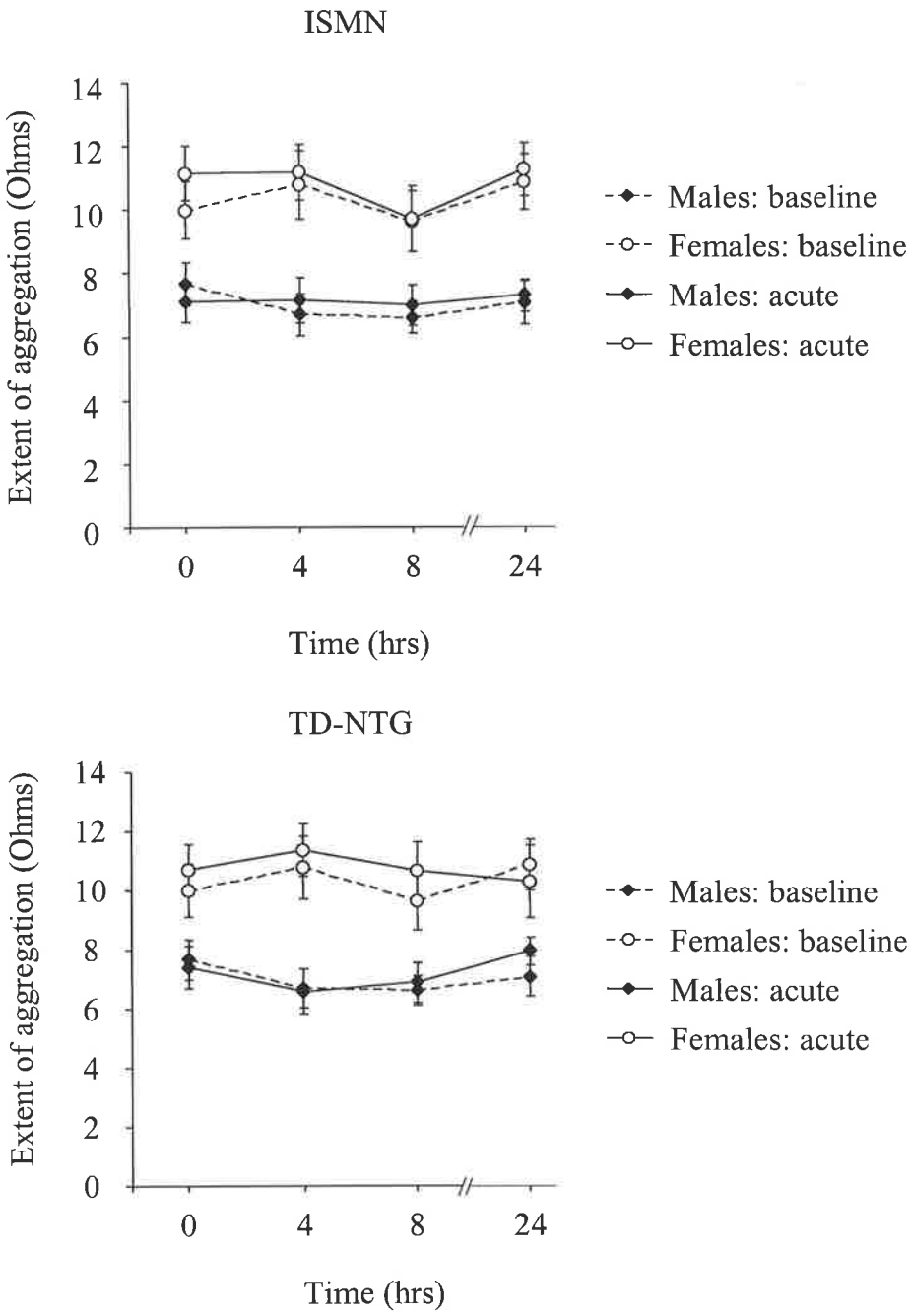


Figure 4.8 Acute effects of ISMN and TD-NTG on ADP-induced aggregation

The anti-aggregatory effects of ISMN (upper panel) and TD-NTG (lower panel) were examined across the time period of 0,4,8 and 24-hrs and according to gender. Levels of significance are shown in Table 4.4. Either 120mg ISMN or 15mg/24-hrs TD-NTG was administered immediately following zero hour blood sampling depending on initial randomization. Each patient had already undergone 7 days of nitrate treatment with either 60mg ISMN or 7.5mg TD-NTG, with a 24-hr nitrate free period just prior to acute phase blood sampling. Gender-related differences in platelet aggregability remained without any significant anti-aggregatory effect of either nitrate preparation being observed. Levels of statistical significance are summarized in Table 4.4.

Table 4.4 Anti-aggregatory effect of acute nitrate pharmacotherapy:- Three-way ANOVA contingency table

	ISMN		TD-NTG	
Determinants	***F***	***p***	***F***	***p***
Time	1.10	0.36	0.44	0.72
Treatment	0.65	0.42	0.63	0.43
Gender	82.7	< 0.01	73.6	< 0.01
Interactions				
Time x Treatment	0.01	0.99	0.07	0.97
Time x Gender	0.53	0.66	0.79	0.50
Treatment x Gender	0.24	0.62	0.08	0.76
Time x Treatment x Gender	0.35	0.78	0.49	0.69

3-way ANOVA examining the significance and interactions of time, treatment groups ("no nitrates"/acute nitrate pharmacotherapy) and gender for each nitrate preparation regarding platelet response to ADP during acute nitrate treatment.

Changes in platelet responsiveness to nitric oxide donors during acute nitrate pharmacotherapy:-

Nitroglycerine

Data representing platelet responsiveness to NTG (100μM) for both nitrate treatment regimens were demonstrated to conform to a Gaussian distribution and have homogeneous variances across the 24-hr time period (ISMN; 0-hrs KS = 0.13, p = ns; 4-hrs KS = 0.08, p = ns; 8-hrs KS = 0.09, p = ns; 24-hrs KS = 0.06, p = ns; Bartlett's statistic = 2.8, p = 0.42), (TD-NTG; 0-hrs KS = 0.1, p = ns; 4-hrs KS = 0.09, p = ns; 8-hrs KS = 0.12, p = ns; 24-hrs KS = 0.09, p = ns; Bartlett's statistic = 1.9 p = 0.57). By 2-way ANOVA and as summarized in Figure 4.9, no significant change in platelet responsiveness towards NTG was observed following the acute treatment with either 120mg ISMN or 15mg/24-hrs TD-NTG. (2-way ANOVA; Time F = 1.6, p = 0.17; Treatment group F = 0.34, p = 0.71; Time x treatment group F = 0.48, p = 0.82; Bartlett's statistic = 8.1, p = 0.71). A 2-way ordinary ANOVA was performed as estimations of platelet responsiveness to NTG at the 24-hr collection time point for 2 subjects during separate treatment regimens were not performed.

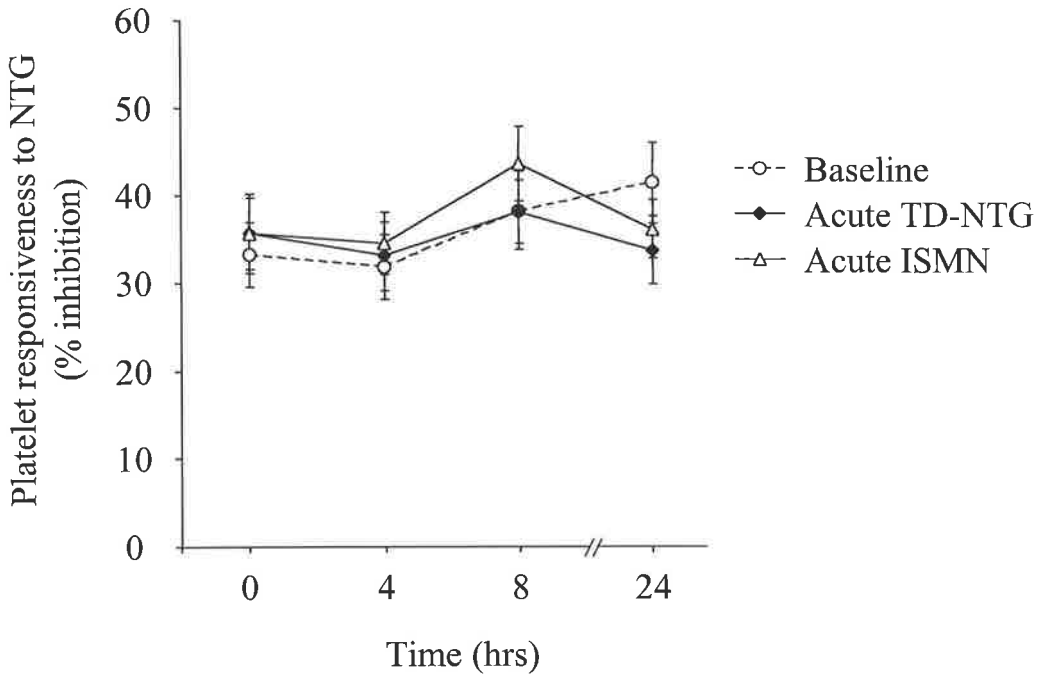


Figure 4.9 Influence of acute nitrate administration on platelet responsiveness to NTG

Acute administration of either 120mg ISMN or 15mg TD-NTG had no significant effect on platelet responsiveness to NTG (100μM). (2-way ANOVA; Time F = 1.6, p =0.17; Treatment group F = 0.34, p = 0.71; Time x Treatment group F = 0.48, p = 0.82). For each subject, nitrate preparations were administered immediately post the zero hour blood sampling point.

Sodium nitroprusside

The degree of change in platelet responsiveness to SNP, a more direct donor of nitric oxide than NTG (Ignarro *et al.*, 2002), during acute administration of both nitrate preparations, was also examined. Having already established that the degree of platelet responsiveness to SNP (10μM) during the "no nitrate" phase conforms to a Gaussian distribution, normality in SNP responsiveness for each nitrate preparation along with a standard deviation assessment was performed. (ISMN; 0-hrs KS = 0.07, p = ns; 4-hrs KS = 0.09, p = ns; 8-hrs KS = 0.19, p = ns; 24-hrs KS = 0.07, p = ns. Bartlett's statistic = 1.26, p = 0.74). (TD-NTG; 0-hrs KS = 0.09, p = ns; 4-hrs KS = 0.1, p = ns; 8-hrs KS = 0.08, p = ns; 24-hrs KS = 0.09, p = ns. Bartlett's statistic = 0.13, p = 0.99). By 2-way ANOVA and as shown in Figure 4.10, no significant change in platelet responsiveness to SNP was observed following acute administration of either nitrate preparation. (2-way ANOVA; Time F = 0.41, p = 0.74; Treatment group F = 0.06, p = 0.94; Time x Treatment group F = 1.03, p = 0.987; Bartlett's statistic = 3.34, p = 0.98). As with the assessment of the effects of platelet responsiveness to NTG (100μM) an ordinary ANOVA was performed (rather than a repeated measures ANOVA) because of missing data from two subjects.

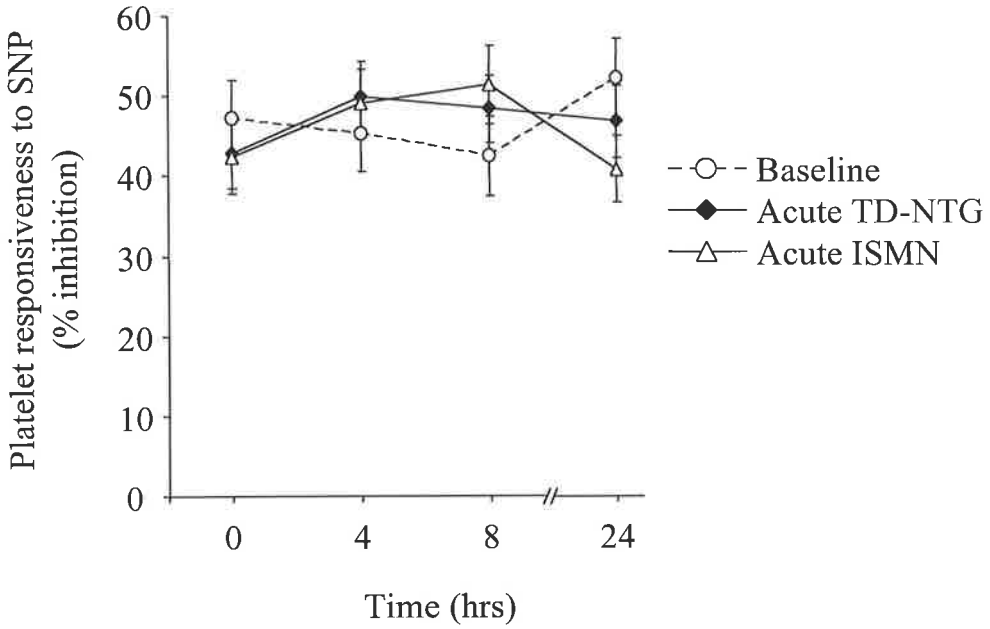


Figure 4.10 Influence of acute nitrate administration on platelet responsiveness to SNP

Acute administration of either 120mg ISMN or 15mg TD-NTG following a 24-hrs non-nitrate period had no significant effect on platelet responsiveness to SNP (10μM). Each nitrate preparation was administrated immediately post zero hr blood sampling (2-way ANOVA; Time F = 0.41, p = 0.74; Treatment group F = 0.06, p = 0.94; Time x Treatment group F = 1.03, p = 0.98).

Acute effects of nitrate pharmacotherapy on superoxide production

The acute administration of ISMN (120mg) and TD-NTG (15mg/12-hr application) was examined for their effects on whole blood superoxide as detected by LDCL.

Pre-aggregation LDCL

The acute effects of nitrate administration on superoxide levels were analyzed using lucigenin (12.5μM). Pre-aggregation LDCL data for both acute nitrate treatment regimens were found to be normally distributed, with the ISMN treatment phase containing heterogeneous variances (ISMN: 0-hrs KS = 0.18, p = ns; 4-hrs KS = 0.23, p = ns; 8-hrs KS = 0.26, p = ns; 24-hrs KS = 0.21, p = ns; Bartlett's statistic = 10.3, p = 0.016). (TD-NTG; 0-hrs KS = 0.18, p = ns; 4-hrs KS = 0.17, p = ns; 8-hrs KS = 0.18, p = ns; 24-hrs KS = 0.15, p = ns; Bartlett's statistic = 6.6, p = 0.08). Therefore as the "no nitrate" and ISMN phases both contained significant differences in the standard deviations across the sampling time points, a log transformation of all the data was performed before ANOVA. By 2-way ANOVA a significant difference between the treatment groups was observed (2-way ANOVA;

Treatment group F = 3.48, p = 0.033; Time F = 1.95, p = 0.12; Treatment group x Time F = 0.69, p = 0.66; Bartlett's statistic = 11.4, p = 0.41). By Bonferroni's multiple post hoc test, there was no significant difference between data points for control and treatment, despite a significant difference between the treatment groups observed within the ANOVA. For a further summary of the acute nitrate effects on pre-aggregation LDCL, see the left panel of Figure 4.11.

Aggregation-associated LDCL

Data representing the extent of aggregation-associated LDCL for both acute nitrate treatment regimens were found to conform to a Gaussian distribution and not to contain any significant standard deviations across the sampling time points within each treatment regimen (ISMN: 0-hrs KS = 0.19, p = ns; 4-hrs KS = 0.12, p = ns; 8-hrs KS = 0.23, p = ns; 24-hrs KS = 0.15, p = ns; Bartlett's statistic = 0.54, p = 0.9) (TD-NTG; 0-hrs KS = 0.16, p = ns; 4-hrs KS = 0.23, p = ns; 8-hrs KS = 0.18, p = ns; 24-hrs KS = 0.19, p = ns: Bartlett's statistic = 2.6, p = 0.46). However, as the "no nitrate" data demonstrated a significant difference between the standard deviations of the time points, all data points for the acute phase of the trial underwent a log transformation. By 2-way ANOVA and as shown in Figure 4.11 right panel, there was a non-significant trend towards a difference between the treatment groups. There was also a significant difference in aggregation-associated LDCL over the 24-hr time period with no significant interaction between either determinant (2-way ANOVA; Treatment group F = 2.4, p = 0.098; Time F = 4.8, p < 0.01; Treatment group x Time F = 0.46, p = 0.83; Bartlett's statistic = 8.87, p = 0.63). For a further summary of the acute nitrate effects on aggregation-associated LDCL see the lower right panel of Figure 4.11.

Summary

Results representing the effects of acute nitrate pharmacotherapy on both pre-aggregation and aggregation-associated LDCL can be summarized as follows:-

- Data representing both the pre-aggregation and aggregation-associated LDCL tended to be lower during the acute TD-NTG pharmacotherapy phase than the ISMN treatment phase.
- LDCL values tended to be the lowest at the 4-hr time-point (cf. significant time variability post-aggregation)

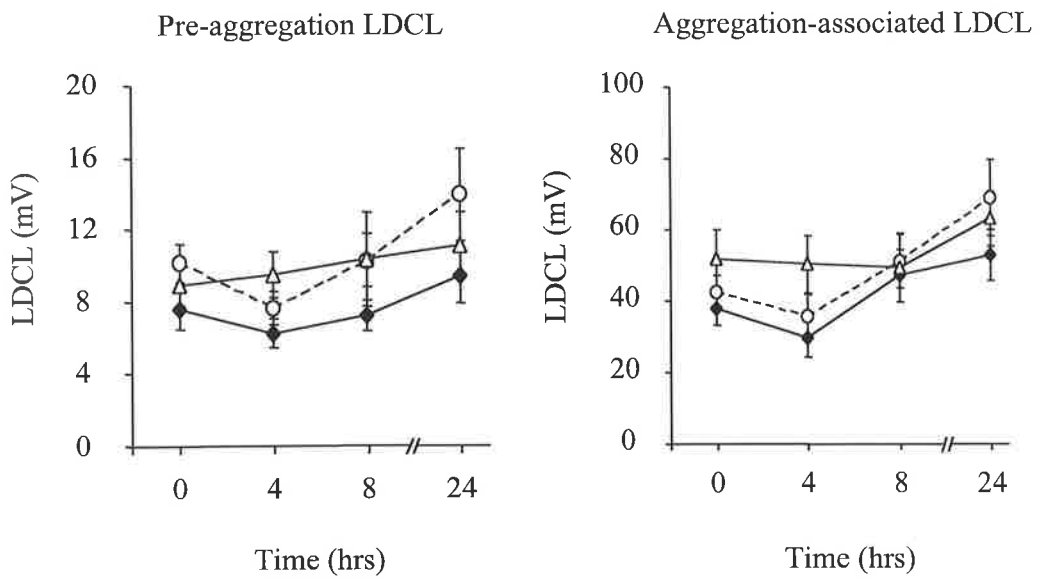


Figure 4.11 Acute administration of nitrates and its effects on whole blood superoxide generation

Figure legend: *Open circle dotted line = LDCL during the "no nitrate" phase of the study. Closed diamond solid line = Acute TD-NTG treatment phase. Open triangle solid line = Acute ISMN treatment phase.* ***left****, pre-aggregation LDCL 2-way ANOVA:- Treatment F = 3.48, p = 0.033; Time F = 1.95, p = 0.12; Treatment x Time F = 0.69, p = 0.66;* ***right*** *aggregation-associated LDCL; Treatment F = 2.4, p = 0.098; Time F = 4.8, p < 0.01; Treatment x Time F = 0.46, p = 0.84.*

[4.5.3.3] Chronic nitrate pharmacotherapy

Anti-aggregatory effects

Having completed the acute phase sampling period of the trial for the first nitrate preparation, each subject then took each nitrate for an additional two weeks in order to examine the chronic nitrates effects on platelet responses to ADP. On the final day of chronic nitrate treatment (Day 28/49) each patient had blood samples taken at 0,4 and 8-hrs, with all subjects being instructed to take his/her final nitrate dose immediately following the zero hour collection.

ISMN

Like the "no nitrate" and acute phases of the trial, platelet response to ADP for each gender during the chronic phase of the investigation conformed to a Gaussian distribution (Appendix Table 22). By 3-way repeated measures ANOVA and as displayed in Figure 4.12, there was no significant variation in ADP response following chronic administration of 120mg ISMN

(Bartlett's statistic = 10.2, p = 0.51). A repeated measures ANOVA was utilized, as all data across the time periods were accounted for.

In much the same way as demonstrated for the acute administration of ISMN, gender differences in aggregability remained, with blood samples from females being significantly more aggregable than those from male subjects. There were no significant differences between the treatment groups ("no nitrate"/chronic ISMN) and cross the 8-hr time course. There were also no significant interactions between any of the determinants. The levels of significance and degree of interaction between these variables are displayed within Table 4.5. ADP-responses were similar in magnitude to those during the corresponding acute phase (Figure 4.8).

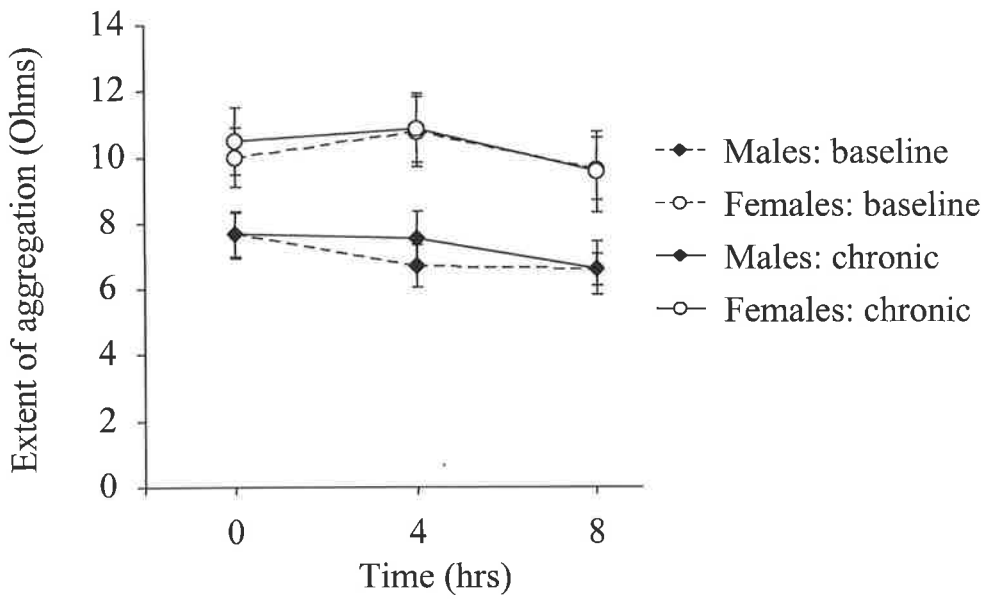


Figure 4.12 Chronic effects of ISMN pharmacotherapy on ADP-induced aggregation

The anti-aggregatory effects of chronic ISMN pharmacotherapy were examined across the period (8 hrs) and gender. Platelets from female subjects were considerably more aggregable than male subjects, with chronic ISMN pharmacotherapy having no significant anti-aggregatory effects (Table 4.5).

TD-NTG

Similar to the results for ISMN, the data populations regarding platelet response to ADP during the chronic phase of the study also conformed to a Gaussian distribution and were effectively matched (Appendix Table 22). By 3-way repeated measures ANOVA and as illustrated in Figure 4.13, chronic administration of TD-NTG had no significant effect on ADP responses (Bartlett's statistic = 8.41, p = 0.67). Mirroring the results observed for the chronic administration of ISMN, platelets from female subjects were significantly more aggregable than platelets from male subjects. (See Table 4.5 for the levels of significance and the interactions between each variable).

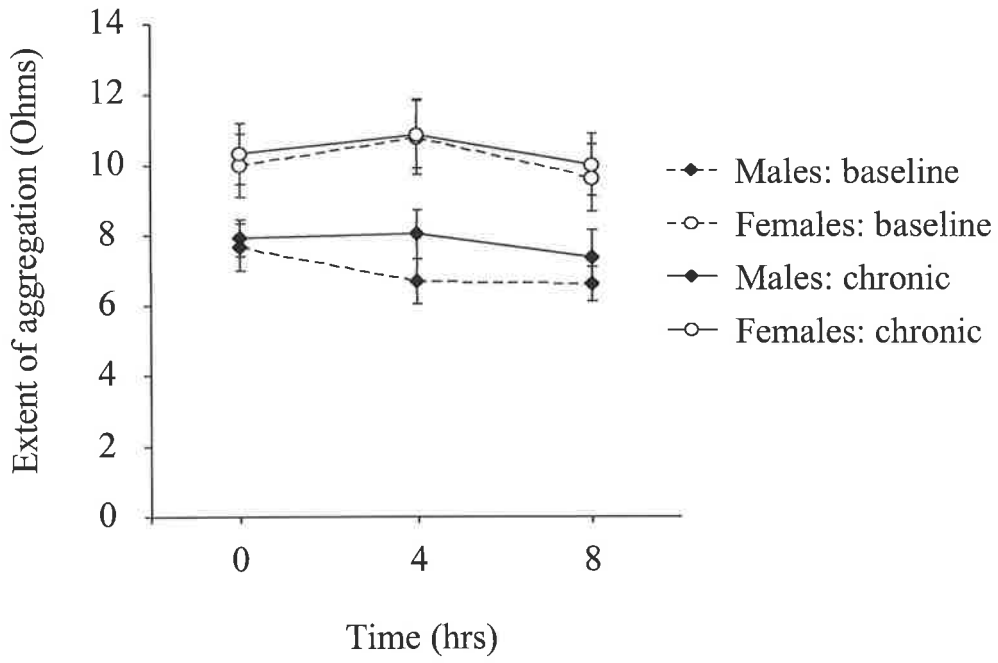


Figure 4.13 Chronic effects of TD-NTG pharmacotherapy on ADP-induced aggregation

The anti-aggregatory effects of chronic TD-NTG pharmacotherapy were examined across the time period (8-hrs) and gender. Platelets from female patients were significantly more aggregable than males with chronic TD-NTG pharmacotherapy having no significant anti-aggregatory effects (Table 4.5).

Table 4.5 Effect of chronic nitrate pharmacotherapy on ADP responses:- three-way ANOVA contingency table

	ISMN		TD-NTG	
Determinants	***F***	***p***	***F***	***p***
Time	1.74	0.18	1.05	0.35
Treatment	0.28	0.59	1.58	0.21
Gender	48.1	< 0.01	57.2	< 0.01
Interactions				
Time x Treatment	0.10	0.90	0.08	0.92
Time x Gender	0.59	0.57	0.70	0.49
Treatment x Gender	0.01	0.89	0.47	0.49
Time x Treatment x Gender	0.16	0.84	0.26	0.77

3-way repeated measures ANOVA examining the significance and interactions of time, treatment groups ("no nitrate"/chronic nitrate pharmacotherapy) and gender for each nitrate preparation regarding platelet response to ADP during chronic nitrate treatment. See also Figure 4.12 and Figure 4.13.

Differences between treatment phases and nitrate preparations

Aggregability

As shown in section 4.5.3.2, platelet response to ADP for either gender remained unchanged across all periods of the investigation. For the purpose of establishing if there was any significant difference across the treatment phases and between the nitrate preparations regarding their effects on platelet aggregability, the extent of platelet aggregation across the respective time periods were pooled for each subject. The pooled data population representing the extent of aggregability was demonstrated to conform to a Gaussian distribution and to contain homogeneous variances (Appendix Table 23). By 3-way ANOVA, no significant differences were observed for platelet aggregability across the treatment phases and between the nitrate preparations, further confirming the absence of any significant anti-aggregatory effect of these nitrate preparations (Bartlett's statistic = 7.83, p = 0.35). However, gender-related differences in platelet aggregability observed throughout each stage of the trial remained. For a further summary see Table 4.6.

Table 4.6 Differences across phases and between nitrate treatments (platelet response to ADP):- three-way ANOVA contingency table

Determinant	*F*	*p*
Therapy	0.05	0.81
Acute/Chronic	0.003	0.95
Gender	37.5	< 0.01
Interactions		
Therapy x Acute/Chronic	0.10	0.75
Therapy x Gender	0.05	0.83
Acute/chronic x Gender	0.58	0.44
Therapy x Acute/Chronic x Gender	0.03	0.86

Differences across the nitrate treatment phases and between the nitrate preparation of ISMN or TD-NTG were analyzed by 3-way ANOVA.

Changes in platelet responsiveness to nitric oxide donors during chronic nitrate pharmacotherapy:-

Nitroglycerine

The influence of chronic administration of either nitrate preparation on platelet responsiveness to NTG (100μM) was also examined. Data populations for platelet responsiveness to NTG during the chronic phase of the trial, conformed to Gaussian distribution and were effectively matched (ISMN; 0-hrs KS = 0.09, p = ns; 4-hrs KS = 0.12, p = ns; 8-hrs KS = 0.05, p = ns: Levene's F-test; F = 4.9, p < 0.01). (TD-NTG; 0-hrs KS = 0.15, p = ns; 4-hrs KS = 0.07, p = ns; 8-hrs KS = 0.12, p = ns: Levene's F-test; F = 5.6, p < 0.01). As displayed in Figure 4.14, no significant difference in platelet responsiveness to NTG (100μM) was observed between the treatment groups or across the time period (2-way repeated measures ANOVA: Treatment group F = 0.66, p = 0.51; Time F = 0.82, p = 0.47; Time x Treatment group F = 0.94, p = 0.46; Bartlett's statistic = 0.9, p = 0.94).

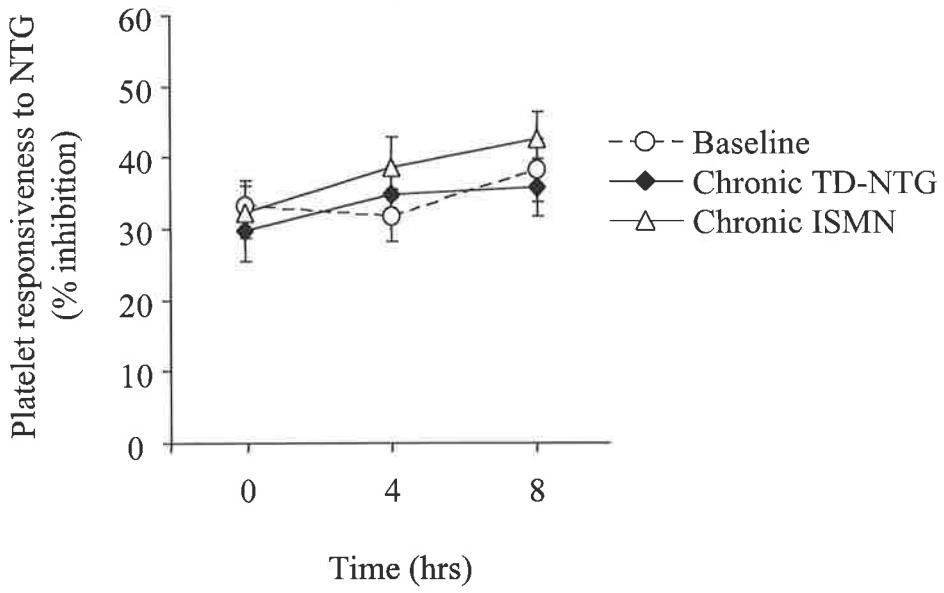


Figure 4.14 Effects of chronic nitrate pharmacotherapy on platelet responsiveness to NTG

Chronic administration of either 120mg ISMN or 15mg TD-NTG for 2 weeks had no significant effect on platelet responsiveness to NTG (100μM). Each nitrate preparation was administered immediately post zero hour blood sampling (2-way repeated measures ANOVA; Treatment group F = 0.66, p = 0.51; Time F = 0.82, p =0.47; Time x Treatment group F = 0.94, p = 0.46).

Differences between treatment phases and nitrate preparations

Platelet responsiveness to NTG

Differences in platelet responsiveness to *in vitro* NTG (100μM) across all phases of the trial and between each nitrate regime were also examined. Given no significant differences in platelet responsiveness to NTG across time was observed throughout the trial periods, NTG responses for each subject at all time points were pooled and analyzed according to treatment phase (acute/chronic) and therapy. The pooled data population representing the extent of platelet responsiveness to NTG was demonstrated to conform to a Gaussian distribution and to contain homogeneous variances (Appendix Table 24). By 2-way ANOVA, there was no detectable difference in platelet responsiveness to NTG across the treatment phases and between the nitrate preparations (2-way ANOVA; Treatment phase F = 0.94, p = 0.33; Therapy F = 0.03, p = 0.85; Treatment phase x Therapy F = 0.12, p = 0.73; Bartlett's statistic = 0.95, p = 0.81).

Changes in platelet responsiveness to nitric oxide donors during chronic nitrate pharmacotherapy:-

Sodium nitroprusside

Changes in platelet responsiveness to SNP (10μM) following chronic administration of either ISMN or TD-NTG was also examined. Platelet responsiveness to SNP for each nitrate treatment regimen was demonstrated to be normally distributed and to be effectively matched between the time points examined (ISMN: 0-hrs KS = 0.18, p = ns; 4-hrs KS = 0.1, p = ns; 8-hrs KS = 0.1, p = ns: Levene's F-test; F = 10.3, p < 0.01) (TD-NTG; 0-hrs KS = 0.12, p = ns; 4-hrs KS = 0.1, p = ns; 8-hrs KS = 0.07, p = ns: Levene's F-test; F = 4.3, p < 0.01). In much the same way as demonstrated for NTG responsiveness and as illustrated in Figure 4.15, no significant effect on platelet responsiveness to SNP (10μM) was observed during chronic nitrate pharmacotherapy (2-way repeated measures ANOVA; Treatment group F = 1.6, p = 0.17; Time F = 0.32, p = 0.73; Time x Treatment group F = 1.6, p = 0.16; Bartlett's statistic = 0.62, p = 0.9).

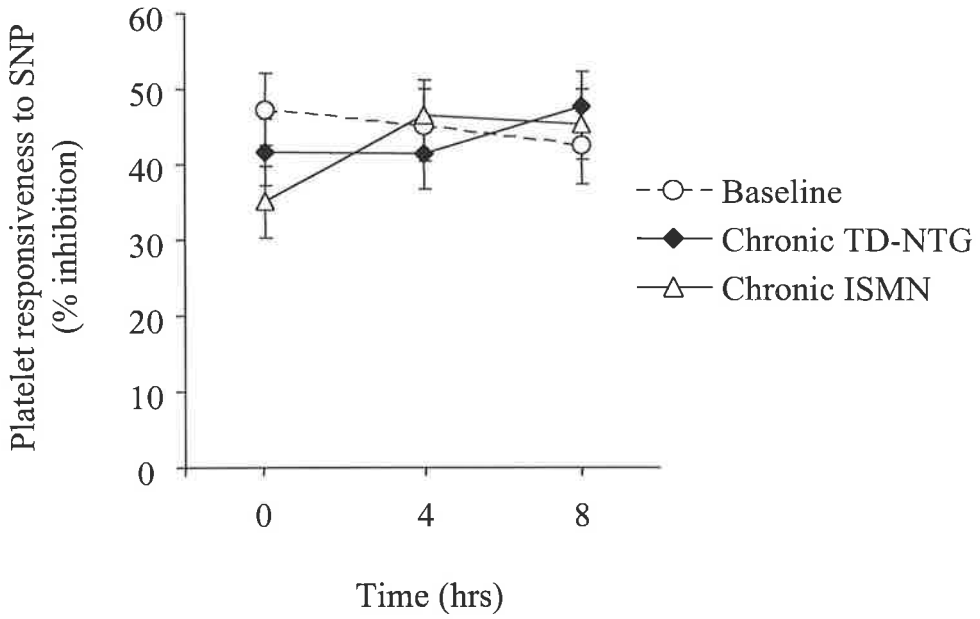


Figure 4.15 Chronic nitrate pharmacotherapy: effects on platelet responsiveness to SNP

Chronic administration of either 120mg ISMN or 15mg TD-NTG for 2 weeks had no significant effect on platelet responsiveness to SNP (10μM). Each nitrate preparation was administered immediately post zero hour blood sampling (2-way repeated measures ANOVA; Treatment group F = 1.6, p = 0.17; Time F = 0.32, p =0.73; Time x Treatment group F = 1.6, p = 0.16).

Differences between treatment phases and nitrate preparations

Platelet responsiveness to SNP

Platelet responsiveness to SNP (10μM) was examined across the treatment phases and between the nitrate preparations used throughout the trial. Results for patient platelet responsiveness to SNP across all time periods was pooled, given no overall significant deviations in platelet responsiveness to SNP were noted. Platelet responsiveness to SNP was then analyzed according to treatment phase (acute/chronic) and nitrate preparation only. The pooled data population representing the extent of platelet responsiveness to SNP conformed to a Gaussian distribution and contained homogeneous variances (Appendix Table 25). By 2-way ANOVA, no significant difference was observed in platelet responsiveness to SNP across the acute and chronic phases of the trial or between the two nitrate preparations (2-way ANOVA; Treatment phase $F = 0.08$, $p = 0.77$; Therapy $F = 0.79$, $p = 0.38$; Treatment phase x therapy $F = 0.002$, $p = 0.96$; Bartlett's statistic = 1.4, $p = 0.7$).

Chronic effects of nitrate pharmacotherapy on superoxide production:-

The effects of chronic nitrate pharmacotherapy with ISMN (120mg) and TD-TN (15mg/12-hr application) on whole blood superoxide were also examined.

Pre-aggregation LDCL

The effects of chronic nitrate pharmacotherapy on whole blood superoxide were examined utilizing lucigenin (12.5μM). Data at each sampling time point for either nitrate preparation conformed to a Gaussian distribution and contained homogeneous variances (ISMN: 0-hrs KS = 0.23, p = ns; 4-hrs KS = 0.15, p = ns; 8-hrs KS = 0.17, p = ns; Bartlett's statistic = 0.59, $p = 0.74$) (TD-NTG: 0-hrs KS = 0.21, p = ns; 4-hrs KS = 0.19, p = ns; 8-hrs KS = 0.17, p = ns; Bartlett's statistic = 4.1, $p = 0.12$). By 2-way ANOVA ("no nitrate" data previously shown to be normally distributed and to contain non-significantly different standard deviations across the time period) no significant difference between the treatment groups or across the 8-hr time period was observed for pre-aggregation LDCL. There was no significant interaction between the two determinants (2-way ANOVA; Treatment group $F = 0.21$, $p = 0.81$; Time $F = 0.6$, $p = 0.54$; Treatment group x Time $F = 1.4$, $p = 0.23$; Bartlett's statistic = 5.3, $p = 0.72$). For a further summary of the effects of chronic nitrate pharmacotherapy on pre-aggregation LDCL see Figure 4.16 left panel.

Aggregation-associated LDCL

Like that of the pre-aggregation LDCL, aggregation-associated LDCL (12.5μM lucigenin) was normally distributed and to have non-significantly different standard deviations across the chronic phase of the trial point (ISMN: 0-hrs KS = 0.15, p = ns; 4-hrs KS = 0.18, p = ns; 8-hrs KS = 0.23, p = ns: Bartlett's statistic = 2.35, p = 0.3) (TD-NTG: 0-hrs KS = 0.19, p = ns; 4-hrs KS = 0.16, p = ns; 8-hrs KS = 0.19, p = ns: Bartlett's statistic = 2.6, p = 0.28). By 2-way ANOVA ("no nitrate" data previously shown to be normally distributed and to contain homogeneous variances across the time period) and as illustrated in Figure 4.16 lower right panel, there was no significant difference between the treatment groups or across the eight-hr time course. No significant interaction was also observed between the two determinants (2-way ANOVA; Treatment group F = 1.3, p = 0.27; Time F = 1.9, p = 0.15; Treatment group x Time F = 0.38, p = 0.81; Bartlett's statistic = 11.8, p = 0.16).

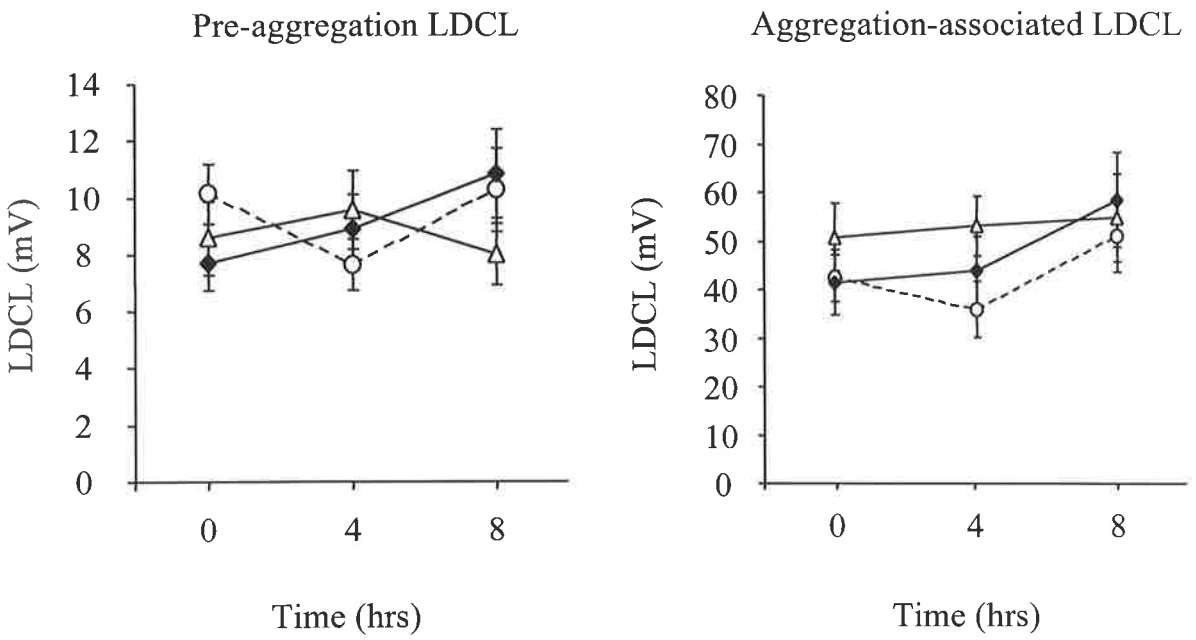


Figure 4.16 Chronic nitrate administration:- effects on whole blood superoxide

Open circle dotted line = LDCL during the "no nitrate" phase of the study. Closed diamond solid line = chronic TD-NTG treatment phase. Open triangle solid line = chronic ISMN phase. Left panel pre-aggregation LDCL 2-way ANOVA; Treatment group F = 0.21, p = 0.81, Time F = 0.6, p = 0.54, Treatment group x time F = 1.4, p = 0.23; right panel aggregation-associated LDCL; Treatment group F = 1.3, p = 0.27; Time F = 1.9, p = 0.15; Treatment group x Time F = 0.38, p = 0.81.

Summary: Luminescence variables

As illustrated within Figure 4.11 and Figure 4.16 the effects of acute and chronic nitrate pharmacotherapy on both the pre-aggregation and aggregation-associated LDCL were essentially similar, with only a trend towards a lower LDCL during acute TD-NTG treatment.

Differences between treatment phases and nitrate preparations regarding luminescence parameters

Pre-aggregation LDCL

Differences between the treatment phases and nitrate regimens across the 8-hr time period were also examined for the pre-aggregation LDCL, utilizing (12.5μM) lucigenin. All data conformed to a Gaussian distribution (Appendix Table 26) and contained homogeneous variances (Bartlett's statistic = 6.2, p = 0.86). By 3-way ANOVA, no significant difference in pre-aggregation LDCL was observed (Appendix Table 27).

Aggregation-associated LDCL

The effect of the treatment phases and nitrate preparations over the 8-hr time period was also examined for the aggregation-associated LDCL (12.5μM lucigenin). All subset data populations conformed to a Gaussian distribution (Appendix Table 28) and contained homogeneous variances (Bartlett's statistic = 13.3, p = 0.27). As described within Appendix Table 29, there was no significant difference in the extent of aggregation-associated LDCL across the treatment phases, between the two regimens (non-significant trend towards a difference) for the 8-hr time period. There were also no significant interactions between any determinants.

LDCL data: lucigenin (125μM)

At the start of the current investigation, superoxide in whole blood was monitored using lucigenin (125μM) in 10 patients. As a number of emerging concerns in the literature stated that "high" concentrations of lucigenin might compound the problem of *in vitro* generation of superoxide by "redox cycling" (Vasquez-Vivar *et al.*, 1997; Liochev *et al.*, 1998; Skatchkov *et al.*, 1999), we opted to change to methodology with lucigenin at 12.5μM.

Results for the 10 patients in whom both the pre-aggregation and aggregation-associated LDCL was determined with lucigenin (125μM) were generally similar to those obtained

utilizing 12.5μM. However, LDCL values were considerably greater. A comparison of zero-hr data in the absence of nitrate therapy is given in Table 4.7.

Table 4.7 Differences in LDCL at the zero hr time point for two concentrations of lucigenin

	Pre-aggregation LDCL	*Post-aggregation LDCL*
125μM lucigenin (n = 10)	75 ± 17	277 ± 49
12.5μM lucigenin (n = 16)	10 ± 1.0	42 ± 5

Mean ± S.E.M LDCL data determined for pre-aggregation and aggregation-associated LDCL at the zero hour time point for two concentrations of lucigenin. Proportional increments in LDCL were similar with both lucigenin concentrations when comparing the pre-aggregation and aggregation-associated LDCL data.

These data therefore suggest that the incremental concentration of lucigenin was associated with about a 7-fold increase in LDCL, but no artifactual exaggeration of the 4-fold increase in superoxide generation seen following aggregation.

[4.5.3.4] Potential "rebound" in platelet aggregation

The "zero hour" phenomenon, representing a reduced exercise capacity prior to nitrate administration during chronic intermittent pharmacotherapy (DeMots and Glasser, 1989), or a "rebound" in anginal symptoms following the withdrawal of nitrates (Ferratini *et al.*, 1989; Figueras *et al.*, 1991), has only been documented previously at a presumptive vascular level. However, the data of Figueras *et al* (1991), for patients with UAP might imply "rebound" at the level of platelet aggregability. A 24-hr nitrate-free period prior to commencement of the chronic phase of the investigation allowed us to examine the hypothesis that a nitrate-free interval during acute pharmacotherapy is associated with platelet hyper-aggregability. Accordingly, for each nitrate preparation platelet aggregability at the zero hour during the acute phase (24-hr nitrate free period prior to sampling) of the investigation, was compared with the extent of aggregability at the zero hour of the chronic phase of the trial.

Firstly and as illustrated in Figure 4.17 (upper left panel), platelet response to ADP during the TD-NTG treatment phase was analyzed according to the treatment phase and gender. No

"rebound" in platelet aggregability during the TD-NTG treatment phase of the study was observed despite a continuation of the gender differences in platelet aggregability (TD-NTG; 2-way repeated measures ANOVA, Treatment phase $F = 0.014$, $p = 0.9$; Gender $F = 19.3$, $p < 0.01$; Treatment phase x Gender $F = 0.47$, $p = 0.49$; Bartlett's statistic = 1.8, $p = 0.6$).

A number of investigators have failed to demonstrate any significant "rebound" in myocardial ischaemia or a "zero hour" effect with the use of ISMN (Chrysant *et al.*, 1993; Parker, 1993). However, there is tentative evidence for the existence of this phenomenon in some patients treated with ISMN (Rehnqvist *et al.*, 1988). Therefore platelet responsiveness during the ISMN treatment phase of the study was also examined for the presence of the "zero hour" effect. As illustrated in Figure 4.17 (upper right panel), no significant "rebound" in platelet aggregability was observed following a nitrate free period for each gender (ISMN; 2-way repeated measures ANOVA; Treatment phase $F = 0.004$, $p = 0.94$; Gender $F = 21.2$, $p < 0.01$; Treatment phase x Gender $F = 0.68$, $p = 0.41$; Bartlett's statistic = 1.65 $p = 0.65$). Gender differences remained with no significant interactions between the two determinants.

Differences between the two nitrate regimens regarding their tendency to induce a "rebound" in platelet aggregability were examined by assessing the change in aggregability for each gender and nitrate used. For each nitrate used, delta aggregability was assessed using a 1-sample *t*-test. No significant change from zero was observed with either nitrate preparation (TD-NTG KS = 0.17, p = ns; ISMN KS = 0.078, p = ns) (1-sample *t*-test:- TD-NTG $t = 0.29$, $p = 0.76$; ISMN $t = 0.059$, $p = 0.95$). For a further summary of the differences between both nitrate regimens and genders see the lower panel of Figure 4.17.

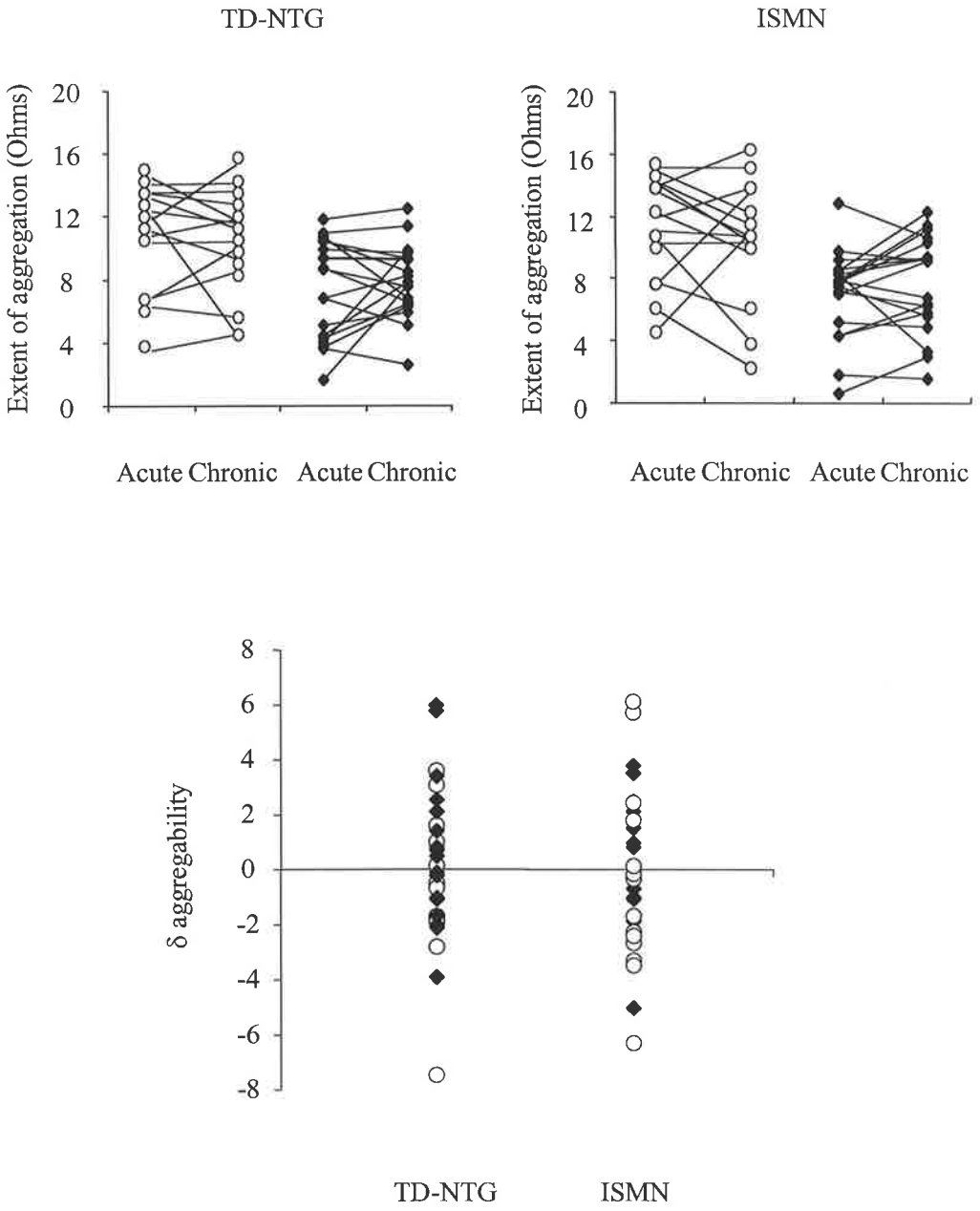


Figure 4.17 "Rebound" in platelet aggregability

A "rebound" in platelet response to ADP was examined during both nitrate treatment phases with no significant "rebound" being observed for either preparation. Open circles = females; closed diamonds = males. ***Upper panels****: Left; TD-NTG; 2-way ANOVA, Treatment phase F = 0.17, p = 0.68; Gender F = 4.3, p = 0.04; Treatment phase x Gender F = 0.04, p = 0.83. Right; ISMN; 2-way ANOVA, Treatment phase F = 0.62, p = 0.43; Gender F = 7.13, p = 0.01; Treatment phase x Gender F = 0.18, p = 0.67.* ***Lower panel****; No significant change from zero was observed for either nitrate preparation (1-sample t-test TD-NTG t = 0.29, p = 0.76; ISMN t = 0.059, p = 0.95).*

[4.5.4] Vasomotor investigations

In a sub-group of trial patients (n = 12; 8 males and 4 females) and in addition to the blood samples taken for the platelet parameters, the acute and chronic effects of ISMN and TD-NTG on selected haemodynamic variables were also examined.

[4.5.4.1] Systolic blood pressure

Diurnal variability in systolic blood pressure

Systolic blood pressure was monitored throughout the "no nitrate" phase of the study (0,4,8 and 24-hrs) in order to determine if there was diurnal variability in systolic blood pressure in the absence of prophylactic nitrate use. Systolic blood pressure readings from these subjects conformed to a Gaussian distribution and contained homogeneous variances (0-hrs KS = 0.14, p = ns; 4-hrs KS = 0.14, p = ns; 8-hrs KS = 0.27, p = ns; 24-hrs = 0.19, p = ns; Bartlett's statistic = 3.4, p = 0.34). A repeated measures ANOVA was not performed as the 24-hr reading from one of the subjects was not performed. By 1-way ANOVA, no significant diurnal variation was observed in the systolic blood pressure of the 10 subjects with SAP (1-way ANOVA F = 1.65, p = 0.19).

Acute nitrate pharmacotherapy

The acute effects of both nitrate preparations on systolic blood pressure were examined. Systolic blood pressures during the acute phase of either nitrate treatment conformed to Gaussian distribution and contained homogeneous variances (ISMN: 0-hrs KS = 0.12, p = ns; 4-hrs KS = 0.19, p = ns; 8-hrs KS = 0.1, p = ns; 24-hrs KS = 0.2, p = ns; Bartlett's statistic = 0.9, p = 0.82) (TD-NTG: 0-hrs KS = 0.19, p = ns; 4-hrs KS = 0.25, p = ns; 8-hrs KS = 0.14, p = ns; 24-hrs KS = 0.21, p = ns; Bartlett's statistic = 3.4, p = 0.33). By 2-way ANOVA, there were no significant differences between the treatment groups but there was a significant decrease in the systolic blood pressure across the 24-hrs time period of the acute phase of the trial (2-way ANOVA; Treatment group F = 0.95, p = 0.38; Time F = 3.78, p = 0.012, Treatment group x Time F = 1.8, p = 0.81; Bartlett's statistic = 8.17, p = 0.69). However, there was a trend for systolic blood pressure at the 4 and 8-hr time points to be lower following nitrate administration than in its absence. For a further summary of the effects of acute nitrate pharmacotherapy on systolic blood pressure see Figure 4.18.

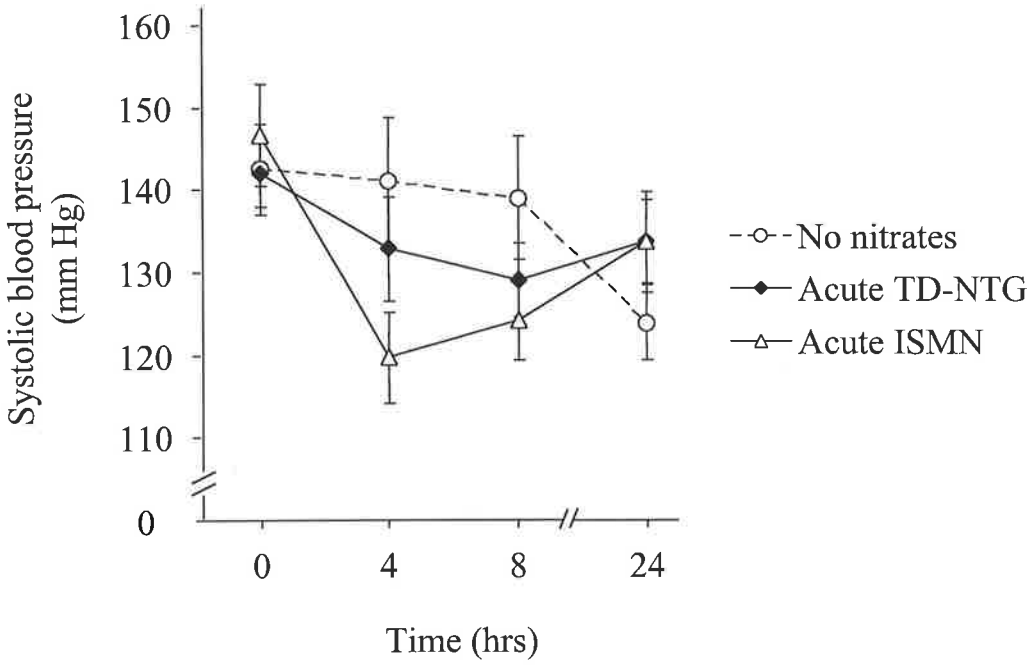


Figure 4.18 Acute effects of nitrate pharmacotherapy on systolic blood pressure

Acute administration of either 120mg ISMN or 15mg TD-NTG had no significant effect on systolic blood pressure. For each subject examined, nitrates were administrated immediately post the zero hour blood collection time points. No significant difference between the treatment groups but rather a significant reduction in systolic blood pressure over time, was observed. (By 2-way ANOVA:- Treatment group F = 0.95, p = 0.38; Time F = 3.78 p = 0.012; Treatment group x Time F = 1.8, p = 0.1).

Chronic nitrate pharmacotherapy

Following 2 weeks of either 120mg ISMN or 15mg/24-hrs (12-hr application) TD-NTG, the chronic effects of nitrate pharmacotherapy on systolic blood pressure was also examined. Systolic blood pressure as demonstrated for the acute phase of the trial were also shown to conform to a Gaussian distribution and to contain homogeneous variances during the chronic phase of the trial (ISMN: 0-hrs KS = 0.1, p = ns; 4-hrs KS = 0.16, p = ns; 8-hrs KS = 0.19, p = ns; Bartlett's statistic = 0.15, p = 0.93) (TD-NTG: 0-hrs KS = 0.09, p = ns; 4-hrs KS = 0.15, p = ns; 8-hrs KS = 0.11, p = ns; Bartlett's statistic = 1.9, p = 0.37). By 2-way ANOVA, chronic pharmacotherapy with either nitrate preparation had no significant effect on systolic blood pressure (2-way ANOVA; Treatment group F = 1.85, p = 0.16; Time F = 1.77, p = 0.17; Treatment group x Time F = 0.38, p = 0.82; Bartlett's statistic = 5.86, p = 0.67). There was no significant decrease in blood pressure over the 8-hr time course directly contrasting to the results observed during acute administration of the nitrate preparations. For a further

summary of the effects of chronic nitrate pharmacotherapy on systolic blood pressure, see Figure 4.19.

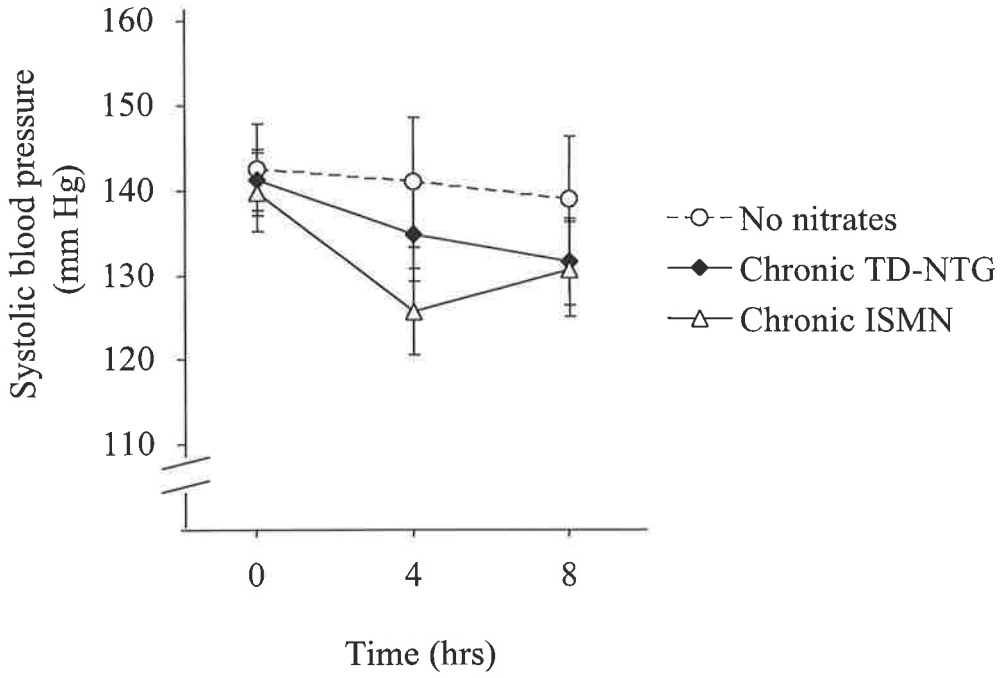


Figure 4.19 Chronic effects of nitrate pharmacotherapy on systolic blood pressure

Chronic administration of either 120mg ISMN or 15mg/24-hrs (12-hr application) for a period of 2 weeks had no significant effect on systolic blood pressure. For each subject examined, nitrates were administered immediately post the zero hour blood collection point (By 2-way ANOVA, Treatment group F = 1.8, p = 0.16, Time F = 1.78, p = 0.17, Treatment group x Time F = 0.38, p = 0.82).

Differences between treatment phases and nitrate preparations: systolic blood pressure

Having established that there were no statistically significant acute or chronic effects of either ISMN/TD-NTG on systolic blood pressure over the respective time periods, potential differences between the trial treatment phases (acute/chronic) and nitrate preparations (ISMN/TD-NTG) on systolic blood pressure for the 8-hr time period were then examined. All data populations were demonstrated to conform to a Gaussian distribution (Appendix Table 30) and to contain homogeneous variances (Bartlett's statistic = 6.3, p = 0.85). As indicated in Table 4.8 there was no significant difference in systolic blood pressure between the acute/chronic phases or between the two nitrate preparations. However, there were significant differences in systolic blood pressure over time. Utilizing Bonferroni's post hoc multiple comparison test the mean systolic blood pressure at the zero hour of the acute ISMN phase of the trial was significantly greater than that of the 4-hr blood pressure reading.

Table 4.8 Differences across phases and between nitrate treatments (systolic blood pressure) three-way ANOVA contingency table

Determinants	F	p
Phase	0.29	0.58
Time	9.70	< 0.01
Treatment	1.90	0.16
Interactions		
Phase x Time	0.79	0.45
Phase x Treatment	0.01	0.91
Time x Treatment	1.56	0.21
Phase x Time x Treatment	0.33	0.72

The differences in systolic blood pressure between treatment phases (acute/chronic) for both nitrate preparations (ISMN/TD-NTG) were examined by 3-way ANOVA (Bartlett's statistic = 6.3, p = 0.85).

[4.5.4.2] Heart rate

Diurnal variability

Along with changes in systolic blood pressure, changes in heart rate post nitrate administration were examined with each nitrate preparation. Firstly, diurnal variability in heart rate was examined. Data representing heart rate was shown to conform to a Gaussian distribution and to contain homogeneous variances (0-hrs KS = 0.25, p = ns; 4-hrs KS = 0.17, p = ns; 8-hrs KS = 0.22, p = ns; 24-hrs = 0.14, p = ns; Bartlett's statistic = 0.96, p = 0.81). Utilizing a 1- way ANOVA, no significant diurnal variability in heart rate was observed (1-way ANOVA:- F = 0.14, p = 0.94).

Acute nitrate pharmacotherapy

Acute administration of either nitrate preparation was examined for the effects on heart rate. Data representing heart rate conformed to a Gaussian distribution and contained homogeneous variances during the acute phase of the investigation (ISMN: 0-hrs KS = 0.19, p = ns; 4-hrs KS = 0.12, p = ns; 8-hrs KS = 0.14, p = ns; 24-hrs KS = 0.17, p = ns; Bartlett's statistic = 1.25, p = 0.74) (TD-NTG: 0-hrs KS = 0.17, p = ns; 4-hrs KS = 0.11, p = ns; 8-hrs KS = 0.15, p = ns; 24-hrs KS = 0.11, p = ns; Bartlett's statistic = 0.74, p = 0.85).

As illustrated in Figure 4.20, the acute administration of either nitrate preparation had no significant effect on heart rate (2-way ANOVA:- Treatment group F = 0.17, p = 0.84; Time F = 0.34, p = 0.78; Treatment group x time F = 0.1, p = 0.99; Bartlett's statistic = 6.1, p = 0.86).

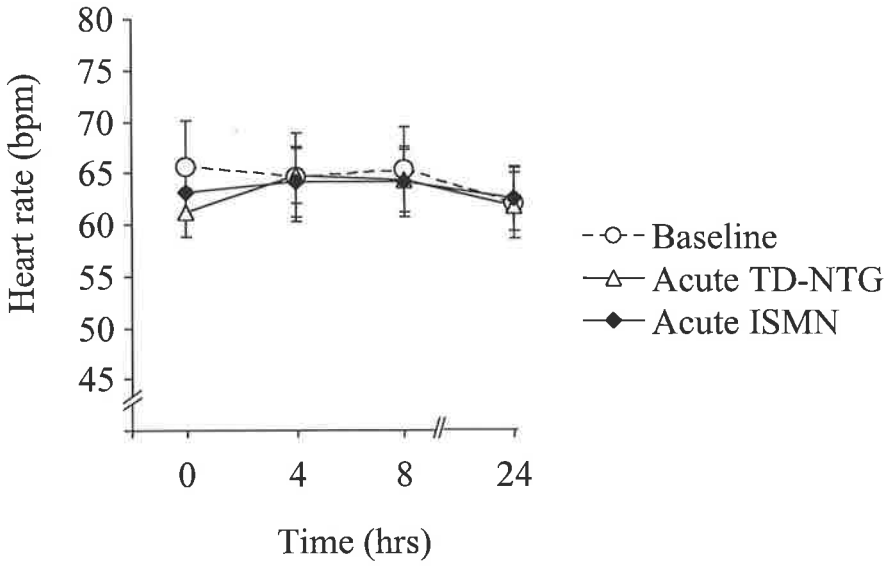


Figure 4.20 Acute effects of nitrate pharmacotherapy on heart rate

Acute administration of either 120mg ISMN or 15mg TD-NTG had no significant effect on heart rate. For each subject examined, nitrates were administrated immediately post the zero hour blood collection time points. (2-way ANOVA:- Treatment group F = 0.17, p = 0.84; Time F = 0.34, p = 0.78; Treatment group x Time F = 0.1, p = 0.99).

Chronic nitrate pharmacotherapy

Chronic administration of either nitrate preparation was also examined for the effects on heart rate. Data representing heart rate conformed to a Gaussian distribution and contained homogeneous variances during the acute phase of the investigation (ISMN: 0-hrs KS = 0.11, p = ns; 4-hrs KS = 0.13, p = ns; 8-hrs KS = 0.16, p = ns; Bartlett's statistic = 0.152, p = 0.96) (TD-NTG: 0-hrs KS = 0.098, p = ns; 4-hrs KS = 0.11, p = ns; 8-hrs KS = 0.19, p = ns; Bartlett's statistic = 0.18, p = 0.91).

As illustrated in Figure 4.21, chronic treatment with either nitrate preparation had no significant effect on heart rate (2-way ANOVA:- Treatment group F = 0.36, p = 0.69; Time F = 0.29, p = 0.75; Treatment group x time F = 0.12, p = 0.97; Bartlett's statistic = 5.7, p = 0.91).

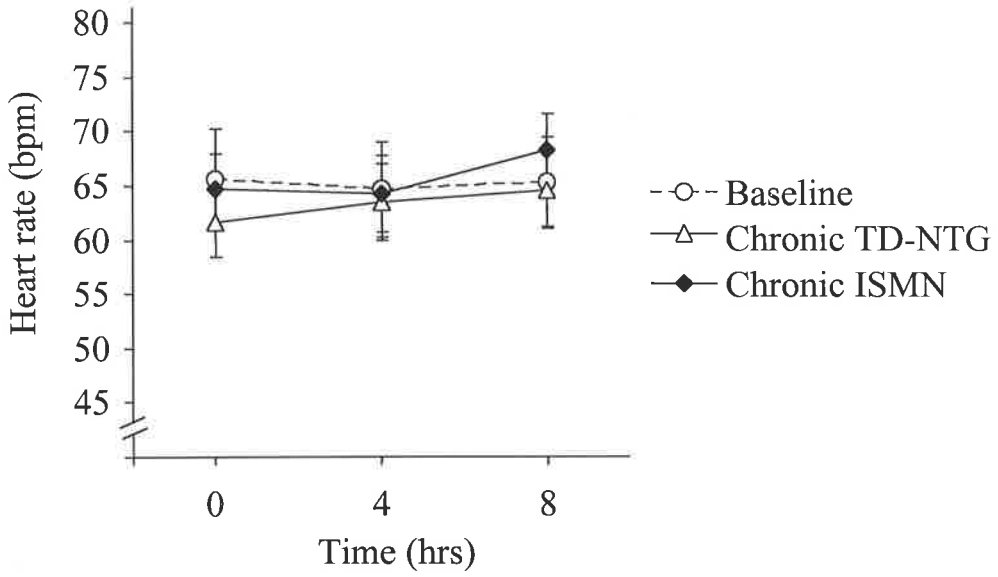


Figure 4.21 Chronic effects of nitrate pharmacotherapy on heart rate

Chronic administration of either 120mg ISMN or 15mg/24-hrs (12-hr application) for a period of 2 weeks had no significant effect on heart rate. For each subject examined, nitrates were administered immediately post the zero hour blood collection point (2-way ANOVA, Treatment group F = 0.37, p = 0.69, Time F = 0.29, p = 0.75, Treatment group x Time F = 0.12, p = 0.97).

[4.5.4.3] Effects on Augmentation index

Diurnal variability

Of the 34 subjects enrolled within the trial, diurnal variability in the AI(x) was examined in 10 subjects. Immediately following blood sampling at zero hour (approximately 8am), 4, 8 and 24-hrs, the AI(x) of 10 subjects was examined. AI(x) at each sampling time point during the "no nitrate" period of the trial conformed to a Gaussian distribution and contained homogeneous variances (0-hrs KS = 0.11, p = ns; 4-hrs KS = 0.16, p = ns; 8-hrs KS = 0.18, p = ns; 24-hrs KS = 0.12, p = ns. Bartlett's statistic = 2.4, p = 0.5). As illustrated in Figure 4.22 and by 1-way ANOVA, no significant diurnal variability in AI(x) was observed (1-way ANOVA; F = 0.88, p = 0.46). A repeated measures ANOVA was not performed as the 24-hr sample was not obtained in one subject.

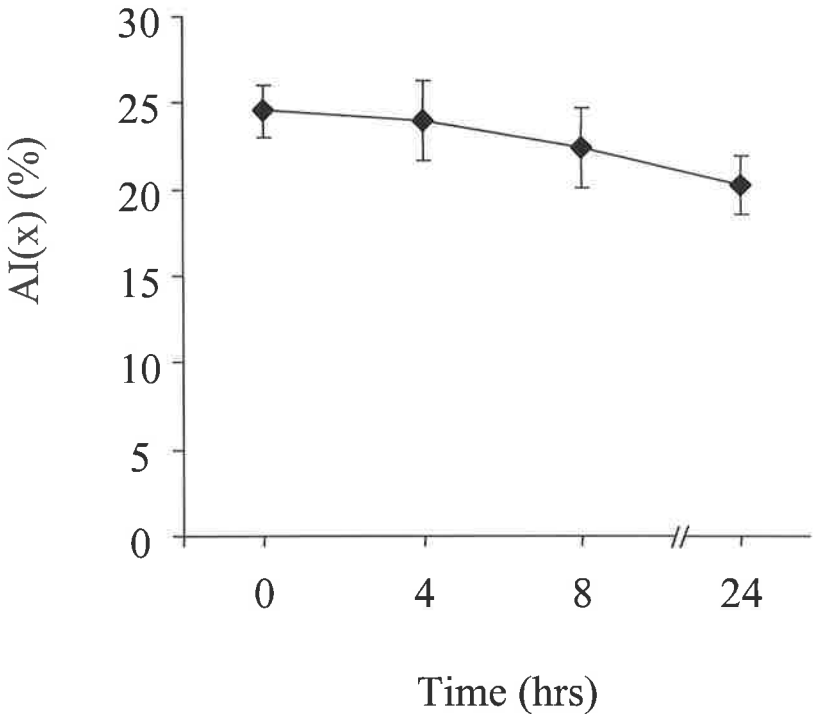


Figure 4.22 Diurnal variability in AI(x)

Variability in AI(x) in 10 subjects during the "no nitrate" collection phase. No significant diurnal variability in AI(x) was observed (1-way ANOVA; F = 0.88, p = 0.46).

Acute nitrate pharmacotherapy

The acute administration of either 120mg ISMN or 15mg/24-hrs TD-NTG following a 24 -hr nitrate free period and 7 days of either 60mg ISMN or 7.5mg/24-hrs TD-NTG was examined for effect on the AI(x) in 12 of the 34 enrolled subjects. AI(x) was shown to conform to a Gaussian distribution and to contain homogeneous variances during the acute phase of the investigation (ISMN 0-hrs KS = 0.13, p = ns; 4-hrs KS = 0.25, p = ns; 8-hrs KS = 0.23, p = ns; 24-hrs KS = 0.24, p = ns. Bartlett's statistic = 0.85, p = 0.84). (TD-NTG 0-hrs KS = 0.16, p = ns; 4-hrs KS = 0.21, p = ns; 8-hrs KS = 0.13, p = ns; 24-hrs KS = 0.17, p = ns. Bartlett's statistic = 1.9, p = 0.58).

As illustrated in Figure 4.23 addition of either nitrate preparation caused a significant reduction in the AI(x) relative to "no nitrates" (2-way ANOVA; Treatment group F = 9.1, p < 0.01; Time F = 29.2, p < 0.01; Treatment group x Time F = 8.4, p < 0.01; Bartlett's statistic = 7.86, p = 0.72). Utilizing Bonferroni's post hoc multiple comparison test a number of significant differences were observed, with the most notable being the AI(x) for 4 and 8-hrs "no nitrates" being significantly higher than that recorded for both nitrate preparations (p <

0.01). There was no significant difference in AI(x) at the zero and 24-hr time points between all treatment groups. There was also no significant difference between the AI(x) for TD-NTG and ISMN treatment groups at 4-hrs post nitrate administration.

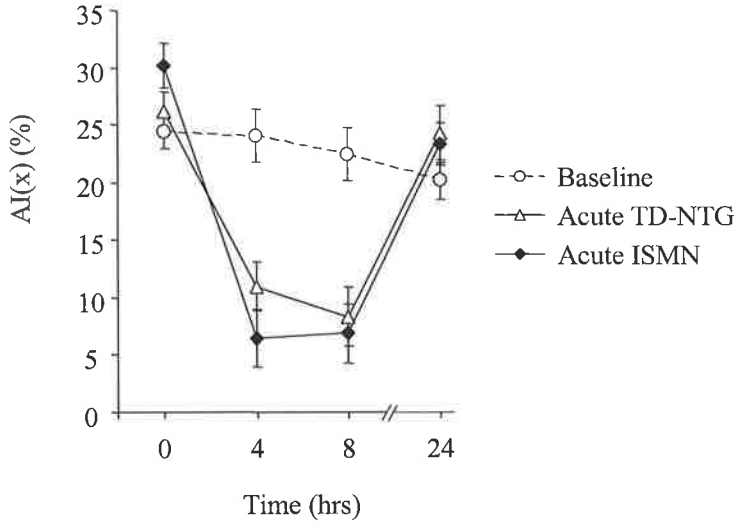


Figure 4.23 Acute nitrate effects on AI(x)

Acute administration of either ISMN (120mg) or TD-NTG 15mg (12-hr application) immediately post the zero hour blood collection time point caused a significant reduction in the AI(x) that remains throughout 4 and 8-hrs (2-way ANOVA; Treatment group $F = 9.2$, $p < 0.01$; Time $F = 29.2$, $p < 0.01$; Treatment group x Time $F = 8.4$, $p < 0.01$).

Chronic nitrate pharmacotherapy

Administration of either ISMN or TD-NTG immediately post the zero hour collection to subjects that had received pharmacotherapy with either nitrate preparation for a period of 2 weeks still demonstrated a pronounced and significant reduction in the AI(x). AI(x)'s were demonstrated to conform to a Gaussian distribution and to contain homogeneous variances (ISMN 0-hrs KS = 0.27, p = ns; 4-hrs KS = 0.17, p = ns; 8-hrs KS = 0.13, p = ns; Bartlett's statistic = 1.7, p = 0.42). (TD-NTG 0-hrs KS = 0.22, p = ns; 4-hrs KS = 0.23, p = ns; 8-hrs KS = 0.14, p = ns. Bartlett's statistic = 2.2, p = 0.34).

Administration of either nitrate preparation immediately post the zero hour blood collection time point caused a significant reduction in the AI(x) relative to "no nitrate" values (Figure 4.24: 2-way ANOVA: Treatment group F = 20.0, p < 0.01, Time F = 5.9, p < 0.01, Treatment group x Time F = 4.78, p < 0.01; Bartlett's statistic = 9.14, p = 0.33). Utilizing Bonferroni's post hoc multiple comparison test, no significant differences in AI(x) were observed at the zero hour time point. Interestingly, at the 4-hr time point, AI(x) for the TD-NTG treatment

phase was not significantly different from that of "no nitrates", contrasting to that of ISMN that was significantly different ($p < 0.05$). At the 8-hr time point, only during the TD-NTG treatment phase was the AI(x) significantly lower than that of "no nitrates" ($p < 0.05$), contrasting this time to the ISMN treatment phase that was not significantly different from "no nitrates".

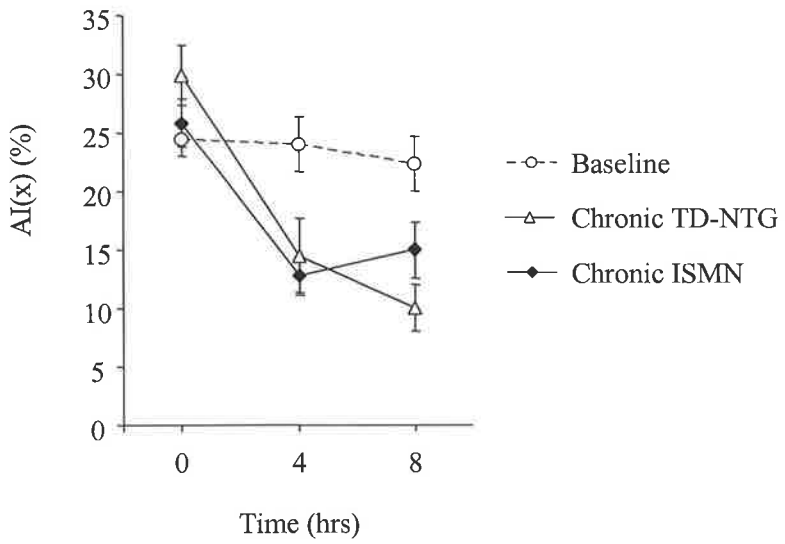


Figure 4.24 Chronic nitrate pharmacotherapy:- effects on AI(x)

Chronic treatment with either ISMN (120mg) or TD-NTG 15mg (12-hr application) immediately post the zero hour blood collection time point caused a significant reduction in the AI(x) that remains throughout 4 and 8hrs. By 2 way ANOVA; Treatment group $F = 20.0$, $p < 0.01$, Time $F = 5.9$, $p < 0.01$, Treatment group x Time $F = 4.8$, $p < 0.01$.

"Rebound" in AI(x)

A 24-hr nitrate-free period (prior to the commencement of the chronic phase of the trial) allowed us to examine whether a nitrate-free period during chronic nitrate pharmacotherapy was associated with a "rebound" in the AI(x). Therefore for each nitrate preparation, the AI(x) at the zero hour during the acute phase (24-hr nitrate free period prior to sampling) of the trial were compared with those at the zero hour of the chronic phase of the investigation.

As illustrated in Figure 4.25 left panel, there was no significant change in AI(x) during the TD-NTG treatment phase of the trial (paired *t*-test t = 1.4, p = 0.18). Analogous results were observed during the ISMN treatment phase, with no significant change in AI(x) being evident (right panel of Figure 4.25, paired *t*-test t = 1.5, p = 0.16).

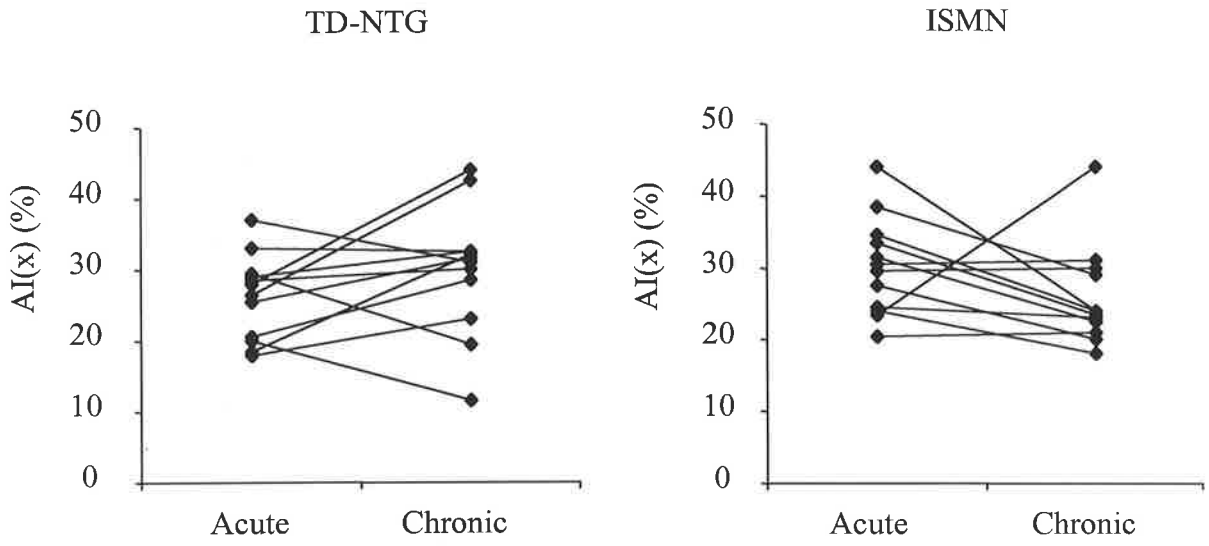


Figure 4.25 "Rebound" in AI(x)

Potential "rebound" in the AI(x) was examined during both nitrate treatment phases with no significant "rebound" being observed for either nitrate preparation. Right panel TD-NTG paired t-test t = 1.4, p = 0.18; left panel ISMN paired t-test t = 1.5, p = 0.16.

Differences between the nitrate therapies/treatment phases regarding AI(x)

For the purposes of establishing if there was any significant difference across the treatment phases and between each nitrate preparation regarding AI(x), a 3-way ANOVA was performed using the 0, 4 and 8-hr time points (Bartlett's statistic = 8.6, p = 0.65). As illustrated in Table 4.9 and Figure 4.26, nitrate administration caused a significant reduction in the AI(x) over time. There was also a significant difference between the acute and chronic treatment phases for both nitrate preparations, indicating tolerance induction. However, there were no significant differences between the nitrate preparations in reducing the AI(x) post administration, both acutely and chronically. Moreover, the triple interaction determinant (p = 0.07) raised the possibility that the effects of ISMN were associated with slightly more marked tolerance induction. Utilizing Bonferroni's post hoc multiple comparison test, there were no significant differences between the AI(x) values for each nitrate preparation and phase, at all the examined time points.

Table 4.9 Three-way ANOVA contingency table

Determinant	*F*	*p*
Time	75.8	< 0.01
Therapy	0.12	0.72
Treatment phase	5.60	0.018
Interactions		
Time x therapy	1.15	0.31
Time x treatment phase	1.70	0.18
Therapy x treatment phase	0.02	0.88
Time x therapy x phase	2.64	0.07

Differences across the nitrate treatment phases and between each nitrate preparation were analyzed by 3-way ANOVA using the time points 0, 4 and 8 hrs only.

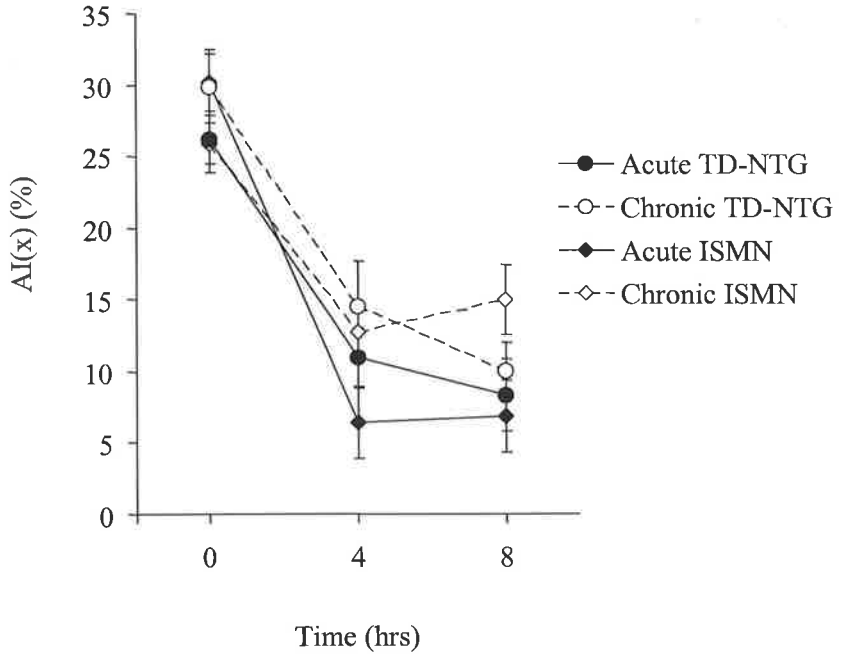


Figure 4.26 Differences between therapies and treatment phases

By 3-way ANOVA (Table 4.6), administration of either nitrate preparation (ISMN or TD-NTG) showed a significant reduction in the AI(x). The effectiveness of reducing the AI(x) was no different between each nitrate preparation but was different between phases of administration, indicating tolerance development (3-way ANOVA; Time $F = 75.8$, $p < 0.01$; Therapy $F = 0.12$, $p = 0.72$; Treatment phase $F = 5.6$, $p = 0.018$: interactions are listed in Table 4.6).

[4.5.5] Platelet and luminescence variables in those subjects who had vascular parameters examined

A discussion and an analysis of the aggregation/LDCL results obtained with subjects that had vascular parameters assessed (n = 12), can be found within the appendix chapter of this thesis. Briefly, the platelet aggregation and luminescence results within this cohort of subjects were similar to those of the total patient cohort. No significant anti-aggregatory effect was observed during both the acute and chronic phases of the trial for either nitrate preparation. Furthermore, the extent of platelet responsiveness to both NTG (100μM) and SNP (10μM) remained unchanged during the different trial phases, a result that parallels the luminescence data. For further commentary and analysis see the Appendix chapter of this thesis.

[4.5.6] AI(x) vs SNP responsiveness

In order to determine if there was any significant correlation between the vascular responsiveness to nitric oxide and platelet responsiveness to nitric oxide, a potential relationship between the AI(x) and platelet responsiveness to SNP at the zero hour of the "no nitrate" collection phase of the trial, was examined. In 10 subjects and as illustrated in Figure 4.27, no significant inter-relationship between AI(x) and platelet SNP (10μM) responsiveness was observed (regression analysis r = -0.13, p = 0.72; run test p = 0.17).

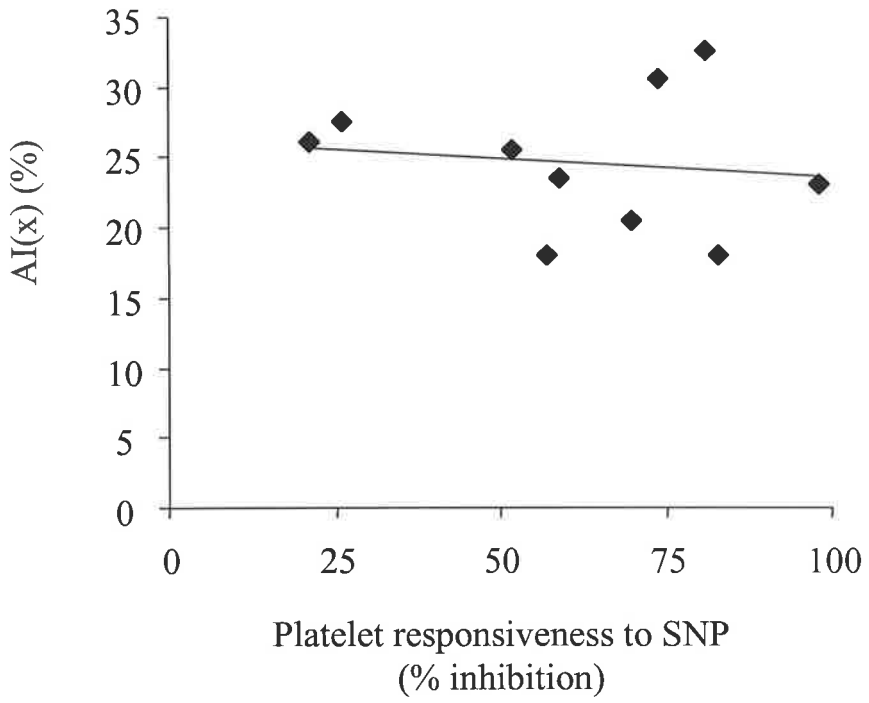


Figure 4.27 AI(x) versus platelet responsiveness to SNP

The AI(x) was not correlated with platelet responsiveness to SNP (10μM). Regression analysis r = -0.13, p = 0.72; run test p = 0.17.

[4.5.7] Platelet and vascular responsiveness to nitroglycerine

Platelet responsiveness to NTG was compared to the 4-hourly AI(x) values during the acute phase of the trial for the TD-NTG preparation in-order to examine the relationship between platelet responsiveness and vascular responsiveness to NTG.

As illustrated in Figure 4.28, there was a trend towards a significant relationship between platelet responsiveness to NTG (100μM) and the % change in AI(x) at 4-hrs post acute TD-NTG administration (Regression analysis, platelet NTG responsiveness vs TD-NTG AI(x) r = 0.54, p = 0.086, run test p = 0.91).

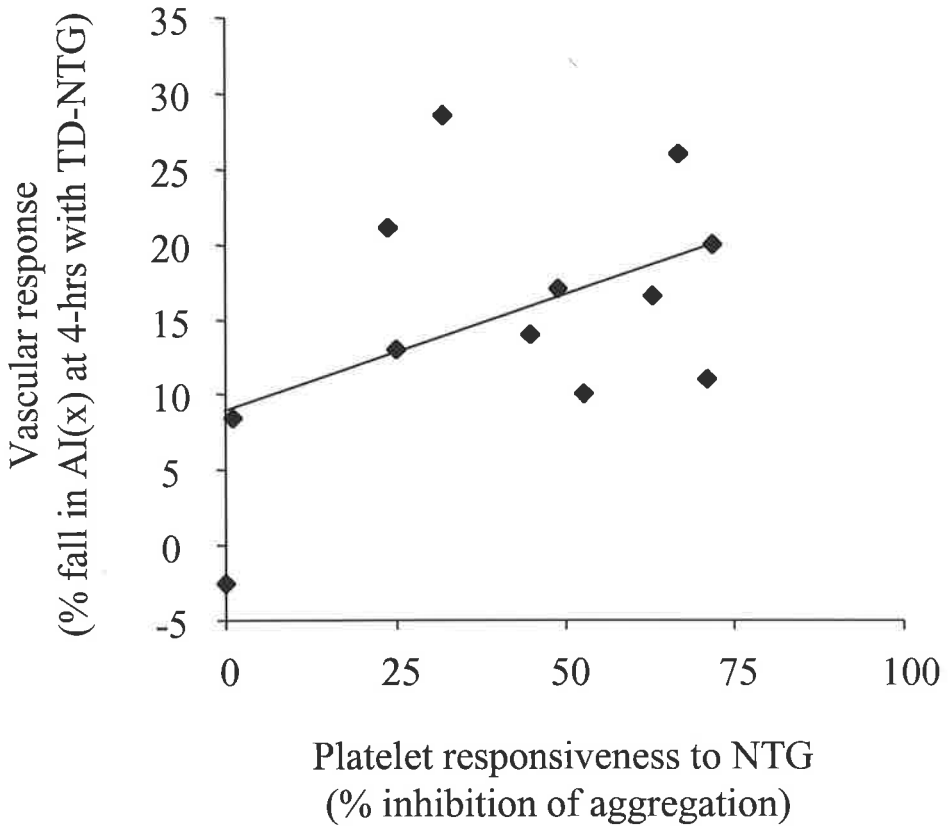


Figure 4.28 Acute platelet responsiveness to NTG (100μM) versus AI(x)

A trend towards a significant relationship existed between platelet responsiveness to NTG (100μM) and the % change in AI(x) at 4-hrs post acute TD-NTG administration (regression analysis, r = 0.54, p = 0.086, run test p = 0.91).

[4.5.8] Relationship between the percentage fall in AI(x) and platelet responsiveness to NTG

Having examined the relationship between platelet responsiveness to NTG and the change in AI(x) at 4-hrs post acute administration of TD-NTG we then wanted to establish if there was a relationship between the fall in AI(x) post nitrate administration and platelet responsiveness to NTG (100μM) at the 0-hr time point. As illustrated in Figure 4.29, there was no significant relationship between the absolute fall in AI(x) post TD-NTG administration and the extent of platelet responsiveness to NTG (100μM) at the 0-hr time point (regression analysis, $r = 0.16$, $p = 0.61$, run test $p = 0.18$).

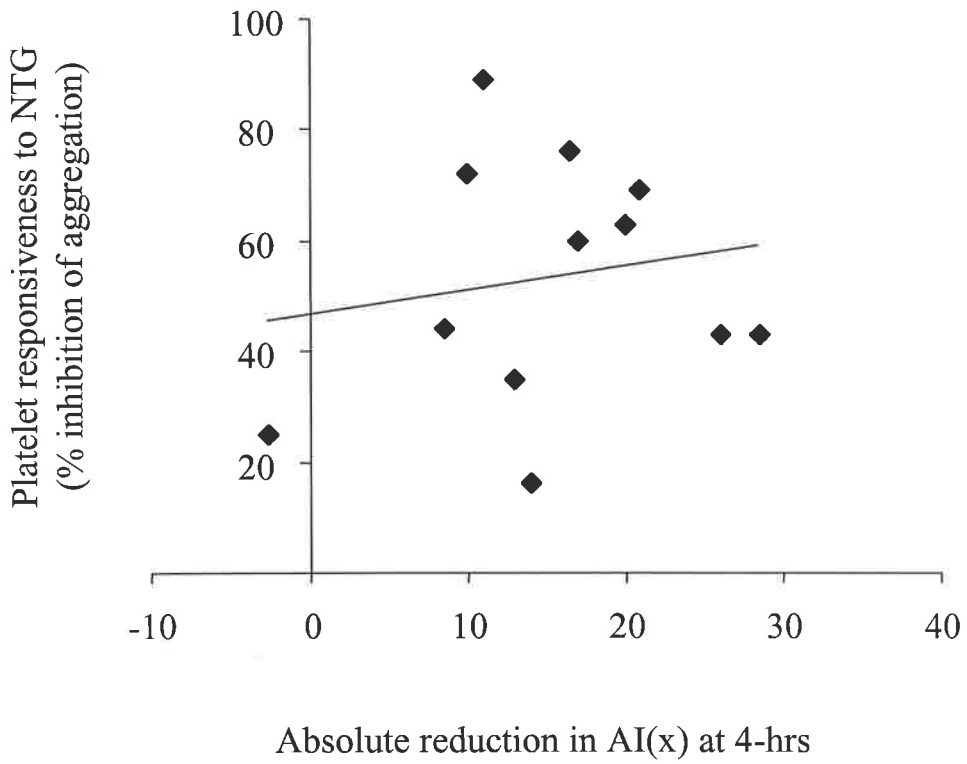


Figure 4.29 Relationship between absolute reduction in AI(x) at 4-hrs and the degree of platelet responsiveness to NTG

No significant relationship was observed between the absolute fall in AI(x) post TD-NTG administration and the extent of platelet responsiveness to NTG (100μM) at the zero-hr time point (regression analysis r = 0.16, p = 0.61, run test = 0.18).

[4.5.9] Acute platelet NTG responsiveness vs acute platelet superoxide

At 4-hrs post administration of either ISMN or TD-NTG a relationship between the degree of platelet responsiveness to NTG (100μM) and whole blood superoxide, was examined.

Platelet responsiveness to NTG vs pre-aggregation LDCL

Utilizing samples from a sub-population of the study cohort, the relationship between the extent of platelet responsiveness to NTG (100μM) at the 4-hr time point post administration of either nitrate preparation, and the degree of pre-aggregation LDCL as detected by lucigenin (12.5μM) was examined. By regression analysis, no significant correlation was evident (regression analysis ISMN $r = -0.31$, $p = 0.24$, run test $p = 0.96$; TD-NTG $r = -0.07$, $p = 0.79$, run test $p = 0.2$). Utilizing ANCOVA both populations of data from each nitrate treatment regimen were found to be significantly different from each other (ANCOVA $F = 4.9$, $p = 0.035$). For a summary of the relationship between platelet responsiveness to NTG and the pre-aggregation LDCL see the lower left panel of Figure 4.30.

Platelet responsiveness to NTG vs aggregation-associated LDCL

As was demonstrated above for the pre-aggregation LDCL data, no significant correlation between the degree of platelet responsiveness to NTG and aggregation-associated LDCL was found for either nitrate preparation (regression analysis ISMN $r = 0.1$, $p = 0.7$, run test $p = 0.23$; TD-NTG $r = 0.02$, $p = 0.93$, run test $p = 0.94$). By ANCOVA both data populations for each nitrate preparation were found to be significantly different from each other (ANCOVA $F = 4.3$, $p = 0.047$). For a further summary see the lower right panel of Figure 4.30.

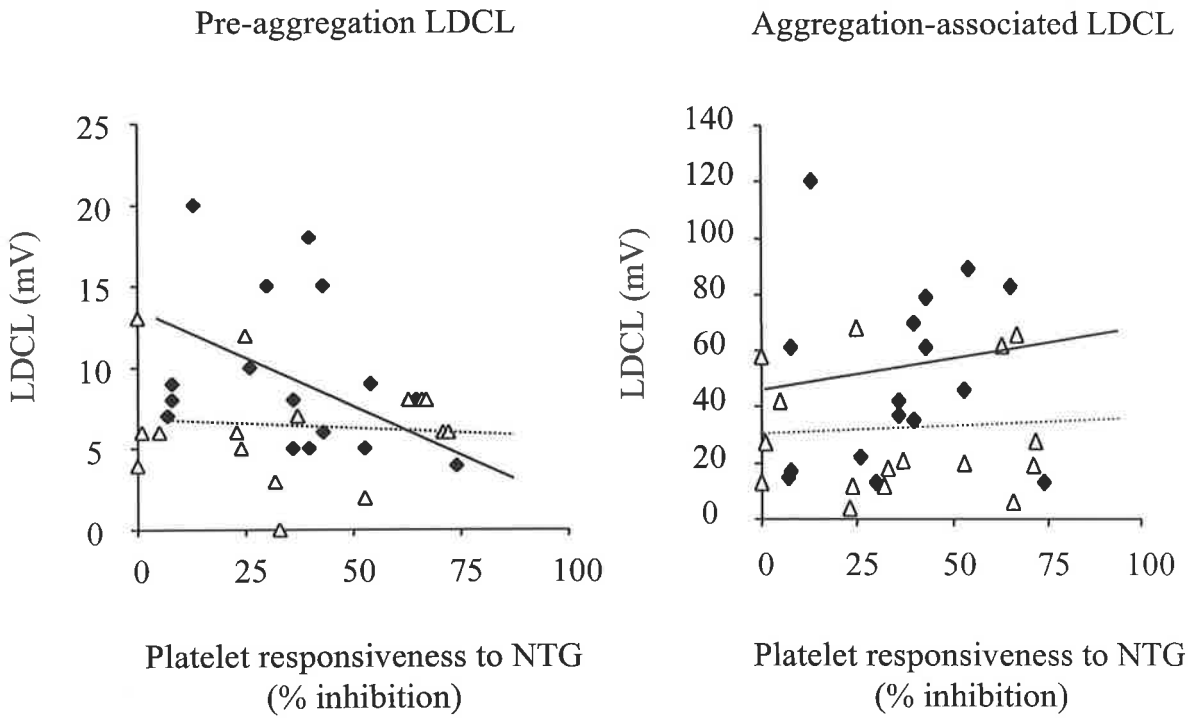


Figure 4.30: Relationship between acute platelet responsiveness to NTG and whole blood superoxide levels

Solid diamonds = ISMN; Open triangles = TD-NTG. ***Left****; platelet NTG responsiveness and pre-aggregation LDCL (regression analysis ISMN r = -0.31, p = 0.24, run test p = 0.96; TD-NTG r = -0.07, p = 0.79, run test p = 0.2).* ***Right****; platelet NTG responsiveness and aggregation-associated LDCL (regression analysis ISMN r = 0.1, p = 0.7, run test p = 0.23; TD-NTG r = 0.02, p = 0.93, run test p = 0.94).*

[4.5.10] Relationship between nitrate effects on AI(x) and whole blood superoxide during the acute phase administration of ISMN and TD-NTG

The degree of change in AI(x) 4-hrs post administration of either nitrate preparation was examined to see if it correlated with the degree of whole blood superoxide.

Pre-aggregation LDCL

As shown in the upper panel of Figure 4.31, no significant relationship between the degree of change in AI(x) and the pre-aggregation LDCL was observed for either nitrate preparation (regression analysis ISMN: $r = 0.007$, $p = 0.98$, run test = 0.93; TD-NTG $r = 0.07$, $p = 0.84$, run test $p = 0.88$). By ANCOVA both data populations were not significantly different from each other (ANCOVA $F = 0.024$, $p = 0.88$).

Aggregation-associated LDCL

Delta AI(x) at four hours post administration of both nitrate preparations were also correlated with the degree of aggregation-associated LDCL (lower panel of Figure 4.31). No significant correlation was found between delta AI(x) and the aggregation-associated LDCL for either nitrate preparation (regression analysis ISMN: $r = 0.2$, $p = 0.6$, run test $p = 0.5$; TD-NTG $r = 0.015$, $p = 0.96$, run test $p = 0.64$). By ANCOVA both data populations were not significantly different from each other (ANCOVA $F = 0.14$, $p = 0.9$).

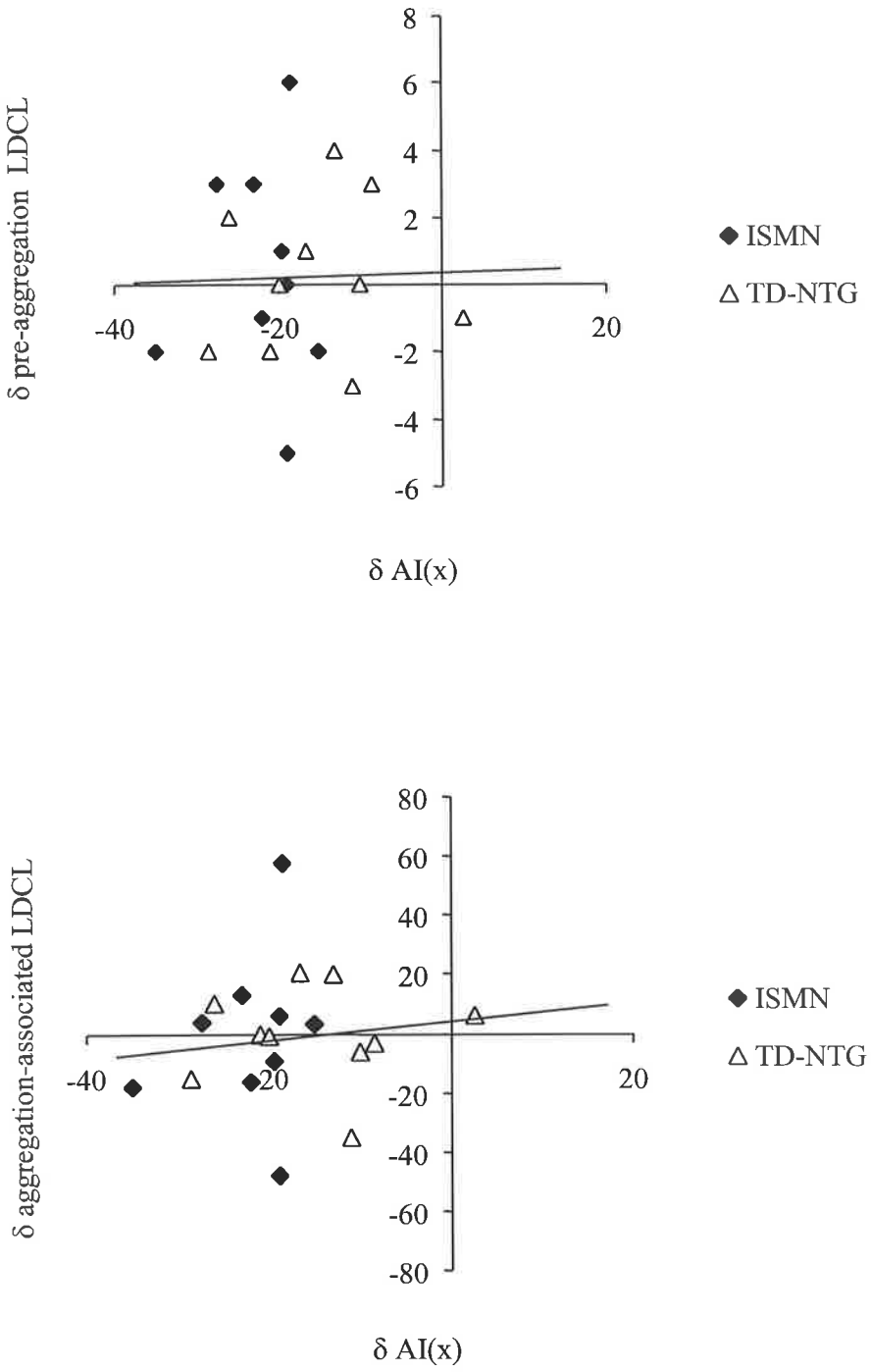


Figure 4.31 Relationship between delta AI(x) and whole blood superoxide

Upper panel: *No significant correlation between delta AI(x) and pre-aggregation LDCL (regression analysis ISMN: r = 0.007, p = 0.98, run test = 0.93; TD-NTG: r = 0.07, p = 0.84, run test p = 0.88).* ***Lower panel:*** *No significant correlation between delta AI(x) and aggregation-associated LDCL (regression analysis ISMN: r = 0.2, p = 0.6, run test = 0.5; TD-NTG: r = 0.015, p = 0.96, run test p = 0.64).*

[4.6] Discussion

Organic nitrates are in widespread use for the treatment/management of patients with SAP, ACSs and heart failure (Darius, 1999; Horowitz, 2000). In addition to their vasodilator properties the therapeutic effects of organic nitrates are related, at least in patients with ACS, to their potent anti-aggregatory effects (Stamler and Loscalzo, 1991).

[4.6.1] Anti-platelet effects of organic nitrates ex vivo

In the present study, we investigated the putative anti-aggregatory effects of both acute and chronic administration of either ISMN (120mg) and TD-NTG (15mg/24hrs-12hr application) in patients with mild to moderate SAP utilizing *ex vivo* methodology. Acute (Figure 4.7) or chronic (Figure 4.11/4.12) administration of either nitrate preparation showed no significant anti-aggregatory effect. Unlike a number of previous studies examining the anti-aggregatory effects of NTG (as outlined in section D.1.3.2 of chapter 1), this is the first study examining both the acute and chronic anti-aggregatory effects ISMN and TD-NTG.

TD-NTG

It is now widely accepted that NTG along with other organic nitrate preparations possess potent anti-platelet properties, initially demonstrated *in vitro* with high concentrations (Mehta and Mehta, 1979; Rolland *et al.*, 1984; Saxon and Kattlove, 1976) but now shown at an *ex vivo* level with therapeutically achievable transdermal dosing regimens (De Caterina *et al.*, 1989; Diodati *et al.*, 1990; Diodati *et al.*, 1995; Karlberg *et al.*, 1992; Wolfram *et al.*, 1996).

Studies demonstrating significant anti-aggregatory effects

Andrews *et al* (1994), investigating the anti-aggregatory effects of TD-NTG were the first to demonstrate a significant anti-aggregatory effect from TD-NTG in healthy volunteers, one and two hours post a single application (0.6mg/hr). The anti-platelet properties of TD-NTG were demonstrated both at a platelet-rich plasma and whole blood level, with the most potent of effects being observed within the former platelet preparation. In contrast to the methods that were used within the current study, Andrews *et al* (1994) added a cGMP phosphodiesterase inhibitor (MB 22948, 100μM) to each blood/platelet sample in order to amplify the anti-aggregatory properties of TD-NTG. The addition of a cGMP phosphodiesterase inhibitor to a blood sample was not performed within the current study and

therefore serves as a possible reason as to why no significant anti-aggregatory effect was observed post TD-NTG administration at either an acute or chronic level.

Lacoste *et al* (1994), examining the anti-aggregatory effects of TD-NTG (0.6mg/hr) in a cohort of SAP patients, also demonstrated a significant anti-aggregatory effect of TD-NTG 3-hrs post administration, a result that was demonstrated without the addition of a cGMP phosphodiesterase inhibitor. Furthermore, TD-NTG was demonstrated to decrease thrombus deposition on pig aorta, both at high and low shear rates, further indicating a significant anti-aggregatory effect. However, unlike the current study in which all subjects were required to continue with their current anti-anginal medications, the study by Lacoste *et al* (1994), required all participating subjects to discontinue their anti-anginal and anti-platelet drugs >2 weeks prior to study commencement (exception, β-adrenoceptor antagonists).

Studies demonstrating no significant anti-aggregatory effect

Apart from the aforementioned studies, a number of previous investigators have demonstrated no significant *ex vivo* anti-aggregatory effect of TD-NTG (Giugliano *et al.*, 1995; Hogan *et al.*, 1989). More recently Bath *et al* (2001), examining the ability of TD-NTG to lower blood pressure and its anti-platelet properties in subjects with acute stroke, demonstrated a significant reduction in systolic blood pressure post nitrate administration but no significant change in heart rate, platelet aggregation or expression of glycoproteins Ia, Ib, IIIa and P-selectin.

ISMN

To date there is limited data examining the *ex vivo* anti-platelet properties of ISMN alone or compared to those of NTG or ISDN, a fact that partly provided the impetus for the current investigation. Wolfram *et al* (1996), examining the *ex vivo* anti-aggregatory effects of TD-NTG (64mg), ISDN (120mg) and ISMN (50mg) in a cohort of healthy subjects demonstrated that all these nitrate preparations were equipotent haemodynamically 90 and 150 minutes post administration. Interestingly, significant *ex vivo* anti-aggregatory effects were observed for the TD-NTG and ISDN, but not for the ISMN preparation, an observation that concurs with the results of Drummer *et al* (1991).

Current study results: lack of significant anti-aggregatory effect

In summary, the balance of evidence in the literature supports the view that both TD-NTG and ISMN demonstrably inhibit platelet aggregation at an *ex vivo* level. A number of possible reasons exist why we did not observe any significant anti-aggregatory effect with either nitrate preparation throughout all phases of the current study. These can be categorized as either clinical or experimental.

Clinical

Most, but not all studies examining the anti-aggregatory properties of TD-NTG/ISMN have been performed on healthy subjects or SAP patients who have had all other anti-anginal/platelet medications withdrawn prior to study commencement. However, the current study was designed to investigate both the acute and chronic incremental effects of TD-NTG/ISMN in subjects with mild to moderate SAP on a background of anti-anginal/platelet medications, a study design that reflects clinical reality. Therefore, it is possible that the absence of any significant anti-aggregatory effect following TD-NTG/ISMN administration may have been due to the lack of significant incremental effects over those of the concomitant medications each subject was receiving.

Throughout the literature a number of hypotheses have been generated addressing the discrepancy between the concentrations of organic nitrates required to cause an anti-aggregatory effect *in vitro* compared with *ex vivo/in vivo*. One such hypothesis includes the synergistic action of NTG and prostacyclin at inhibiting platelet aggregation (Stamler and Loscalzo, 1991). De Caterina *et al* (1998), observed that the IC_{50} for prostacyclin-induced inhibition of platelet aggregation was significantly reduced following ISDN administration, a result that concurs with the observations of others (Anfossi *et al.*, 1993; Klockenbusch and Schror, 1993; Schror *et al.*, 1988; Willis *et al.*, 1989). Moreover, Salvemini *et al* (1996), examining the role of prostacyclin in nitric oxide-dependent inhibition of platelet aggregation, demonstrated *in vitro* that nitric oxide released from NTG activates cyclo-oxygenase (COX) in endothelial cells leading to release of prostacyclin. The addition of endothelial cells (previously shown to release prostacyclin in response to administration of therapeutically relevant concentrations of nitric oxide donors), caused a ten-fold shift to the left of the concentration-response curve for NTG-induced inhibition of thrombin induced platelet aggregation. Furthermore, the addition of indomethacin was demonstrated to inhibit

the prostacyclin-mediated component of the inhibition of platelet aggregation, to a level that was achieved with the nitric oxide donor alone, implying an important role for COX/prostacyclin/cAMP in the anti-aggregatory effects of nitric oxide donors (Salvemini *et al.*, 1996).

In the current study, 88% of subjects were receiving aspirin throughout the trial. Reductions in prostacyclin release resulting from the use of aspirin and hence a reduction in the effectiveness of TD-NTG/ISMN may serve as a reason for the absence of any significant anti-aggregatory effect. However, it seems unlikely that this hypothesis would function as the sole reason why no significant anti-aggregatory effect was observed as nitrate administration caused profound reductions in the AI(x) (Figure 4.23/4.24):- prostacyclin would presumably potentate the vasodilator and anti-aggregatory effects of nitrate to similar extents. Examination of the hypothesis that aspirin reduces the *ex vivo* anti-aggregatory effects of organic nitrates was beyond the scope of the current investigation. However, this hypothesis does warrant further examination given some of the aforementioned evidence and the observation that indomethacin pretreatment of human umbilical arteries significantly attenuates NTG induced vascular relaxation *in vitro* (Klockenbusch and Schror, 1993). One may speculate that alterations in the AI(x) post nitrate administration may have been even more pronounced if these subjects were not receiving aspirin.

Many of the subjects in the current study also continued to receive prophylactic anti-anginal treatment with other agents (notably non-dihydropyridine calcium antagonists in 68% of subjects) throughout the protocol. Calcium antagonists have moderate anti-aggregatory effects (section C.18 of chapter 1). No clinical studies have been performed to date to determine whether these effects are additive over those of organic nitrates.

Experimental

The findings of a series of experiments examining the anti-aggregatory effects of NTG in subjects with UAP or acute MI, performed by Diodati *et al* (1990) may also serve as a potential reason for the absence of any detectable anti-aggregatory effect of TD-NTG/ISMN administration. For each subject, acute NTG infusion was titrated to achieve a 10% decrease in the mean arterial blood pressure (mean infusion rate 1.19 ± 0.2μg/kg/min). This dosing regimen caused a significant inhibition of ADP or thrombin-induced platelet aggregation, but

represents a far greater rate of NTG systemic administration than occurred in the current study. Specifically, in the study of Diodati *et al* (1990), approximately 5mg of NTG would have been infused intravenously per hour (assuming mean subject weight of 70Kg) compared with approximately 0.6mg/hr released into the skin for the TD-NTG preparation utilized in the current study. Furthermore, Diodati *et al* (1990) examined platelet function at the bedside. If platelet samples were left at room temperature for a period of 25 minutes prior to experimentation, the anti-aggregatory effect of NTG was not detected. In the current study, assessment of platelet function was performed 10 to 30 minutes post sampling. While a longer "lag time" between venesection and assessment of aggregation does not necessarily preclude detection of effects, previous "positive" studies of this type, for example in platelet-rich plasma (Chirkov *et al.*, 1993) have followed administration of large doses of NTG.

Finally, it has previously been shown (Willoughby *et al.*, 1996) that the anti-aggregatory effects of NTG *in vitro* are markedly potentiated when platelet aggregation is induced by a mixture of platelet agonists rather than by ADP alone. It was speculated that this multi-agonist induced aggregation more closely mimics *in vivo* circumstances than ADP alone. The multi-agonist model was not utilized in the current study, but might have facilitated detection of NTG effects.

Summary

Within the current study, both acute and chronic administration of either TD-NTG/ISMN was not shown to have significant *ex vivo* anti-aggregatory effects despite profound effects on the vasomotor responses. There are two possible reasons;

- Firstly, the vasomotor effects of the nitrate preparations far exceed their anti-aggregatory effects.
- Secondly, it remains probable that the methodology utilized herein (*ex vivo* aggregometry) is sub-optimal for detection of the anti-aggregatory effects occurring *in vivo*.

[4.6.2] "Rebound" platelet hyper-aggregability

The mechanism/s involved in the phenomenon of "rebound" in ischaemic events upon nitrate withdrawal or during a nitrate-free interval are unclear. Some investigations have suggested a

heightened sensitivity to vasoconstrictors may serve as a potential mechanism (Hebert and Lam, 2000). As discussed in chapter 1 (section D.3.3), the development of tolerance to organic nitrates is associated with activation of vasoconstrictor mechanisms (renin/angiotensin system) (Munzel *et al.*, 1995a), effectively counteracting the vasodilator properties of organic nitrates, a phenomenon also referred to as pseudotolerance. Utilizing a dog model, Munzel and Bassenge (1996) demonstrated "rebound" vasoconstriction of the anterior circumflex artery upon sudden withdrawal of chronic NTG, a phenomenon that was limited by ACE inhibition (enalapril 1mg/kg/24-hrs). Moreover, there is also a suggestion that catecholamines may play some role in the phenomenon of "rebound". Holdright *et al* (1993) demonstrated no "rebound" during intermittent TD-NTG pharmacotherapy in patients with SAP that were undergoing a β-adrenergic pharmacotherapy. No clinically based studies have evaluated the role of platelets in "rebound". However, Figueras *et al* (1991) observed that cessation of NTG infusion in patients with unstable angina frequently caused rapid re-emergence of ischaemia; this might well reflect incremental platelet aggregation.

No "rebound" platelet hyper-aggregability during the nitrate-free interval was observed within the current study. This is the first investigation in human subjects examining possible "rebound" in platelet aggregability during a nitrate free period with nitrate preparations/doses, analogous with that previously demonstrated to illicit the "zero hour" phenomenon of a reduced exercise capacity prior to nitrate administration (DeMots and Glasser, 1989).

Only one study to date (pig model), has investigated the hypothesis that acute withdrawal of NTG (TD-NTG 0.8mg/h for 48-hrs) was associated with platelet hypersensitivity (Hebert and Lam, 2000). Platelet thrombus formation on aortic media and the extent of whole blood platelet aggregation was significantly inhibited following chronic exposure to TD-NTG (48-hrs). However, two hours post nitrate withdrawal both platelet deposition and the extent of platelet aggregation remained significantly decreased. This result contrasted to the mean arterial pressures that were significantly elevated when compared to the "no nitrate" pressures. Moreover, following two hours post withdrawal of NTG treatment, Hebert and Lam (2000) demonstrated a super-sensitive response to a bolus angiotensin II, manifesting as a significant increase in mean arterial pressure compared to pressures during the "no nitrate" phase, suggesting that augmented responsiveness of the vessel wall to vasoconstrictors rather

than platelet hyper-activity is a mechanism behind the "rebound" phenomenon post nitrate withdrawal.

[4.6.3] Sensitivity to nitric oxide donors

Nitrate tolerance

The continuous use of organic nitrates usually results in the development of nitrate tolerance with evidence of its development being more readily observed at the vascular rather than the platelet level. Throughout the literature there are only a limited number of studies addressing the phenomenon at the platelet level. Baines *et al* (1994), utilizing anaesthetized mini-pigs demonstrated a significant attenuation in the preload effects of NTG, a result that paralleled a fall in platelet cGMP. This result was in concordance with the observations of Bassenge and Fink (1996), where vascular changes were demonstrated to parallel platelet changes. Chirkov *et al* (1997), examining the phenomenon of nitrate tolerance at the platelet level in a cohort of patients with SAP, demonstrated a significant rightward shift in the concentration-response curve following sublingual administration of a single dose of NTG (300μg). Significantly greater concentrations of NTG added *in vitro* were required to reverse ADP-induced platelet aggregation, indicating tolerance development. Within the same study, the extent of platelet responsiveness to NTG added *in vitro* (100μM) was significantly diminished following intravenous infusion of NTG (5μg/min for 24hrs) compared to platelet responsiveness to NTG prior to infusion (Chirkov *et al.*, 1997).

In the current study, the extent of platelet responsiveness to *in vitro* NTG (100μM) remained unchanged during both the acute and chronic treatment phases of ISMN/TD-NTG pharmacotherapy (Figure 4.9/4.14). However, with applanation tonometry as a means of assessing the vasomotor function of each nitrate preparation, a significant attenuation in the AI(x) was observed following chronic administration of either nitrate preparation (Figure 4.24), indicating nitrate tolerance development at the vascular level. Platelet responsiveness to *in vitro* NTG (100μM) in subjects assessed via applanation tonometry, were similar to those subjects in whom the vascular responsiveness to ISMN/TD-NTG were not assessed (Appendix).

Interestingly, the monitoring of systolic blood pressure and heart rate throughout all phases of the trial did not demonstrate haemodynamic tolerance (Table 4.8 and Figures 4.20/4.21).

Therefore, pulse wave analysis during chronic nitrate exposure may serve as a more sensitive means of assessing nitrate effect, rather than changes in blood pressure.

A number of studies to date have examined the effects of organic nitrates and other sources of nitric oxide donors on AI(x) (Chowienczyk *et al.*, 1999; O'Rourke and Mancia, 1999; Wilkinson *et al.*, 2002). The current study is the first to demonstrate tolerance development following chronic exposure to ISMN/TD-NTG in a cohort of SAP patients as detected by pulse wave analysis. It is also akin to the observations of others in which tolerance development to NTG and other nitrovasodilators and the augmentation thereof, was assessed using more invasive techniques (venous occlusion plethysmography) (Gori *et al.*, 2001; Milone *et al.*, 1999b; Watanabe *et al.*, 1998a).

Platelet vasomotor inter-relationship

The current study is the first investigation examining the possible relationship between the development of tolerance at the platelet and vascular level in humans with mild to moderate SAP. Whilst demonstrating marked haemodynamic tolerance in rats following prolonged NTG administration, Booth *et al* (1996) was unable to demonstrate a reduction in platelet cGMP or rat bleeding time. Akin to these results, Hebert *et al* (1997) demonstrated persistent inhibition of platelet deposition on porcine aortic media utilizing a dosing regimen that resulted in marked haemodynamic tolerance (TD-NTG; 0.8mg/hr/48-hrs). Moreover, no significant correlation was observed between the extent of platelet responsiveness to *in vitro* NTG (100μM) and AI(x) 4 hrs post administration of TD-NTG during the acute phase of the trial (Figure 4.28). This reinforces the observation that tolerance development at the platelet level does not parallel tolerance development at the vascular level.

Nitrate tolerance and superoxide

Several studies to date have reported that sustained exposure to NTG results in increased superoxide generation (Dikalov *et al.*, 1999; Dikalov *et al.*, 1998a; Munzel *et al.*, 1996a; Munzel *et al.*, 2000b; Munzel *et al.*, 1995b). These observations lead to the development of the "oxidative stress" hypothesis as a means to explain the phenomenon behind nitrate tolerance development (Section D.3.2.2 of chapter 1). As such, one of the purposes of the current investigation was to examine changes in whole blood superoxide levels during both acute and chronic exposure to ISMN/TD-NTG.

Acute (Figure 4.11) or chronic (Figure 4.16) administration of either nitrate preparation was demonstrated not to cause any significant change in the extent of pre-aggregation or aggregation-associated LDCL. Furthermore, there was no significant relationship between the extent of platelet responsiveness to NTG and the degree of either pre-aggregation or aggregation-associated LDCL (Figure 4.30), suggesting that acute administration of organic nitrates in the dose utilized, is not associated with a sudden rise in superoxide.

Concluding that superoxide has no role in the phenomenon of organic nitrate tolerance induction, utilizing the results observed herein, must however be made with caution as no significant tolerance towards either nitrate preparation was observed at the platelet level. One is unable to totally rule out involvement of superoxide in the phenomenon of nitrate tolerance as the luminescent variables assessed herein were also demonstrated to be intimately associated with platelet aggregation (section II chapter 2) and independent of any vascular source of superoxide. Furthermore, superoxide was not assessed in all subjects. Despite these limitations, tolerance towards both nitrate preparations was observed at a vascular level with no significant changes in the luminescent variables (Appendix Figure 4).

Utilizing platelet samples from 10 normal male volunteers McVeigh *et al* (2002), recently demonstrated that 3 days of continuous TD-NTG (10mg/24hrs) resulted in a significant increase in platelet derived superoxide (detected using 20μM lucigenin). This increase was accompanied by an increase in platelet NADH oxidase activity (McVeigh *et al.,* 2002). Utilizing applanation tonometry, a significant effect of NTG was apparent on pulse waveform morphology after 3 day of NTG exposure despite evidence of tolerance induction, a result that is in concordance with our observations. Moreover, the traditional haemodynamic parameters measuring NTG efficacy, systolic/diastolic blood pressure and heart rate remained unchanged following chronic exposure to NTG. Results that are in concordance with our observations. However, no significant change in LDCL was observed following the acute or chronic administration of TD-NTG or ISMN. Reasons for this discrepancy lie in the differences between the two studies. All experiments performed by McVeigh *et al* (2002) were undertaken on a cohort of male NVs not receiving any concurrent medications. Furthermore, the non-intermittent dosing regimen caused profound nitrate tolerance, and therefore results are not clinically relevant given intermittent dosing recommendations (Thadani *et al.*, 1986; Parker *et al.*, 1995). Finally, McVeigh *et al* (2002) did not assess the functional significance of an increased platelet derived superoxide. Within the current study

intermittent dosing with TD-NTG was not associated with a significant increase in LDCL but was also not associated with a significant reduction in platelet aggregability.

Nitric oxide resistance

Within chapter 3, an ACS as distinct from SAP was associated with a significantly reduced responsiveness in the anti-aggregatory effects of SNP (10μM). Given the study design within chapter 3, the influence of prior/continuous nitrate consumption on the extent of platelet responsiveness to SNP may very well be dose-dependent and would remain somewhat obscured within a multivariate analysis.

Throughout the literature there is *in vitro* evidence demonstrating that NOS is regulated by both endogenous and exogenous sources of nitric oxide in a negative feedback dependent mechanism (reviewed extensively by Griscavage *et al.*, 1995). More recently Gori *et al* (2001), in a cohort of healthy subjects, demonstrated that continuous treatment with TD-NTG (0.6mg/h for 6 days) resulted in a marked inhibition of acetylcholine-induced increases in forearm blood flow as measured using strain gauge plethysmography, consistent with "endothelial dysfunction" induced in parallel with nitrate tolerance. Moreover, *L*-NMMA induced vasoconstriction was significantly blunted in volunteers that had been treated with TD-NTG, suggesting that NTG pharmacotherapy has an inhibitory effect on basal as well as on stimulated vascular nitric oxide.

The current study was designed to examine the extent of platelet responsiveness to both NTG and SNP in the presence and absence of two nitrate preparations (ISMN/TD-NTG). As illustrated in Figure 4.10/4.15, acute or chronic administration of either ISMN or TD-NTG had no significant effect on the extent of platelet responsiveness to SNP (10μM). These results firstly demonstrate that there was no cross-tolerance to a more direct donor of nitric oxide following either acute or chronic exposure to either nitrate preparation, a result that confirms the observations of Chirkov *et al* (1997). Secondly, the results also cast doubt on the hypothesis that reduced platelet responsiveness to nitric oxide results from a negative feedback inhibition of NOS, given prolonged exposure to exogenous sources of nitric oxide (TD-NTG/ISMN) were unable to influence the extent of platelet responsiveness to nitric oxide. However, at no stage throughout the duration of the trial was the function of platelet derived NOS directly examined.

Adding further weight against the hypothesis of a negative feedback inhibition of NOS serving as a mechanism behind a reduced responsiveness to nitric oxide at the platelet level, McVeigh *et al* (2002), recently demonstrated that non-intermittent TD-NTG was associated with a significant increase in platelet-derived nitric oxide. These results were confirmed utilizing a nitric oxide sensitive electrode and through the measurements of nitrite, suggesting that platelet NOS activity increases following prolonged exposure to NTG.

[4.6.4] Inter-relationship between platelet and vascular responsiveness to nitric oxide

Apart from measuring the degree of *ex vivo* anti-aggregatory effects of commonly utilized nitrate preparations, the current study was also designed to examine the relationship between individual platelet and vasomotor responsiveness to nitric oxide.

Despite only running the comparison in a small cohort (Figure 4.27), no significant relationship between the extent of platelet responsiveness to SNP and AI(x) was observed. Furthermore, the reduction in AI(x) at 4 hrs post administration of TD-NTG was not related to the extent of platelet responsiveness to NTG, at the zero hour time point (Figure 4.29). Given the small number of subjects analyzed, the significance of this relationship is unclear and further exploration of this relationship is required.

[4.6.5] Superoxide and vascular responsiveness to nitric oxide

Within section I of chapter 2, a trend towards a significant relationship between the extent of platelet responsiveness to SNP and the degree of change in platelet responsiveness to SNP post administration of SOD/catalase, was observed in those subjects with a history of angina pectoris (SAP/ACS). These results suggested that the superoxide anion might play a role in the phenomenon of attenuated platelet responsiveness to nitric oxide. However, within section III of chapter 2 no significant relationship was observed between the extent of platelet responsiveness to SNP and both pre-aggregation and aggregation-associated LDCL, implying that superoxide's role in the phenomenon of platelet hypo-responsiveness to nitric oxide is more complex than simply of a scavenger of nitric oxide.

As illustrated in Figure 4.31 there was no significant relationship between the change in AI(x) 4-hrs post administration of either nitrate preparation and the degree of change in either pre-aggregation or aggregation-associated LDCL. These results suggest that there is no

relationship between the extent of vascular responsiveness to nitric oxide and the degree of superoxide detected within a whole blood sample. Reasons for the absence of any significant relationship between vascular responsiveness to nitric oxide and superoxide levels are numerous and may include Type II error.

[4.7] Limitations of the study

The current study has a number of major objectives centered upon the assumption that the dosing regimens for TD-NTG and ISMN respectively, would be sufficient to permit not only detection but also quantitation of their anti-aggregatory effects. This proved to be incorrect. However, the study in no way implies that organic nitrates lack anti-aggregatory effects.

While the inability to measure anti-aggregatory effects *ex vivo* limits the utility of the study, it achieved a number of important objectives, including evaluation of the (lack of) effects of chronic nitrate administration on tolerance at the platelet level, despite induction of tolerance at the vascular level. In this regard, there are a number of caveats.

First, a single index of vasomotor responsiveness to nitrate was utilized. Changes in AI(x) do not necessarily parallel those of the venodilator effects of nitrates, or even necessarily the coronary vasomotor effects, all of which may mediate changes in anginal threshold. Secondly, one objective of the study was to determine whether there are any substantial differences in the extent of tolerance induction between TD-NTG and ISMN. The study was designed on the assumption that both preparations would be (initially) approximately equipotent. At least measured by changes in AI(x), this proved to be correct. However, there was a trend towards greater attenuation of ISMN than with TD-NTG. Previous clinical studies with ISMN have generally utilized end-points such as exercise duration (Chrysant *et al.*, 1993) rather than measurements of any vasomotor changes. There are no clinical comparisons between nitrate preparations as regards the extent of tolerance induction. However, in view of the recent claims, largely from Bassenge's group (Mullenheim *et al.*, 2001), that pentaerythritol tetranitrate is largely "immune" to tolerance induction, it is increasingly relevant to compare effects of other nitrate preparations. Changes in AI(x), examined in a somewhat larger cohort of patients could provide a component of such an analysis. It must also be stated that whereas changes in platelet response to NTG *ex vivo* can be taken as a measure of "true" nitrate tolerance, it is possible that a component of

attenuation of AI(x) effects might have been due to increased concentrations or effects of vasoconstrictor agents ("pseudo-tolerance").

[4.8] Conclusions

In a cohort of patients with mild to moderate SAP, neither acute nor chronic administration of either ISMN or TD-NTG induced a detectable change in the degree of *ex vivo* platelet aggregation in the doses examined. Neither nitrate preparation induced a desensitization of platelets to nitric oxide as donated *in vitro* by either NTG (100μM) or SNP (10μM), nor was there any evidence of "rebound" in platelet hyper-aggregability during the nitrate free periods. The effects of both ISMN and TD-NTG on wave reflection were found to be similar and provided evidence of continued vasodilatation during chronic nitrate administration, despite evidence of partial tolerance development.

[4.9] Chapter summary

This study is the first comparing the acute and chronic effects of clinically similar doses of prophylactic nitrate preparations. We demonstrated that both acute and chronic administration of either SR isosorbide 5'mono-nitrate (120mg) or TD-NTG (15mg/24-hrs (12-hr application)), induced no significant *ex vivo* anti-aggregatory effect within a cohort of SAP patients with mild to moderate (Canadian Cardiovascular Class II-III) SAP, with no rebound platelet hyper-aggregability being observed during nitrate free periods. Moreover, the absence of any significant *ex vivo* anti-aggregatory response to either ISMN or TD-NTG was observed despite a significant vascular effect (reduction in the AI(x)), as measured in a sub-population of studied subjects.

The study provided important information about the phenomenon of nitrate tolerance induction. Both preparations (ISMN and TD-NTG) exerted similar vasomotor effects at baseline; these vasomotor effects were significantly attenuated during chronic therapy. This haemodynamic tolerance occurred without any tolerance at the platelet level (an important aspect of nitrate safety) or without any changes in rates of superoxide production (an important aspect of understanding the mechanisms of tolerance induction).

Chapter 5

Studies examining the pathophysiological and anti-aggregatory effects of nitric oxide: General discussion and future directions

General discussion and future directions

[5.1] Introduction

Platelet hyper-aggregability, combined with hypo-responsiveness to exogenous nitric oxide, observed in patients with angina pectoris or risk factors for CAD, reflects an impaired physiological response to endogenously derived nitric oxide. As such, both phenomena, viewed together or separately, represent a mechanism behind the increased risk of thrombotic events. With this in mind, the studies described herein were designed for the specific purposes of: -

- Characterizing further the phenomena of platelet hyper-aggregability and platelet hypo-responsiveness to the anti-aggregatory effects of nitric oxide. Further to this, experiments were also designed to examine the role of superoxide within both phenomena.

- Examining potential determinants of platelet hypo-responsiveness to nitric oxide

- Examining the anti-aggregatory and vasomotor effects of acute and chronic nitrate pharmacotherapy in a cohort of SAP patients, with special attention being paid to the effects of nitrate treatment on platelet aggregability, platelet responsiveness to nitric oxide and platelet aggregation following acute nitrate withdrawal.

[5.2] Studies examining the phenomena of platelet hyper-aggregability and hypo-responsiveness to donors of nitric oxide

[5.2.1] Study purpose and results

Previous investigations demonstrated that platelets from patients with SAP are hyper-aggregable to ADP and resistant to the anti-aggregatory effects of nitric oxide (Chirkov *et al.*, 1993; Chirkov *et al.*, 1996), a phenomenon that is also observed in platelets from subjects with risk factors for CAD (Anfossi *et al.*, 1998; Woods *et al.*, 1993). The current study examined further the phenomena of platelet hyper-aggregability and hypo-responsiveness to nitric oxide in subjects with both SAP and ACS, compared to a cohort of normal subjects. The current study was also designed to examine the role of the ROS superoxide within both phenomena.

In patients with SAP and ACS, as distinct from a population of NVs, platelet aggregability was increased with inhibition of platelet aggregation by either NTG or SNP being significantly attenuated. The addition of SOD/catalase (serving as scavengers of superoxide and hydrogen peroxide) significantly inhibited ADP-induced platelet aggregation in those subjects with SAP and ACS, but not in normals only. However, baseline platelet aggregation was not significantly correlated with change in aggregation post SOD/catalase, implying that superoxide is not the only factor related to the phenomenon of platelet hyper-aggregability but nevertheless is important in the regulation of platelet aggregation in SAP and ACS patients only.

Overall, addition of SOD/catalase to a whole blood sample did not significantly alter the extent of platelet responsiveness to SNP. However, when the data were analyzed according to presence of myocardial ischaemia (SAP/ACS versus NV), a significant relationship between platelet responsiveness to SNP and change in SNP responsiveness post superoxide scavenging was observed within the angina pectoris cohort. Moreover, a bidirectional relationship was observed between platelet responsiveness to SNP and change in SNP responsiveness post SOD/catalase administration. These results imply a significant role for superoxide in the phenomenon of platelet hypo-responsiveness to nitric oxide in subjects with acute/chronic ischaemic heart disease.

Following these initial investigations it was apparent that a more direct measure of superoxide within a whole blood sample was required to further examine the mechanism/s behind the phenomena of platelet hyper-aggregability and hypo-responsiveness to nitric oxide. Utilizing LDCL, superoxide was detected prior to induction of platelet aggregation within whole blood samples. Induction of platelet aggregation resulted in an aggregation-associated increase in LDCL, an effect that was related to the extent of platelet aggregation. Furthermore, a lag period between the induction of platelet aggregation and the commencement of the aggregation-associated LDCL was also observed and found to be a function of the concentration of ADP used and the extent of platelet aggregation prior the commencement of the aggregation-associated LDCL.

Utilizing LDCL for the detection of superoxide in whole blood, baseline and aggregation-associated LDCL were found to be significantly greater within ACS patients than in the SAP and NV cohorts. However, there was no significant relationship between platelet aggregation

or platelet responsiveness to SNP, when compared to baseline or aggregation-associated LDCL. When LDCL was expressed relative to aggregation/SNP responsiveness, in ACS patients, release of superoxide per unit of inhibition of aggregation, was significantly greater than in SAP patients.

Overall these results suggest that the mechanism/s involved in the regulation of platelet aggregation for patients with CAD and hypo-responsiveness to donors of nitric oxide are more complex than a simple model of excess of superoxide.

[5.2.2] Implications

The results obtained from the three studies described in chapter 2 represent a further characterization of the phenomena of platelet hyper-aggregability and hypo-responsiveness to the anti-aggregatory effects of nitric oxide donors. The results also represent a series of preliminary investigations examining the role of superoxide within the two phenomena and as such the results have a number of important implications.

These include: -

- Superoxide plays an important regulatory role in platelet aggregation in subjects with acute and chronic ischaemic heart disease. As the concentration of superoxide was found to be elevated in subjects with ACS, a direct benefit from strategies designed to decrease superoxide release and/or increase superoxide clearance may limit platelet activation in these subjects and hence ameliorate their clinical status and improve their prognosis.

- The observation of a bidirectional relationship between baseline SNP responsiveness and change in SNP responsiveness post SOD/catalase administration observed in the cohort of subjects with a history of angina pectoris, suggests that a beneficial effect may also be observed with analogous strategies in patients with attenuated platelet responsiveness to SNP. Conversely, such treatment may be detrimental in patients with a normal or "good" SNP responsiveness. This hypothesis has obvious implications as regards to the overall "neutral" results of recently published interventions utilizing dietary supplementation with various anti-oxidants (e.g. HOPE, Heart Protection Study).

- Within a whole blood sample, induction of platelet aggregation is associated with an increase in superoxide generation and as such emphasizes the potential for release of superoxide at the sites of vascular injury where platelet aggregation plays an integral part in thrombus formation.

[5.3] Studies examining the clinical determinants of platelet hypo-responsiveness to nitric oxide

[5.3.1] Study purpose and results

As identified in Chapter 2, platelets from patients with an ACS were demonstrated to be hyper-aggregable to ADP and hypo-responsive to the anti-aggregatory effects of exogenous nitric oxide, phenomena previously observed in patients with SAP. It is within this context that the current study was designed to examine the clinical determinants of platelet hypo-responsiveness to nitric oxide in patients with SAP and ACS relative to cohorts of NIPs and NVs. The current study was also undertaken to investigate whether platelet responsiveness to nitric oxide was related to the underlying diagnosis, extent of fixed CAD, related to the severity of angina (SAP subjects), or associated with particular coronary risk factors or pharmacotherapy.

As described within chapter 2, platelets from patients with ACS were significantly more aggregable following ADP administration and significantly hypo-responsive to the anti-aggregatory effects of SNP or NTG relative to those from subjects with SAP or a cohort of NIPs/NVs. Furthermore, whilst examining differences in aggregability across the subject cohorts, a gender-dependent differential anti-aggregatory effect of aspirin was observed. By multivariate analysis, the diagnosis of an ACS was an independent predictor of attenuated platelet responsiveness to nitric oxide. Furthermore, the presence of nitric oxide resistance was independent of classical coronary risk factors and the majority of forms of pharmacotherapy. Notable exceptions were perhexiline and statin pharmacotherapy, both of which were associated with a decrease incidence of platelet hypo-responsiveness to nitric oxide.

[5.3.2] Implications

This is the first analysis of this type in the literature. Implications include: -

- Most importantly, patients theoretically most in need of the beneficial anti-platelet/vasomotor effects of nitric oxide, i.e. patients with ACS or severe SAP, also demonstrate the greatest resistance to the anti-aggregatory effects of nitric oxide, a phenomenon that was not associated with any of the classical coronary risk factors. As such, these patients are likely to respond sub-optimally to the actions of exogenously donated nitric oxide.

- The finding that pharmacotherapy with statins and with perhexiline was associated with a lower incidence of an attenuated platelet responsiveness to nitric oxide, provides an impetus for the prospectively designed evaluation of their effects on the anti-aggregatory effects of nitric oxide, including evaluation of relevant mechanisms.

- The observation of a gender-dependent differential anti-aggregatory effect of aspirin has a number of important implications. Firstly, it may serve as an explanation for attenuation of benefit in some primary/secondary prevention ischaemic event trials, where females constituted a significant proportion of the total number of participants. Secondly, it raises the possibility that females may be hypo-responsive to other anti-aggregatory agents, and that this may contribute to the "Yently syndrome" of poor outcome in females with coronary disease.

[5.4] Studies evaluating the anti-aggregatory and arterial vasomotor effects of prophylactic nitrate pharmacotherapy

[5.4.1] Study purpose and results

Organic nitrates are in widespread use for the treatment of subjects with symptomatic IHD and heart failure. Apart from their vasomotor properties, organic nitrates exert potent anti-aggregatory effects *in vivo*, effects that have also been demonstrated *ex vivo*, but mainly with high dose nitrate regimens. The current study was undertaken to examine the *ex vivo* anti-aggregatory effects of ISMN and TD-NTG during both acute and chronic administration in subjects with SAP. Furthermore, the study was designed to examine the effects on platelet

responsiveness to nitric oxide and to correlate the nitrates' effects on platelet function and arterial wave reflection as one measure of vasomotor response.

Neither ISMN nor TD-NTG induced any significant change in the platelet response to ADP, NTG or SNP, or in the levels of superoxide, as detected using LDCL, during all experimental time periods. Moreover, there was no evidence of platelet hyper-aggregability upon nitrate withdrawal implying no role for platelet hyper-aggregability in the "zero-hour" phenomenon. In contrast to these results, both nitrate preparations were associated with decreased AI(x) 4 and 8 hrs post acute dosing, an effect that did not differ markedly between nitrate preparations, but was shown to be attenuated following chronic nitrate exposure, implying tolerance development.

[5.4.2] Implications

Neither nitrate regimen examined within chapter 4 produced detectable *ex vivo* anti-aggregatory effects. This implies a deficiency of methodology rather than a biological effect. Furthermore, the results from an examination of the anti-aggregatory and arterial vasomotor function following prophylactic nitrate pharmacotherapy have a number of potentially important implications.

These include: -

- As neither nitrate regimen was demonstrated to alter ADP-induced platelet aggregation or platelet responsiveness to nitric oxide during both chronic pharmacotherapy and nitrate free periods, these suggest that neither the "zero-hour" phenomenon nor other reflections of nitrate "rebound" states, reflect changes in platelet aggregability.

- Both nitrate regimens induced similar effects on pulse wave reflection, a phenomenon that was shown to be attenuated during chronic nitrate pharmacotherapy with either preparation. These results provide further evidence that regimens incorporating "nitrate-free" periods do not circumvent the development of partial nitrate tolerance.

[5.5] Major study limitations

A number of possible study limitations may be identified; most of which have been discussed within each relevant chapter of this thesis. However, major study limitations that influence the results across all experimental chapters can be summarized as follows: -

- Throughout all the experimental phases described in the thesis, *in vitro* administration of a nitric oxide donor and the apparent attenuation in platelet responsiveness towards these agents within samples may not precisely reflect the *in vivo* extent of platelet resistance towards nitric oxide.

- One of the major findings of this thesis has been the observation that platelets from patients with symptomatic and asymptomatic ischaemic heart disease are hyper-aggregable in response to relatively low concentration of ADP. In an *in vivo* situation, platelets are exposed to more that one agonist at any one time, with some agonists acting in concert (Huang and Detwiler 1981; Alarayyed *et al.*, 1995). Moreover, when a combination of platelet agonists (adrenaline, serotonin and thrombin, in subthreshold concentrations) were utilized (Willoughby *et al.*,1996), platelets from both SAP and normal subjects demonstrated increased platelet sensitivity to the anti-aggregatory effects of NTG or PGE_1. The use of ADP as a single pro-aggregant is therefore convenient, although un-physiological. This may be particularly relevant to the failure to observe *ex vivo* effects of nitrate therapy in Chapter 4.

- Experiments using ADP-induced whole blood platelet aggregometry and platelets from patients with ACS automatically biases investigations into platelet function towards those subjects who were treated less aggressively, excluding subjects not receiving ADP receptor and/or GPIIb/IIIa receptor antagonists. By (inevitably) excluding these subjects from the current series of experiments an important cohort of patients is missing, who theoretically, may have demonstrated extreme attenuation of platelet responsiveness to nitric oxide, compared to the overall cohort of ACS patients examined herein.

- *Ex vivo* estimation of the levels of whole blood superoxide via the use of LDCL also constitutes a study limitation. As eluded to in the introduction of chapter 2 (section 2.2.8), *ex vivo* estimations of superoxide levels are not fully representative of the levels found *in*

vivo, as alternative sources (vascular endothelium or shear stress related) are absent in blood samples. Thus, interpretation of the exact role of superoxide modulating the phenomena of platelet hyper-aggregability and hypo-responsiveness to nitric oxide must be made with caution.

[5.6] Future directions/experiments

[5.6.1] Studies addressing the phenomenon of platelet hyper-aggregability

Some potential future studies examining the phenomenon of platelet hyper-aggregability can be summarized as follows : -

A) Experiments addressing mechanism/s: -

- Several questions remain unresolved. Is the phenomenon of platelet hyper-aggregability restricted to hyper-sensitivity towards (low concentrations of) ADP alone, or is it related to some or all potential mechanisms of platelet activation (alternative platelet agonists, shear stress)?

- Is the phenomenon of platelet hyper-aggregability related in part to dysfunction of the platelet guanylate cyclase-cGMP system, known to modulate platelet aggregation? Is hyper-aggregability only a manifestation of dysfunctional platelet regulation?

- Although the current results have implicated superoxide as an important modulator of hyper-aggregability, the signal transduction pathway for superoxide release, the component of intra-platelet derived superoxide, and the potential amenability of these superoxide pathways to pharmacological manipulation, remain to be explored.

B) Clinical issues

- As described above, studies were performed on patients. This process engendered selection bias. For example, it was impossible to assess initial hyper-aggregability once patients had received agents such as ADP receptor antagonists and/or GPIIb/IIIa inhibitors. In order to test the hypothesis that the extent of hyper-aggregability is correlated with clinical status, data from such patients would need to be collected prior to inception of incremental therapy, despite the practical limitations inherent in this aim. This issue is also relevant to investigations of platelet responsiveness to nitric oxide, by

virtue of exclusion of the "worst" patients in the ACS cohort, and by virtue of current strategies to utilize perhexiline selectively for patients with persistent symptoms.

- The findings related to gender-specific effects of aspirin are of great potential interest, but were a secondary, non-hypothesis-based observation within the current study. Despite the strength of the observation, prospective investigations specifically testing this hypothesis, both with more extensive patient cohorts, and with specifically designed studies, would be of great importance. Furthermore, there is an issue of selection bias here: potentially, patients failing aspirin therapy would be more likely to receive additional agents which might exclude them from the current investigations: the current findings may therefore understate actual differences.

[5.6.2] Studies addressing the phenomenon of platelet hypo-responsiveness to donors of nitric oxide

Some potential future studies examining the phenomenon of platelet hypo-responsiveness to nitric oxide can be summarized as follows.

Delineation of the nexus between hyper-aggregability and hypo-responsiveness to nitric oxide

As discussed previously, it is difficult to characterize fully two abnormalities that exert physiologically opposite effects: it could, for example, be postulated that hypo-responsiveness to nitric oxide donors is an inevitable consequence of exaggerated responsiveness to ADP, via functional antagonism. Because the current methodology does not permit construction of a complete ADP concentration-response curve within the period of normal platelet function (*ex vivo*), this issue can be evaluated best by techniques such as ANCOVA, a convoluted method.

Given that it is difficult to measure precisely the physiological effects of nitric oxide, independent of the impact of ADP, there is increased need to measure such effects, biochemically. In particular, a greater emphasis on the measurement of soluble guanylate cyclase activation would be useful in providing parallel biochemical data.

Evaluation of the relationship between platelet hypo-responsiveness to nitric oxide and:-

- Hypo-responsiveness to nitric oxide within the vasculature
- "Endothelial dysfunction"

As previously outlined in chapter 1, "endothelial dysfunction" is generally defined on the basis of attenuation of the vasomotor response to nitric oxide-releasing stimuli, such as acetylcholine or shear stress. Although initial investigators assumed that affected blood vessels maintained normal responses to exogenous nitric oxide donors (Ludmer *et al.*, 1987), there is now a large body of evidence to indicate that in most circumstances there is an attenuation of vascular responses to exogenous nitric oxide. Of particular interest have been the observations of Schachinger *et al.*, (2001), who demonstrated that diminution of coronary arterial responsiveness to nitric oxide donors was predictive of future cardiac events.

Nevertheless, no studies to date have simultaneously evaluated platelet and vascular responsiveness to nitric oxide. This type of experiment would be challenging, as it begs the question of the ideal source of vasculature, given that not all blood vessels respond identically to nitric oxide donors. Potential methodologies might include intra-coronary injection with quantitative coronary angiography, measurement of forearm blood flow with venous occlusion plethysmography and/or measurement of arterial compliance with applanation tonometry and pulse wave analysis. If such studies were to reveal close correlations between platelet and vascular responsiveness to nitric oxide, a prognostic impact of abnormalities in platelet responsiveness would be established by this association. Nevertheless, there are many differences in the biochemistry of platelet and vascular responses to nitric oxide (notably the exposure of platelets to high concentrations of superoxide during inflammation and incipient aggregation). Therefore, no close correlation need exist.

Establishments of a nexus between platelet hypo-responsiveness to nitric oxide and "endothelial dysfunction" would have other notable corollaries:

- This might stimulate further evaluation (currently very limited) of the potential role of platelet nitric oxide synthase dysfunction in coronary risk.
- Improved platelet response to nitric oxide could be established, via prospective studies, as a surrogate for reversal of endothelial dysfunction.

- Studies evaluating acute and chronic vascular and platelet responses to nitric oxide donors.

As described in Chapter 4, our evaluation of the effects of cutaneous NTG or oral ISMN via *ex vivo* platelet aggregometry in whole blood was inadequate, almost certainly because of insensitive *ex vivo* methodology. There is no implication that NTG lacks therapeutically relevant anti-aggregatory effects.

Willoughby *et al.*, (1996) have described marked potentiation of the *ex vivo* effects of NTG on platelet aggregation, utilizing multiple agonists rather than ADP alone to induce aggregation. This more "physiological" method of inducing aggregation needs to be utilized in pilot studies, in order to determine whether it is adequately sensitive for the planned study design. If the multiple aggregation method proves sub-optimal, none of the previously described method for measuring platelet responses to nitric oxide are suitable for large-scale evaluations such as were attempted in Chapter 4; it would be possible essentially only to quantitate platelet responses to high doses of organic nitrates.

On the other hand, applanation tonometry and pulse wave analysis proved adequately sensitive to quantitate organic nitrate effects on arterial compliance. The current investigations in Chapter 4 represent the first example of the use of this technique to evaluate the phenomena of nitrate tolerance and rebound; the sensitivity and reproducibility of the technique should lead to its more widespread use in such evaluations. Specifically, it appears that the effects of both NTG and ISMN on augmentation index in patients with stable angina, are sufficiently great for changes in response of the order of 20% to be reliably detected.

Therapeutic amelioration of platelet resistance to the anti-aggregatory effects of nitric oxide

The current studies have established a basis for platelet resistance to nitric oxide: "scavenging" by superoxide anion. In conjunction with a (reversible?) dysfunction of platelet soluble guanylate cyclase (Chirkov *et al.*, 1999), it remains to be established whether the relative role of these two anomalies are constant, and/or whether they may be selectively targeted by therapeutic interventions.

For example, we observed that therapy with perhexiline is associated with a lower than expected prevalence of nitric oxide resistance (Chapter 3). Prospective experiments confirmed sensitization of nitric oxide by perhexiline, apparently mediated primarily by restoration of soluble guanylate cyclase responsiveness. On the other hand, it might be anticipated that ACE inhibitors, which inhibit the expression of NAD(P)H oxidase subunits, might improve platelet function by reducing superoxide levels, as (acutely) might ascorbic acid (Ellis *et al.*, 2001). There is a high priority to evaluate the impact on platelet function both established (e.g. ACE inhibitors, statins) and candidate (e.g. folic acid, sepiapterin and anti-oxidants) cardioprotective agents, in order to determine to what extent their (putative) beneficial effects may reside at the platelet level, and to limit unnecessary polypharmacy.

Chapter 6

Bibliography

Abernethy, D.R. and J.B. Schwartz, 1999, Calcium-antagonist drugs, N Engl J Med, 341, 1447-57.

Abou-Mohamed, G., W.H. Kaesemeyer, R.B. Caldwell and R.W. Caldwell, 2000, Role of L-arginine in the vascular actions and development of tolerance to nitroglycerin, Br J Pharmacol, 130, 211-8.

Abrams, J., 1986, Tolerance to organic nitrates, Circulation, 74, 1181-5.

Abrams, J., 1991, The mystery of nitrate resistance, Am J Cardiol, 68, 1393-6.

Abrams, J., 1992, Mechanisms of action of the organic nitrates in the treatment of myocardial ischemia, Am J Cardiol, 70, 30B-42B.

Abrams, J., 1995, The role of nitrates in coronary heart disease, Arch Intern Med, 155, 357-64.

Abrams, J., 1996, Beneficial actions of nitrates in cardiovascular disease, Am J Cardiol, 77, 31C-7C.

Abrams, J., 2000, Medical therapy of unstable angina and non-Q-wave myocardial infarction, Am J Cardiol, 86, 24J-33J.

Abu-Soud, H.M. and D.J. Stuehr, 1993, Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer, Proc Natl Acad Sci U S A, 90, 10769-72.

Adams, M.R., R. McCredie, W. Jessup, J. Robinson, D. Sullivan and D.S. Celermajer, 1997, Oral L-arginine improves endothelium-dependent dilatation and reduces monocyte adhesion to endothelial cells in young men with coronary artery disease, Atherosclerosis, 129, 261-9.

Adams, M.R., J. Robinson, R. McCredie, J.P. Seale, K.E. Sorensen, J.E. Deanfield and D.S. Celermajer, 1998, Smooth muscle dysfunction occurs independently of impaired endothelium-dependent dilation in adults at risk of atherosclerosis, J Am Coll Cardiol, 32, 123-7.

Afanas'ev, I.B., 2001, Lucigenin chemiluminescence assay for superoxide detection, Circ Res, 89, E46.

Afanas'ev, I.B., E.A. Ostrachovitch and L.G. Korkina, 1999, Lucigenin is a mediator of cytochrome C reduction but not of superoxide production, Arch Biochem Biophys, 366, 267-74.

Afanas'ev, I.B., E.A. Ostrakhovitch, E.V. Mikhal'chik and L.G. Korkina, 2001, Direct enzymatic reduction of lucigenin decreases lucigenin-amplified chemiluminescence produced by superoxide ion, Luminescence, 16, 305-7.

Ago, T., H. Nunoi, T. Ito and H. Sumimoto, 1999, Mechanism for phosphorylation-induced activation of the phagocyte NADPH oxidase protein $p47^{phox}$. Triple replacement of serines 303, 304, and 328 with aspartates disrupts the SH3 domain-mediated intramolecular interaction in $p47^{phox}$, thereby activating the oxidase, J Biol Chem, 274, 33644-53.

Aikawa, M., E. Rabkin, S. Sugiyama, S.J. Voglic, Y. Fukumoto, Y. Furukawa, M. Shiomi, F.J. Schoen and P. Libby, 2001, An HMG-CoA Reductase Inhibitor, Cerivastatin, Suppresses Growth of Macrophages Expressing Matrix Metalloproteinases and Tissue Factor *In vivo* and *In vitro*, Circulation, 103, 276-283.

Aiken, J.W., R.R. Gorman and R.J. Shebuski, 1979, Prevention of blockage of partially obstructed coronary arteries with prostacyclin correlates with inhibition of platelet aggregation, Prostaglandins, 17, 483-94.

Alarayyed, N.A., B.R. Graham, B.N.C. Pritchard and C.C.T. Smith, 1995, The potentiation of adrenaline-induced *in vitro* platelet aggregation by ADP, collagen and serotonin and its inhibition by naftopidil and doxazosin in normal human subjects, Br J Clinc Pharmacol, 39, 369-74.

Alberio, L. and G.L. Dale, 1999, Review article: platelet-collagen interactions: membrane receptors and intracellular signaling pathways, Eur J Clin Invest, 29, 1066-76.

Albert, M.A., E. Danielson, N. Rifai and P.M. Ridker, 2001, Effect of statin therapy on C-reactive protein levels: the pravastatin inflammation/CRP evaluation (PRINCE): a randomized trial and cohort study, JAMA, 286, 64-70.

Alderton, W.K., C.E. Cooper and R.G. Knowles, 2001, Nitric oxide synthases: structure, function and inhibition, Biochem J, 357, 593-615.

Ambrosio, G., P. Golino, I. Pascucci, M. Rosolowsky, W.B. Campbell, F. DeClerck, I. Tritto and M. Chiariello, 1994, Modulation of platelet function by reactive oxygen metabolites, Am J Physiol, 267, H308-18.

Ambrosioni, E., C. Borghi and B. Magnani, 1995, The effect of the angiotensin-converting-enzyme inhibitor zofenopril on mortality and morbidity after anterior myocardial infarction. The Survival of Myocardial Infarction Long-Term Evaluation (SMILE) Study Investigators, N Engl J Med, 332, 80-5.

Amin-Hanjani, S., N.E. Stagliano, M. Yamada, P.L. Huang, J.K. Liao and M.A. Moskowitz, 2001, Mevastatin, an HMG-CoA reductase inhibitor, reduces stroke damage and upregulates endothelial nitric oxide synthase in mice, Stroke, 32, 980-6.

Anderson, H.V., R.L. Kirkeeide, A. Krishnaswami, L.A. Weigelt, M. Revana, H.F. Weisman and J.T. Willerson, 1994a, Cyclic flow variations after coronary angioplasty in humans: clinical and angiographic characteristics and elimination with 7E3 monoclonal antiplatelet antibody, J Am Coll Cardiol, 23, 1031-7.

Anderson, H.V., M. Revana, O. Rosales, L. Brannigan, Y. Stuart, H. Weisman and J.T. Willerson, 1992, Intravenous administration of monoclonal antibody to the platelet GP IIb/IIIa receptor to treat abrupt closure during coronary angioplasty, Am J Cardiol, 69, 1373-6.

Anderson, T.J., 1999, Assessment and treatment of endothelial dysfunction in humans, J Am Coll Cardiol, 34, 631-8.

Anderson, T.J., E. Elstein, H. Haber and F. Charbonneau, 2000, Comparative study of ACE-inhibition, angiotensin II antagonism, and calcium channel blockade on flow-mediated vasodilation in patients with coronary disease (BANFF study), J Am Coll Cardiol, 35, 60-6.

Anderson, T.J., I.T. Meredith, P. Ganz, A.P. Selwyn and A.C. Yeung, 1994b, Nitric oxide and nitrovasodilators: similarities, differences and potential interactions, J Am Coll Cardiol, 24, 555-66.

Anderson, T.J., I.T. Meredith, A.C. Yeung, B. Frei, A.P. Selwyn and P. Ganz, 1995a, The effect of cholesterol-lowering and antioxidant therapy on endothelium-dependent coronary vasomotion, N Engl J Med, 332, 488-93.

Anderson, T.J., A. Uehata, M.D. Gerhard, I.T. Meredith, S. Knab, D. Delagrange, E.H. Lieberman, P. Ganz, M.A. Creager and A.C. Yeung., 1995b, Close relation of endothelial function in the human coronary and peripheral circulations, J Am Coll Cardiol, 26, 1235-41.

Andersson, T.L., J. Matz, G.A. Ferns and E.E. Anggard, 1994, Vitamin E reverses cholesterol-induced endothelial dysfunction in the rabbit coronary circulation, Atherosclerosis, 111, 39-45.

Andrews, N.P., D.S. Goldstein and A.A. Quyyumi, 1999a, Effect of systemic alpha-2 adrenergic blockade on the morning increase in platelet aggregation in normal subjects, Am J Cardiol, 84, 316-20.

Andrews, R., J.A. May, J. Vickers and S. Heptinstall, 1994, Inhibition of platelet aggregation by transdermal glyceryl trinitrate, Br Heart J, 72, 575-9.

Andrews, R.K., Y. Shen, E.E. Gardiner, J.F. Dong, J.A. Lopez and M.C. Berndt, 1999b, The glycoprotein Ib-IX-V complex in platelet adhesion and signaling, Thromb Haemost, 82, 357-64.

Andrews, T.C., K. Raby, J. Barry, C.L. Naimi, E. Allred, P. Ganz and A.P. Selwyn, 1997, Effect of cholesterol reduction on myocardial ischemia in patients with coronary disease, Circulation, 95, 324-8.

Anfossi, G., P. Massucco, E. Mularoni, F. Cavalot, L. Mattiello and M. Trovati, 1993, Organic nitrates and compounds that increase intraplatelet cyclic guanosine monophosphate (cGMP) levels enhance the antiaggregating effects of the stable prostacyclin analogue iloprost, Prostaglandins Leukot Essent Fatty Acids, 49, 839-45.

Anfossi, G., E.M. Mularoni, S. Burzacca, M.C. Ponziani, P. Massucco, L. Mattiello, F. Cavalot and M. Trovati, 1998, Platelet resistance to nitrates in obesity and obese NIDDM, and normal platelet sensitivity to both insulin and nitrates in lean NIDDM, Diabetes Care, 21, 121-6.

Antiplatelet Trialists' Collaboration., 1988, Secondary prevention of vascular disease by prolonged antiplatelet treatment, Br Med J (Clin Res Ed), 296, 320-31.

Antiplatelet Trialists' Collaboration., 1994, Collaborative overview of randomised trials of antiplatelet therapy-II: Maintenance of vascular graft or arterial patency by antiplatelet therapy, BMJ, 308, 159-68.

Antony, I., G. Lerebours and A. Nitenberg, 1996, Angiotensin-converting enzyme inhibition restores flow-dependent and cold pressor test-induced dilations in coronary arteries of hypertensive patients, Circulation, 94, 3115-22.

Anversa, P., W. Cheng, Y. Liu, A. Leri, G. Redaelli and J. Kajstura, 1998, Apoptosis and myocardial infarction, Basic Res Cardiol, 93 Suppl 3, 8-12.

Aoki, H., M. Inoue, T. Mizobe, M. Harada, H. Imai and A. Kobayashi, 1997a, Platelet function is inhibited by nitric oxide liberation during nitroglycerin-induced hypotension anaesthesia, Br J Anaesth, 79, 476-81.

Aoki, I., N. Aoki, K. Kawano, K. Shimoyama, A. Maki, M. Homori, A. Yanagisawa, M. Yamamoto, Y. Kawai and K. Ishikawa, 1997b, Platelet-dependent thrombin generation in patients with hyperlipidemia, J Am Coll Cardiol, 30, 91-6.

Arbustini, E., B. Dal Bello, P. Morbini, A.P. Burke, M. Bocciarelli, G. Specchia and R. Virmani, 1999, Plaque erosion is a major substrate for coronary thrombosis in acute myocardial infarction, Heart, 82, 269-72.

Armstrong, M.L., D. Brand, A.J. Emmett, J.L. Hodge, G.S. Kellaway, P. Mestitz, M. Reefman and D.C. Wallace, 1974, A multicentre trial of perhexiline maleate, beta-blocker and placebo in angina pectoris, Med J Aust, 2, 389-93.

Armstrong, P.W., J.A. Armstrong and G.S. Marks, 1980, Pharmacokinetic-hemodynamic studies of intravenous nitroglycerin in congestive cardiac failure, Circulation, 62, 160-6.

Armstrong, R.A., 1996, Platelet prostanoid receptors, Pharmacol Ther, 72, 171-91.

Arnelle, D.R. and J.S. Stamler, 1995, NO^+, NO, and NO^- donation by S-nitrosothiols: implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation, Arch Biochem Biophys, 318, 279-85.

Arstall, M.A., J. Yang, I. Stafford, W.H. Betts and J.D. Horowitz, 1995, N-acetylcysteine in combination with nitroglycerin and streptokinase for the treatment of evolving acute myocardial infarction. Safety and biochemical effects, Circulation, 92, 2855-62.

Aruoma, O.I., B. Halliwell, B.M. Hoey and J. Butler, 1989, The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid, Free Radic Biol Med, 6, 593-7.

Ascherio, A., E.B. Rimm, M.A. Hernan, E. Giovannucci, I. Kawachi, M.J. Stampfer and W.C. Willett, 1999, Relation of consumption of vitamin E, vitamin C, and carotenoids to risk for stroke among men in the United States, Ann Intern Med, 130, 963-70.

Aslan, M., T.M. Ryan, B. Alder, T.M. Townes, D.A. Parks, J.A. Thompson, A. Tousson, M.T. Gladwin, R.P. Patel, M.M. Tarpey, I. Batinic-Haberie, C.R. White and B.A. Freeman, 2001, Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease, Proc Natl Acad Sci USA, 98, 15215-20.

Asmar, R., 1999, Arterial stiffness and pulse wave velocity (Elsevier, Amsterdam).

Aszodi, A., A. Pfeifer, M. Ahmad, M. Glauner, X.H. Zhou, L. Ny, K.E. Andersson, B. Kehrel, S. Offermanns and R. Fassler, 1999, The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function, Embo J, 18, 37-48.

Atherton, J.J., H. Thomson, T.D. Moore, K.N. Wright, G.W.F. Muehle, L.E. Fitzpatrick and M.P. Frenneaux, 1997, Diastolic ventricular interaction. A possible mechanism for abnormal vascular responses during volume unloading in heart failure, Circulation, 96, 4273-4279.

Ault, K.A., C.P. Cannon, J. Mitchell, J. McCahan, R.P. Tracy, W.F. Novotny, J.D. Reimann and E. Braunwald, 1999, Platelet activation in patients after an acute coronary syndrome: results from the TIMI-12 trial. Thrombolysis in Myocardial Infarction, J Am Coll Cardiol, 33, 634-9.

Aviram, M., 1989, Modified forms of low density lipoprotein affect platelet aggregation *in vitro*, Thromb Res, 53, 561-7.

Aviram, M., B. Fuhrman, S. Keidar, I. Maor, M. Rosenblat, G. Dankner and G. Brook, 1989, Platelet-modified low density lipoprotein induces macrophage cholesterol accumulation and platelet activation, J Clin Chem Clin Biochem, 27, 3-12.

Axelsson, K.L. and R.G. Andersson, 1983, Tolerance towards nitroglycerin, induced *in vivo*, is correlated to a reduced cGMP response and an alteration in cGMP turnover, Eur J Pharmacol, 88, 71-9.

Babior, B.M., 1999, NADPH oxidase: an update, Blood, 93, 1464-76.

Badimon, J.J., L. Badimon, V.T. Turitto and V. Fuster, 1991, Platelet deposition at high shear rates is enhanced by high plasma cholesterol levels. *In vivo* study in the rabbit model, Arterioscler Thromb, 11, 395-402.

Badimon, L., V. Turitto, J.A. Rosemark, J.J. Badimon and V. Fuster, 1987, Characterization of a tubular flow chamber for studying platelet interaction with biologic and prosthetic materials: deposition of indium 111-labeled platelets on collagen, subendothelium, and expanded polytetrafluoroethylene, J Lab Clin Med, 110, 706-18.

Baigent, C., R. Collins, P. Appleby, S. Parish, P. Sleight and R. Peto, 1998, ISIS-2: 10 year survival among patients with suspected acute myocardial infarction in randomised comparison of intravenous streptokinase, oral aspirin, both, or neither. The ISIS-2 (Second International Study of Infarct Survival) Collaborative Group, BMJ, 316, 1337-43.

Baines, C.P., R.S. Szwarc and H.A. Ball, 1994, Parallel tolerance between platelet cyclic GMP and preload effects of nitroglycerin in anaesthetized mini-pigs, Br J Pharmacol, 113, 334-5.

Barath, P., M.C. Fishbein, J. Cao, J. Berenson, R.H. Helfant and J.S. Forrester, 1990, Detection and localization of tumor necrosis factor in human atheroma, Am J Cardiol, 65, 297-302.

Barnard, E.A. and J. Simon, 2001, An elusive receptor is finally caught: $P2Y_{12}$, an important drug target in platelets, Trends Pharmacol Sci, 22, 388-91.

Barrett, J.S., G. Murphy, K. Peerlinck, I. De Lepeleire, R.J. Gould, D. Panebianco, E. Hand, H. Deckmyn, J. Vermylen and J. Arnout, 1994, Pharmacokinetics and pharmacodynamics of MK-383, a selective non-peptide platelet glycoprotein-IIb/IIIa receptor antagonist, in healthy men, Clin Pharmacol Ther, 56, 377-88.

Barry, W.H., J.D. Horowitz and T.W. Smith, 1985, Comparison of negative inotropic potency, reversibility, and effects on calcium influx of six calcium channel antagonists in cultured myocardial cells, Br J Pharmacol, 85, 51-9.

Bass, D.A., P. Olbrantz, P. Szejda, M.C. Seeds and C.E. McCall, 1986, Subpopulations of neutrophils with increased oxidative product formation in blood of patients with infection, J Immunol, 136, 860-6.

Bassenge, E. and B. Fink, 1996, Tolerance to nitrates and simultaneous upregulation of platelet activity prevented by enhancing antioxidant state, Naunyn Schmiedebergs Arch Pharmacol, 353, 363-7.

Bassenge, E., N. Fink, M. Skatchkov and B. Fink, 1998, Dietary supplement with vitamin C prevents nitrate tolerance, J Clin Invest, 102, 67-71.

Bassenge, E. and D.J. Stewart, 1986, Effects of nitrates in various vascular sections and regions, Z Kardiol, 75 Suppl 3, 1-7.

Bassenge, E. and J. Zanzinger, 1992, Nitrates in different vascular beds, nitrate tolerance, and interactions with endothelial function, Am J Cardiol, 70, 23B-29B.

Bath, P.M., R. Pathansali, R. Iddenden and F.J. Bath, 2001, The effect of transdermal glyceryl trinitrate, a nitric oxide donor, on blood pressure and platelet function in acute stroke, Cerebrovasc Dis, 11, 265-72.

Bauer, J.A. and H.L. Fung, 1991, Differential hemodynamic effects and tolerance properties of nitroglycerin and an S-nitrosothiol in experimental heart failure, J Pharmacol Exp Ther, 256, 249-54.

Bauersachs, J., A. Bouloumie, D. Fraccarollo, K. Hu, R. Busse and G. Ertl, 1999, Endothelial dysfunction in chronic myocardial infarction despite increased vascular endothelial nitric oxide synthase and soluble guanylate cyclase expression: role of enhanced vascular superoxide production, Circulation, 100, 292-8.

Bayer, B.L., K.E. Blass and W. Forster, 1979, Anti-aggregatory effect of prostacyclin (PGI_2) *in vivo*, Br J Pharmacol, 66, 10-2.

Bayraktutan, U., L. Blayney and A.M. Shah, 2000, Molecular characterization and localization of the NAD(P)H oxidase components $gp91^{phox}$ and $p22^{phox}$ in endothelial cells, Arterioscler Thromb Vasc Biol, 20, 1903-11.

Beaughard, M., M. Brasset, G. John and R. Massingham, 1995, Failure of calcium channel blockade to reduce platelet-mediated cyclic flow variations in dogs with coronary stenosis and endothelial injury, J Cardiovasc Pharmacol, 26, 577-83.

Becker, R.C., E.G. Bovill, J.M. Corrao, S.P. Ball, K. Ault, K. Mann and R.P. Tracy, 1994, Platelet Activation Determined by Flow Cytometry Persists Despite Antithrombotic Therapy in Patients with Unstable Angina and Non-Q-Wave Myocardial Infarction, J Thromb Thrombolysis, 1, 95-100.

Bellosta, S., D. Via, M. Canavesi, P. Pfister, R. Fumagalli, R. Paoletti and F. Bernini, 1998, HMG-CoA reductase inhibitors reduce MMP-9 secretion by macrophages, Arterioscler Thromb Vasc Biol, 18, 1671-8.

Bendall, J.K., A.C. Cave, C. Heymes, N. Gall and A.M. Shah, 2002, Pivotal role of a $gp91^{phox}$-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice, Circulation, 105, 293-6.

Bennett, B.M., D.C. Leitman, H. Schroder, J.H. Kawamoto, K. Nakatsu and F. Murad, 1989, Relationship between biotransformation of glyceryl trinitrate and cyclic GMP accumulation in various cultured cell lines, J Pharmacol Exp Ther, 250, 316-23.

Bennett, B.M., B.J. McDonald, R. Nigam and W.C. Simon, 1994, Biotransformation of organic nitrates and vascular smooth muscle cell function, Trends Pharmacol Sci, 15, 245-9.

Bennett, B.M., H. Schroder, L.D. Hayward, S.A. Waldman and F. Murad, 1988, Effect of *in vitro* organic nitrate tolerance on relaxation, cyclic GMP accumulation, and guanylate cyclase activation by glyceryl trinitrate and the enantiomers of isoidide dinitrate, Circ Res, 63, 693-701.

Bennett, C.L., J.M. Connors, J.M. Carwile, J.L. Moake, W.R. Bell, S.R. Tarantolo, L.J. McCarthy, R. Sarode, A.J. Hatfield, M.D. Feldman, C.J. Davidson, H.M. Tsai and E.L. Michalets, 2000, Thrombotic Thrombocytopenic Purpura Associated with Clopidogrel, N Engl J Med, 342, 1773-1777.

Bennett, C.L., P.D. Weinberg, K. Rozenberg-Ben-Dror, P.R. Yarnold, H.C. Kwaan and D. Green, 1998, Thrombotic thrombocytopenic purpura associated with ticlopidine. A review of 60 cases, Ann Intern Med, 128, 541-4.

Benrahmoune, M., P. Therond and Z. Abedinzadeh, 2000, The reaction of superoxide radical with N-acetylcysteine, Free Radic Biol Med, 29, 775-82.

Berger, G., J.M. Masse and E.M. Cramer, 1996, Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX, and V, Blood, 87, 1385-95.

Berkels, R., A. Bertsch, T. Zuther, S. Dhein, K. Stockklauser, P. Rosen and R. Rosen, 1997, Evidence for a NO synthase in porcine platelets which is stimulated during activation/aggregation, Eur J Haematol, 58, 307-13.

Berkenboom, G., D. Fontaine, P. Unger, S. Baldassarre, N. Preumont and J. Fontaine, 1999, Absence of nitrate tolerance after long-term treatment with ramipril: an endothelium-dependent mechanism, J Cardiovasc Pharmacol, 34, 547-53.

Berkenboom, G., P. Unger and J. Fontaine, 1989, Atherosclerosis and responses of human isolated coronary arteries to endothelium-dependent and -independent vasodilators, J Cardiovasc Pharmacol, 14 Suppl 11, S35-9.

Berliner, J.A., M. Navab, A.M. Fogelman, J.S. Frank, L.L. Demer, P.A. Edwards, A.D. Watson and A.J. Lusis, 1995, Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics, Circulation, 91, 2488-96.

Bertrand, M.E., V. Legrand, J. Boland, E. Fleck, J. Bonnier, H. Emmanuelson, M. Vrolix, L. Missault, S. Chierchia, M. Casaccia, L. Niccoli, A. Oto, C. White, M. Webb-Peploe, E. Van Belle and E.P. McFadden, 1998, Randomized multicenter comparison of conventional anticoagulation versus antiplatelet therapy in unplanned and elective coronary stenting. The full anticoagulation versus aspirin and ticlopidine (FANTASTIC) study, Circulation, 98, 1597-603.

Beyer, W., J. Imlay and I. Fridovich, 1991, Superoxide dismutases, Prog Nucleic Acid Res Mol Biol, 40, 221-53.

Bhatt, D.L., S.P. Marso, A.M. Lincoff, K.E. Wolski, S.G. Ellis and E.J. Topol, 2000, Abciximab reduces mortality in diabetics following percutaneous coronary intervention, J Am Coll Cardiol, 35, 922-8.

Bhunia, A.K., H. Han, A. Snowden and S. Chatterjee, 1997, Redox-regulated signaling by lactosylceramide in the proliferation of human aortic smooth muscle cells, J Biol Chem, 272, 15642-9.

Billah, M.M., E.G. Lapetina and P. Cuatrecasas, 1980, Phospholipase A_2 and phospholipase C activities of platelets. Differential substrate specificity, Ca^{2+} requirement, pH dependence, and cellular localization, J Biol Chem, 255, 10227-31.

Birkebaek, N.H., H. Vejby-Christensen, P. Jakobsen and K. Winther, 1988, The effect of nifedipine and captopril on platelet activation and prostanoid production in essential hypertension, J Hypertens Suppl, 6, S378-80.

Bjorkman, D.J., 1998, The effect of aspirin and nonsteroidal anti-inflammatory drugs on prostaglandins, Am J Med, 105, 8S-12S.

Blann, A.D., G.Y. Lip and R. Fijnheer, 1999, Significance of soluble P-selectin, von Willebrand factor, and other adhesion molecules in hypercholesterolemia and peripheral artery disease, Circulation, 99, 2478-9.

Blann, A.D., C. Steele and C.N. McCollum, 1997, The influence of smoking on soluble adhesion molecules and endothelial cell markers, Thromb Res, 85, 433-8.

Blann, A.D. and M.A. Waite, 1996, von Willebrand factor and soluble E-selectin in hypertension: influence of treatment and value in predicting the progression of atherosclerosis, Coron Artery Dis, 7, 143-7.

Blockmans, D., H. Deckmyn and J. Vermylen, 1995, Platelet activation, Blood Rev, 9, 143-56.

Blum, A., L. Hathaway, R. Mincemoyer, W.H. Schenke, M. Kirby, G. Csako, M.A. Waclawiw, J.A. Panza and R.O. Cannon, 3rd, 2000, Oral L-arginine in patients with coronary artery disease on medical management, Circulation, 101, 2160-4.

Boden, W.E., R.J. Krone, R.E. Kleiger, D. Oakes, H. Greenberg, E.J. Dwyer, Jr., J.P. Miller, J. Abrams, J. Coromilas and R. Goldstein, 1991, Electrocardiographic subset analysis of diltiazem administration on long-term outcome after acute myocardial infarction. The Multicenter Diltiazem Post-Infarction Trial Research Group, Am J Cardiol, 67, 335-42.

Boden, W.E., W.H. van Gilst, R.G. Scheldewaert, I.R. Starkey, M.F. Carlier, D.G. Julian, A. Whitehead, M.E. Bertrand, J.J. Col, O.L. Pedersen, K.I. Lie, J.P. Santoni and K.M. Fox, 2000, Diltiazem in acute myocardial infarction treated with thrombolytic agents: a randomised placebo-controlled trial. Incomplete Infarction Trial of European Research Collaborators Evaluating Prognosis post-Thrombolysis (INTERCEPT), Lancet, 355 (9217), 1751-6.

Body, S.C., 1996, Platelet activation and interactions with the microvasculature, J Cardiovasc Pharmacol, 27 Suppl 1, S13-25.

Boesgaard, S., J. Aldershvile and H.E. Poulsen, 1992, Preventive administration of intravenous N-acetylcysteine and development of tolerance to isosorbide dinitrate in patients with angina pectoris, Circulation, 85, 143-9.

Boesgaard, S., J. Aldershvile, H.E. Poulsen, S. Christensen, H. Dige-Petersen and J. Giese, 1993a, N-acetylcysteine inhibits angiotensin converting enzyme *in vivo*, J Pharmacol Exp Ther, 265, 1239-44.

Boesgaard, S., J. Aldershvile, H.E. Poulsen, S. Loft, M.E. Anderson and A. Meister, 1994, Nitrate tolerance *in vivo* is not associated with depletion of arterial or venous thiol levels, Circ Res, 74, 115-20.

Boesgaard, S., H.E. Poulsen, J. Aldershvile, S. Loft, M.E. Anderson and A. Meister, 1993b, Acute effects of nitroglycerin depend on both plasma and intracellular sulfhydryl compound levels *in vivo*. Effect of agents with different sulfhydryl-modulating properties, Circulation, 87, 547-53.

Boger, R.H., S.M. Bode-Boger, A. Szuba, P.S. Tsao, J.R. Chan, O. Tangphao, T.F. Blaschke and J.P. Cooke, 1998, Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia, Circulation, 98, 1842-7.

Boger, R.H., S.M. Bode-Boger, P.S. Tsao, P.S. Lin, J.R. Chan and J.P. Cooke, 2000, An endogenous inhibitor of nitric oxide synthase regulates endothelial adhesiveness for monocytes, J Am Coll Cardiol, 36, 2287-95.

Booth, B.P., S. Jacob, J.A. Bauer and H.L. Fung, 1996, Sustained antiplatelet properties of nitroglycerin during hemodynamic tolerance in rats, J Cardiovasc Pharmacol, 28, 432-8.

Born, G.V.R., 1962, Aggregation of blood platelets by adenosine diphosphate and its reversal, Nature, 194, 27-29.

Boulos, C., H. Jiang and M. Balazy, 2000, Diffusion of peroxynitrite into the human platelet inhibits cyclooxygenase via nitration of tyrosine residues, J Pharmacol Exp Ther, 293, 222-9.

Bouloumie, A., J. Bauersachs, W. Linz, B.A. Scholkens, G. Wiemer, I. Fleming and R. Busse, 1997, Endothelial dysfunction coincides with an enhanced nitric oxide synthase expression and superoxide anion production, Hypertension, 30, 934-41.

Bramwell, J.C. and A.V. Hill, 1922, Velocity of transmission of the pulse and elasticity of arteries, Lancet, 1 (3250), 891-892.

Brands, M.W. and S.M. Fitzgerald, 1998, Acute endothelium-mediated vasodilation is not impaired at the onset of diabetes, Hypertension, 32, 541-7.

Brar, S.S., T.P. Kennedy, A.B. Sturrock, T.P. Huecksteadt, M.T. Quinn, T.M. Murphy, P. Chitano and J.R. Hoidal, 2002, NADPH oxidase promotes NF-kappaB activation and proliferation in human airway smooth muscle, Am J Physiol Lung Cell Mol Physiol, 282, L782-95.

Brass, L.F., J.A. Hoxie and D.R. Manning, 1993, Signaling through G proteins and G protein-coupled receptors during platelet activation, Thromb Haemost, 70, 217-23.

Brass, L.F., C.C. Shaller and E.J. Belmonte, 1987, Inositol 1,4,5-triphosphate-induced granule secretion in platelets. Evidence that the activation of phospholipase C mediated by platelet thromboxane receptors involves a guanine nucleotide binding protein-dependent mechanism distinct from that of thrombin, J Clin Invest, 79, 1269-75.

Bredt, D.S., 1999, Endogenous nitric oxide synthesis: biological functions and pathophysiology, Free Radic Res, 31, 577-96.

Bredt, D.S., P.M. Hwang, C.E. Glatt, C. Lowenstein, R.R. Reed and S.H. Snyder, 1991, Cloned and expressed nitric oxide synthase structurally resembles cytochrome P_{450} reductase, Nature, 351, 714-8.

Brezinski, D.A., G.H. Tofler, J.E. Muller, S. Pohjola-Sintonen, S.N. Willich, A.I. Schafer, C.A. Czeisler and G.H. Williams, 1988, Morning increase in platelet aggregability. Association with assumption of the upright posture, Circulation, 78, 35-40.
Brien, J.F., B.E. McLaughlin, T.H. Breedon, B.M. Bennett, K. Nakatsu and G.S. Marks, 1986, Biotransformation of glyceryl trinitrate occurs concurrently with relaxation of rabbit aorta, J Pharmacol Exp Ther, 237, 608-14.

Brien, J.F., B.E. McLaughlin, S.M. Kobus, J.H. Kawamoto, K. Nakatsu and G.S. Marks, 1988, Mechanism of glyceryl trinitrate-induced vasodilation. I. Relationship between drug biotransformation, tissue cyclic GMP elevation and relaxation of rabbit aorta, J Pharmacol Exp Ther, 244, 322-7.

Briheim, G., O. Stendahl and C. Dahlgren, 1984, Intra- and extracellular events in luminol-dependent chemiluminescence of polymorphonuclear leukocytes, Infect Immun, 45, 1-5.

Britten, M.B., A.M. Zeiher and V. Schachinger, 1999, Clinical importance of coronary endothelial vasodilator dysfunction and therapeutic options, J Intern Med, 245, 315-27.

Brown, A.S., M.A. Moro, J.M. Masse, E.M. Cramer, M. Radomski and V. Darley-Usmar, 1998, Nitric oxide-dependent and independent effects on human platelets treated with peroxynitrite, Cardiovasc Res, 40, 380-8.

Brown, B.G., E. Bolson, R.B. Petersen, C.D. Pierce and H.T. Dodge, 1981, The mechanisms of nitroglycerin action: stenosis vasodilatation as a major component of the drug response, Circulation, 64, 1089-97.

Brown, M.J., J.D. Horowitz and M.L. Mashford, 1976, A double-blind trial of perhexiline maleate in the prophylaxis of angina pectoris, Med J Aust, 1, 260-3.

Bruemmer, D., U. Riggers, J. Holzmeister, M. Grill, F. Lippek, U. Settmacher, V. Regitz-Zagrosek, E. Fleck and K. Graf, 2001, Expression of CD40 in vascular smooth muscle cells and macrophages is associated with early development of human atherosclerotic lesions, Am J Cardiol, 87, 21-7.

Brune, B. and E.G. Lapetina, 1989, Activation of a cytosolic ADP-ribosyltransferase by nitric oxide-generating agents, J Biol Chem, 264, 8455-8.

Bruning, T.A., P.C. Chang, M.J. Kemme, P. Vermeij, M. Pfaffendorf and P.A. van Zwieten, 1996, Comparison of cholinergic vasodilator responses to acetylcholine and methacholine in the human forearm, Blood Press, 5, 333-41.

Brunton, T.L., 1867, On the use of nitrite of amyl in angina pectoris, Lancet, 2, 97-98.

Buczynski, A., B. Wachowicz, K. Kedziora-Kornatowska, W. Tkaczewski and J. Kedziora, 1993, Changes in antioxidant enzymes activities, aggregability and malonyldialdehyde concentration in blood platelets from patients with coronary heart disease, Atherosclerosis, 100, 223-8.

Buffon, A., G. Liuzzo, L.M. Biasucci, P. Pasqualetti, V. Ramazzotti, A.G. Rebuzzi, F. Crea and A. Maseri, 1999, Preprocedural serum levels of C-reactive protein predict early complications and late restenosis after coronary angioplasty, J Am Coll Cardiol, 34, 1512-21.

Buffon, A., L.M. Biasucci, G. Liuzzo, G. D'Onofrio, F. Crea and A. Maseri, 2002, Widespread coronary inflammation in unstable angina, N Engl J Med, 347, 5-12.

Bult, H., J.M. Bosmans, C.J. Vrints and A.G. Herman, 1995, Isosorbide-dinitrate and SIN-1 as dilators of human coronary arteries and platelet inhibitors, J Cardiovasc Pharmacol, 25, 572-8.

Busse, R. and A. Mulsch, 1990, Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin, FEBS Lett, 265, 133-6.

Butler, A.R., F.W. Flitney and D.L. Williams, 1995, NO, nitrosonium ions, nitroxide ions, nitrosothiols and iron-nitrosyls in biology: a chemist's perspective, Trends Pharmacol Sci, 16, 18-22.

Butler, R., A.D. Morris, J.J. Belch, A. Hill and A.D. Struthers, 2000, Allopurinol normalizes endothelial dysfunction in type 2 diabetics with mild hypertension, Hypertension, 35, 746-51.

Butt, E., K. Abel, M. Krieger, D. Palm, V. Hoppe, J. Hoppe and U. Walter, 1994, cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) *in vitro* and in intact human platelets, J Biol Chem, 269, 14509-17.

Cabassi, A., E.C. Dumont, H. Girouard, J.F. Bouchard, M. Le Jossec, D. Lamontagne, J.G. Besner and J. de Champlain, 2001, Effects of chronic N-acetylcysteine treatment on the actions of peroxynitrite on aortic vascular reactivity in hypertensive rats, J Hypertens, 19, 1233-44.

Caccese, D., D. Pratico, A. Ghiselli, S. Natoli, P. Pignatelli, V. Sanguigni, L. Iuliano and F. Violi, 2000, Superoxide anion and hydroxyl radical release by collagen-induced platelet aggregation -role of arachidonic acid metabolism, Thromb Haemost, 83, 485-90.

Cahilly, C., C.M. Ballantyne, D.S. Lim, A. Gotto and A.J. Marian, 2000, A variant of $p22^{phox}$, involved in generation of reactive oxygen species in the vessel wall, is associated with progression of coronary atherosclerosis, Circ Res, 86, 391-5.

Cai, H., N. Duarte, D.E. Wilcken and X.L. Wang, 1999, NADH/NADPH oxidase $p22^{phox}$ C242T polymorphism and coronary artery disease in the Australian population, Eur J Clin Invest, 29, 744-8.

Cai, H. and D.G. Harrison, 2000, Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress, Circ Res, 87, 840-4.

Cairns, J.A., M. Gent, J. Singer, K.J. Finnie, G.M. Froggatt, D.A. Holder, G. Jablonsky, W.J. Kostuk, L.J. Melendez and M.G. Myers, 1985, Aspirin, sulfinpyrazone, or both in unstable angina. Results of a Canadian multicenter trial, N Engl J Med, 313, 1369-75.

Caldefie-Chezet, F., S. Walrand, C. Moinard, A. Tridon, J. Chassagne and M.P. Vasson, 2002, Is the neutrophil radical oxygen species production measured by luminol and lucigenin chemiluminescence intra or extracellular? Comparison with DCFH-DA flow cytometry and cytochrome c reduction, Clinica Chimica Acta, Article in press.

Calver, A., J. Collier, A. Leone, S. Moncada and P. Vallance, 1993, Effect of local intra-arterial asymmetric dimethylarginine (ADMA) on the forearm arteriolar bed of healthy volunteers, J Hum Hypertens, 7, 193-4.

Cameron, J.D. and A.M. Dart, 1994, Exercise training increases total systemic arterial compliance in humans, Am J Physiol, 266, H693-701.

Cameron, J.D., G.L. Jennings and A.M. Dart, 1996, Systemic arterial compliance is decreased in newly-diagnosed patients with coronary heart disease: implications for prediction of risk, J Cardiovasc Risk, 3, 495-500.

Cameron, J.D., B.P. McGrath and A.M. Dart, 1998, Use of radial artery applanation tonometry and a generalized transfer function to determine aortic pressure augmentation in subjects with treated hypertension, J Am Coll Cardiol, 32, 1214-20.

Campeau, L., 1976, Letter: grading of angina pectoris, Circulation, 54, 522-3.

Cannon, C.P., C.H. McCabe, R.G. Wilcox, A. Langer, A. Caspi, P. Berink, J. Lopez-Sendon, J. Toman, A. Charlesworth, R.J. Anders, J.C. Alexander, A. Skene and E. Braunwald, 2000, Oral glycoprotein IIb/IIIa inhibition with orbofiban in patients with unstable coronary syndromes (OPUS-TIMI 16) trial, Circulation, 102, 149-56.

Cardillo, C., C.M. Kilcoyne, R.O. Cannon, 3rd and J.A. Panza, 1998, Racial differences in nitric oxide-mediated vasodilator response to mental stress in the forearm circulation, Hypertension, 31, 1235-9.

Cardillo, C., C.M. Kilcoyne, R.O. Cannon and J.A. Panza, 1999, Attenuation of cyclic nucleotide-mediated smooth muscle relaxation in blacks as a cause of racial differences in vasodilator function, Circulation, 99, 90-5.

Cardillo, C., C.M. Kilcoyne, R.O. Cannon, 3rd, A.A. Quyyumi and J.A. Panza, 1997, Xanthine oxidase inhibition with oxypurinol improves endothelial vasodilator function in hypercholesterolemic but not in hypertensive patients, Hypertension, 30, 57-63.

Cardinal, D.C. and R.J. Flower, 1980, The electronic aggregometer: a novel device for assessing platelet behavior in blood, J Pharmacol Methods, 3, 135-58.

Carmo, L.G., M. Hatmi, D. Rotilio and B.B. Vargaftig, 1985, Platelet desensitization induced by arachidonic acid is not due to cyclo-oxygenase inactivation and involves the endoperoxide receptor, Br J Pharmacol, 85, 849-59.

Carulli, G., S. Minnucci, M.L. Gianfaldoni, C. Angiolini, A. Azzara and F. Ambrogi, 1995, Interactions between platelets and neutrophils in essential thrombocythaemia. Effects on neutrophil chemiluminescence and superoxide anion generation, Eur J Clin Invest, 25, 929-34.

Carvalho, A.C., R.W. Colman and R.S. Lees, 1974, Platelet function in hyperlipoproteinemia, N Engl J Med, 290, 434-8.

Catella-Lawson, F. and L.J. Crofford, 2001, Cyclooxygenase inhibition and thrombogenicity, Am J Med, 110 Suppl 3A, 28S-32S.

Cattaneo, M., B. Akkawat, A. Lecchi, C. Cimminiello, A.M. Capitanio and P.M. Mannucci, 1991, Ticlopidine selectively inhibits human platelet responses to adenosine diphosphate, Thromb Haemost, 66, 694-9.

Cattaneo, M. and C. Gachet, 2001, The platelet ADP receptors, Haematologica, 86, 346-8.

Celermajer, D.S., K.E. Sorensen, C. Bull, J. Robinson and J.E. Deanfield, 1994, Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction, J Am Coll Cardiol, 24, 1468-74.

Celermajer, D.S., K.E. Sorensen, D. Georgakopoulos, C. Bull, O. Thomas, J. Robinson and J.E. Deanfield, 1993, Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults, Circulation, 88, 2149-55.

Celermajer, D.S., K.E. Sorensen, V.M. Gooch, D.J. Spiegelhalter, O.I. Miller, I.D. Sullivan, J.K. Lloyd and J.E. Deanfield, 1992, Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis, Lancet, 340 (8828), 1111-5.

Chakhtoura, E.Y., F.E. Shamoon, J.I. Haft, G.R. Obiedzinski, A.J. Cohen and R.M. Watson, 2000, Comparison of platelet activation in unstable and stable angina pectoris and correlation with coronary angiographic findings, Am J Cardiol, 86, 835-839.

Chan, A.W. and D.J. Moliterno, 2001, Defining the role of abciximab for acute coronary syndromes: lessons from CADILLAC, ADMIRAL, GUSTO IV, GUSTO V, and TARGET, Curr Opin Cardiol, 16, 375-83.

Chan, J.R., R.H. Boger, S.M. Bode-Boger, O. Tangphao, P.S. Tsao, T.F. Blaschke and J.P. Cooke, 2000, Asymmetric dimethylarginine increases mononuclear cell adhesiveness in hypercholesterolemic humans, Arterioscler Thromb Vasc Biol, 20, 1040-6.

Chang, J., J.H. Musser and H. McGregor, 1987, Phospholipase A_2: function and pharmacological regulation, Biochem Pharmacol, 36, 2429-36.

Charo, I.F., R.M. Scharborough, C.P. du Mee, D. Wolf, D.R. Phillips and R.L. Swift, 1992, Pharmacodynamics of the GPIIb-IIIa antagonist integrilin: Phase I clinical studies in normal healthy volunteers, Circulation, 86, I-260.

Chen, L.Y. and J.L. Mehta, 1996, Further evidence of the presence of constitutive and inducible nitric oxide synthase isoforms in human platelets, J Cardiovasc Pharmacol, 27, 154-8.

Chen, L.Y. and J.L. Mehta, 1997, Downregulation of nitric oxide synthase activity in human platelets by nitroglycerin and authentic nitric oxide, J Investig Med, 45, 69-74.

Chen, L.Y., P. Mehta and J.L. Mehta, 1996, Platelet inhibitory effect of nitroglycerin in platelet-rich plasma: relevance of glutathione-s-transferases in plasma, J Investig Med, 44, 561-5.

Chen, Z., J. Zhang and J.S. Stamler, 2002, Identification of the enzymatic mechanism of nitroglycerine biactivation, Proc Natl Acad Sci USA, 99, 8306-11.

Chew, D.P., D.L. Bhatt, S. Sapp and E.J. Topol, 2001, Increased Mortality With Oral Platelet Glycoprotein IIb/IIIa Antagonists : A Meta-Analysis of Phase III Multicenter Randomized Trials, Circulation, 103, 201-206.

Chew, D.P. and D.J. Moliterno, 2001, GP IIb/IIIa inhibitors in coronary artery disease management: what the latest trials tell us, Cleve Clin J Med, 68, 1017-23.

Chirico, S., C. Smith, C. Marchant, M.J. Mitchinson and B. Halliwell, 1993, Lipid peroxidation in hyperlipidaemic patients. A study of plasma using an HPLC-based thiobarbituric acid test, Free Radic Res Commun, 19, 51-7.

Chirkov, Y.Y., N.N. Belushkina, I.A. Tyshchuk, I.S. Severina and J.D. Horowitz, 1991, Increase in reactivity of human platelet guanylate cyclase during aggregation potentiates the disaggregating capacity of sodium nitroprusside, Clin Exp Pharmacol Physiol, 18, 517-24.

Chirkov, Y.Y., L.P. Chirkova and J.D. Horowitz, 1996, Suppressed anti-aggregating and cGMP-elevating effects of sodium nitroprusside in platelets from patients with stable angina pectoris, Naunyn Schmiedebergs Arch Pharmacol, 354, 520-5.

Chirkov, Y.Y., L.P. Chirkova and J.D. Horowitz, 1997, Nitroglycerin tolerance at the platelet level in patients with angina pectoris, Am J Cardiol, 80, 128-31.

Chirkov, Y.Y., L.P. Chirkova, R.E. Sage and J.D. Horowitz, 1995, Impaired responsiveness of platelets from patients with stable angina pectoris to antiaggregating and cyclicAMP-elevating effects of prostaglandin E_1, J Cardiovasc Pharmacol, 25, 961-6.

Chirkov, Y.Y., A.S. Holmes, L.P. Chirkova and J.D. Horowitz, 1999, Nitrate resistance in platelets from patients with stable angina pectoris, Circulation, 100, 129-34.

Chirkov, Y.Y., A.S. Holmes, S.R. Willoughby, S. Stewart, R.D.Wuttke, P.R. Sage, J.D. Horowitz, 2001, Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets, J Am Coll Cardiol, 37, 1851-7.

Chirkov, Y.Y. and J.D. Horowitz, 1996, N-Acetylcysteine potentiates nitroglycerin-induced reversal of platelet aggregation, J Cardiovasc Pharmacol, 28, 375-80.

Chirkov, Y.Y., J.I. Naujalis, S. Barber, R.E. Sage, D.W. Gove, J.K. Brealey and J.D. Horowitz, 1992, Reversal of human platelet aggregation by low concentrations of nitroglycerin *in vitro* in normal subjects, Am J Cardiol, 70, 802-6.

Chirkov, Y.Y., J.I. Naujalis, R.E. Sage and J.D. Horowitz, 1993, Antiplatelet effects of nitroglycerin in healthy subjects and in patients with stable angina pectoris, J Cardiovasc Pharmacol, 21, 384-9.

Chowienczyk, P.J., R.P. Kelly, H. MacCallum, S.C. Millasseau, T.L. Andersson, R.G. Gosling, J.M. Ritter and E.E. Anggard, 1999, Photoplethysmographic assessment of pulse wave reflection: blunted response to endothelium-dependent $beta_2$-adrenergic vasodilation in type II diabetes mellitus, J Am Coll Cardiol, 34, 2007-14.

Christiansen, N.O., 1988, A time-course study on superoxide generation and protein kinase C activation in human neutrophils, FEBS Lett, 239, 195-8.

Chrysant, S.G., S.P. Glasser, N. Bittar, F.E. Shahidi, K. Danisa, R. Ibrahim, L.E. Watts, R.J. Garutti, R. Ferraresi and R. Casareto, 1993, Efficacy and safety of extended-release isosorbide mononitrate for stable effort angina pectoris, Am J Cardiol, 72, 1249-56.

Chung, S.J., S. Chong, P. Seth, C.Y. Jung and H.L. Fung, 1992, Conversion of nitroglycerin to nitric oxide in microsomes of the bovine coronary artery smooth muscle is not primarily mediated by glutathione-S-transferases, J Pharmacol Exp Ther, 260, 652-9.

Cifuentes, M.E., F.E. Rey, O.A. Carretero and P.J. Pagano, 2000, Upregulation of $p67^{phox}$ and $gp91^{phox}$ in aortas from angiotensin II-infused mice, Am J Physiol Heart Circ Physiol, 279, H2234-40.

Cinar, M.G., S. Ulker, G. Alper and A. Evinc, 2001, Effect of dietary vitamin E supplementationon vascular reactivity of thoracic aorta in streptozotocin-diabetic rats, Pharmacology, 62, 56-64.

Clark, R.A. and S.J. Klebanoff, 1980, Neutrophil-platelet interaction mediated by myeloperoxidase and hydrogen peroxide, J Immunol, 124, 399-405.

Clarkson, P., D.S. Celermajer, A.E. Donald, M. Sampson, K.E. Sorensen, M. Adams, D.K. Yue, D.J. Betteridge and J.E. Deanfield, 1996, Impaired vascular reactivity in insulin-dependent diabetes mellitus is related to disease duration and low density lipoprotein cholesterol levels, J Am Coll Cardiol, 28, 573-9.

Clarkson, P., D.S. Celermajer, A.J. Powe, A.E. Donald, R.M. Henry and J.E. Deanfield, 1997, Endothelium-dependent dilatation is impaired in young healthy subjects with a family history of premature coronary disease, Circulation, 96, 3378-83.

Clarkson, P., M.J. Mullen, A.E. Donald, A.J. Powe, H. Thomson, S.A. Thorne, T. Bull and J.E. Deanfield, 2001, The effect of amlodipine on endothelial function in young adults with a strong family history of premature coronary artery disease: a randomised double blind study, Atherosclerosis, 154, 171-7.

Clemetson, K.J., 1997, Platelet GPIb-V-IX complex, Thromb Haemost, 78, 266-70.

Cleophas, T.J., M.G. Niemeyer, A.H. Zwinderman and E.E. van der Wall, 2000, Isosorbide mononitrate 30% immediate-release 70% sustained-release formulation: a review. DUMQOL (DUtch Mononitrate Quality of Life) Study Group, Angiology, 51, 631-8.

Cole, P.L., A.D. Beamer, N. McGowan, C.O. Cantillon, K. Benfell, R.A. Kelly, L.H. Hartley, T.W. Smith and E.M. Antman, 1990, Efficacy and safety of perhexiline maleate in refractory angina. A double-blind placebo-controlled clinical trial of a novel antianginal agent, Circulation, 81, 1260-70.

Coller, B.S., 1985, A new murine monoclonal antibody reports an activation-dependent change in the conformation and/ or microenvironment of the platelet glycoprotein IIb/IIIa, J Clinc Invest, 76, 101-108.

Coller, B.S., 1995, Blockade of platelet GPIIb/IIIa receptors as an antithrombotic strategy, Circulation, 92, 2373-80.

Coller, B.S., 2001, Anti-GPIIb/IIIa drugs: current strategies and future directions, Thromb Haemost, 86, 427-43.
Coller, B.S., J.D. Folts, L.E. Scudder and S.R. Smith, 1986, Antithrombotic effect of a monoclonal antibody to the platelet glycoprotein IIb/IIIa receptor in an experimental animal model, Blood, 68, 783-6.

Coller, B.S., E.I. Peerschke, L.E. Scudder and C.A. Sullivan, 1983, A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa, J Clin Invest, 72, 325-38.

Coller, B.S. and L.E. Scudder, 1985, Inhibition of dog platelet function by *in vivo* infusion of $F(ab')_2$ fragments of a monoclonal antibody to the platelet glycoprotein IIb/IIIa receptor, Blood, 66, 1456-9.

Cooke, J.P., 2000, Does ADMA cause endothelial dysfunction?, Arterioscler Thromb Vasc Biol, 20, 2032-7.

Cortellaro, M., C. Boschetti, E. Cofrancesco, C. Zanussi, M. Catalano, G. de Gaetano, L. Gabrielli, B. Lombardi, G. Specchia, and L. Tavazzi, 1992, The PLAT Study: hemostatic function in relation to atherothrombotic ischemic events in vascular disease patients. Principal results. PLAT Study Group. Progetto Lombardo Atero-Trombosi (PLAT) Study Group, Arterioscler Thromb, 12, 1063-70.

Cosentino, F., J.E. Barker, M.P. Brand, S.J. Heales, E.R. Werner, J.R. Tippins, N. West, K.M. Channon, M. Volpe and T.F. Luscher, 2001, Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice, Arterioscler Thromb Vasc Biol, 21, 496-502.

Cotter, G., E. Metzkor-Cotter, E. Kaluski, A. Blatt, I. Litinsky, Y. Baumohl, Y. Moshkovitz, Z. Vered, R. Zaidenstein and A. Golik, 1998, Usefulness of losartan, captopril, and furosemide in preventing nitrate tolerance and improving control of unstable angina pectoris, Am J Cardiol, 82, 1024-9.

Coughlin, S.R., 1999, How the protease thrombin talks to cells, Proc Natl Acad Sci U S A, 96, 11023-7.

Coughlin, S.R., 2000, Thrombin signalling and protease-activated receptors, Nature, 407, 258-64.

Cowan, J.C., J.P. Bourke, D.S. Reid and D.G. Julian, 1987, Prevention of tolerance to nitroglycerin patches by overnight removal, Am J Cardiol, 60, 271-5.

Cramer, E.M., G.F. Savidge, W. Vainchenker, M.C. Berndt, D. Pidard, J.P. Caen, J.M. Masse and J. Breton-Gorius, 1990, Alpha-granule pool of glycoprotein IIb-IIIa in normal and pathologic platelets and megakaryocytes, Blood, 75, 1220-7.

Creager, M.A., J.P. Cooke, M.E. Mendelsohn, S.J. Gallagher, S.M. Coleman, J. Loscalzo and V.J. Dzau, 1990, Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans, J Clin Invest, 86, 228-34.

Creager, M.A., S.J. Gallagher, X.J. Girerd, S.M. Coleman, V.J. Dzau and J.P. Cooke, 1992, L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans, J Clin Invest, 90, 1248-53.

Crisby, M., G. Nordin-Fredriksson, P.K. Shah, J. Yano, J. Zhu and J. Nilsson, 2001, Pravastatin treatment increases collagen content and decreases lipid content, inflammation, metalloproteinases, and cell death in human carotid plaques: implications for plaque stabilization, Circulation, 103, 926-33.

Croft, K.D., L.J. Beilin, R. Vandongen, I. Rouse and J. Masarei, 1990, Leukocyte and platelet function and eicosanoid production in subjects with hypercholesterolaemia, Atherosclerosis, 83, 101-9.

Cross, A.R., 2000, $p40^{phox}$ participates in the activation of NADPH oxidase by increasing the affinity of $p47^{phox}$ for flavocytochrome b_{558}, Biochem J, 349, 113-117.

Crossman, D.C., S.W. Larkin, R.W. Fuller, G.J. Davies and A. Maseri, 1989, Substance P dilates epicardial coronary arteries and increases coronary blood flow in humans, Circulation, 80, 475-84.

Dakak, N., N. Makhoul, M.Y. Flugelman, A. Merdler, H. Shehadeh, A. Schneeweiss, D.A. Halon and B.S. Lewis, 1990, Failure of captopril to prevent nitrate tolerance in congestive heart failure secondary to coronary artery disease, Am J Cardiol, 66, 608-13.

Dallegri, F., A. Ballestrero, L. Ottonello and F. Patrone, 1989, Platelets as scavengers of neutrophil-derived oxidants: a possible defence mechanism at sites of vascular injury, Thromb Haemost, 61, 415-8.

Danesh, J., P. Whincup, M. Walker, L. Lennon, A. Thomson, P. Appleby, J.R. Gallimore and M.B. Pepys, 2000, Low grade inflammation and coronary heart disease: prospective study and updated meta-analyses, BMJ, 321, 199-204.

Daniel, J.L., C. Dangelmaier, J. Jin, B. Ashby, J.B. Smith and S.P. Kunapuli, 1998, Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets, J Biol Chem, 273, 2024-9.

Dardik, R., D. Varon, I. Tamarin, A. Zivelin, O. Salomon, B. Shenkman and N. Savion, 2000, Homocysteine and oxidized low density lipoprotein enhanced platelet adhesion to endothelial cells under flow conditions: distinct mechanisms of thrombogenic modulation, Thromb Haemost, 83, 338-44.

Darius, H., 1999, Role of nitrates for the therapy of coronary artery disease patients in the years beyond 2000, J Cardiovasc Pharmacol, 34 Suppl 2, S15-20.

Dart, A.M., F. Lacombe, J.K. Yeoh, J.D. Cameron, G.L. Jennings, E. Laufer and D.S. Esmore, 1991, Aortic distensibility in patients with isolated hypercholesterolaemia, coronary artery disease, or cardiac transplant, Lancet, 338 (8762), 270-3.

Davi, G., P. Alessandrini, A. Mezzetti, G. Minotti, T. Bucciarelli, F. Costantini, F. Cipollone, G.B. Bon, G. Ciabattoni and C. Patrono, 1997, *In vivo* formation of 8-Epi-prostaglandin F_2 alpha is increased in hypercholesterolemia, Arterioscler Thromb Vasc Biol, 17, 3230-5.

Davi, G., M. Averna, I. Catalano, C. Barbagallo, A. Ganci, A. Notarbartolo, G. Ciabattoni and C. Patrono, 1992, Increased thromboxane biosynthesis in type IIa hypercholesterolemia, Circulation, 85, 1792-8.

Davi, G., M. Averna, S. Novo, C.M. Barbagallo, A. Mogavero, A. Notarbartolo and A. Strano, 1989, Effects of synvinolin on platelet aggregation and thromboxane B_2 synthesis in type IIa hypercholesterolemic patients, Atherosclerosis, 79, 79-83.

Davi, G., I. Catalano, M. Averna, A. Notarbartolo, A. Strano, G. Ciabattoni and C. Patrono, 1990, Thromboxane biosynthesis and platelet function in type II diabetes mellitus, N Engl J Med, 322, 1769-74.

Davi, G., G. Ciabattoni, A. Consoli, A. Mezzetti, A. Falco, S. Santarone, E. Pennese, E. Vitacolonna, T. Bucciarelli, F. Costantini, F. Capani and C. Patrono, 1999a, *In vivo* formation of 8-iso-prostaglandin F_2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation, Circulation, 99, 224-9.

Davi, G., G. Ciabattoni, A. Consoli, A. Mezzetti, A. Falco, S. Santarone, E. Pennese, E. Vitacolonna, T. Bucciarelli, F. Costantini, F. Capani and C. Patrono, 1999b, *In vivo* formation of 8-iso-prostaglandin F_2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation, Circulation, 99, 224-9.

Davies, M.J., 1994, Pathology of arterial thrombosis, Br Med Bull, 50, 789-802.

Davies, M.J., 1995, Acute coronary thrombosis -the role of plaque disruption and its initiation and prevention, Eur Heart J, 16 Suppl L, 3-7.

Davies, M.J., 2000, Pathophysiology of acute coronary syndromes, Indian Heart J, 52, 473-9.

Davies, M.J., P.D. Richardson, N. Woolf, D.R. Katz and J. Mann, 1993, Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content, Br Heart J, 69, 377-81.

DAVIT II, 1990, Effect of verapamil on mortality and major events after acute myocardial infarction (the Danish Verapamil Infarction Trial II -DAVIT II), Am J Cardiol, 66, 779-85.

de Beer, F.C., C.R. Hind, K.M. Fox, R.M. Allan, A. Maseri and M.B. Pepys, 1982, Measurement of serum C-reactive protein concentration in myocardial ischaemia and infarction, Br Heart J, 47, 239-43.

de Boer, A.C., A.G. Turpie, R.W. Butt, R.V. Johnston and E. Genton, 1982, Platelet release and thromboxane synthesis in symptomatic coronary artery disease, Circulation, 66, 327-33.

De Caterina, R., D. Giannessi, W. Bernini, G. Lazzerini, A. Mazzone and M. Lombardi, 1989, *In vivo* actions of organic nitrates on platelet function in humans, Z Kardiol, 78 Suppl 2, 56-60.

De Caterina, R., D. Giannessi, F. Crea, S. Chierchia, W. Bernini, P. Gazzetti and A. L'Abbate, 1984, Inhibition of platelet function by injectable isosorbide dinitrate, Am J Cardiol, 53, 1683-7.

De Caterina, R., D. Giannessi, A. Mazzone and W. Bernini, 1988, Mechanisms for the *in vivo* antiplatelet effects of isosorbide dinitrate, Eur Heart J, 9 Suppl A, 45-9.

De Caterina, R., M. Lombardi, W. Bernini, A. Mazzone, D. Giannessi, E. Moscarelli, M. Weiss and G. Lazzerini, 1990, Inhibition of platelet function during *in vivo* infusion of isosorbide mononitrates: relationship between plasma drug concentration and hemodynamic effects, Am Heart J, 119, 855-62.

de Graaf, J.C., J.D. Banga, S. Moncada, R.M. Palmer, P.G. de Groot and J.J. Sixma, 1992, Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions, Circulation, 85, 2284-90.

De Keulenaer, G.W., D.C. Chappell, N. Ishizaka, R.M. Nerem, R.W. Alexander and K.K. Griendling, 1998, Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase, Circ Res, 82, 1094-101.

De la Lande, I.S., T. Philp, I. Stafford and J.D. Horowitz, 1996, Lack of inhibition of glyceryl trinitrate by diphenyleneiodonium in bovine coronary artery, Eur J Pharmacol, 314, 347-50.

De la Lande, I.S., I. Stafford and J.D. Horowitz, 1999, Tolerance induction by transdermal glyceryl trinitrate in rats, Eur J Pharmacol, 374, 71-5.

De Padua Mansur, A., B. Caramelli, C.B. Vianna, D. Chamone and J.A. Ramires, 1997, Smoking and lipoprotein abnormalities on platelet aggregation in coronary heart disease, Int J Cardiol, 62, 151-4.

Del Maschio, A., V. Evangelista, G. Rajtar, Z.M. Chen, C. Cerletti and G. De Gaetano, 1990, Platelet activation by polymorphonuclear leukocytes exposed to chemotactic agents, Am J Physiol, 258, H870-9.

Del Principe, D., A. Menichelli, W. De Matteis, M.L. Di Corpo, S. Di Giulio and A. Finazzi-Agro, 1985, Hydrogen peroxide has a role in the aggregation of human platelets, FEBS Lett, 185, 142-6.

DeLeo, F.R. and M.T. Quinn, 1996, Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins, J Leukoc Biol, 60, 677-91.

DeMots, H. and S.P. Glasser, 1989, Intermittent transdermal nitroglycerin therapy in the treatment of chronic stable angina, J Am Coll Cardiol, 13, 786-95.

Desco, M.C., M. Asensi, R. Marquez, J. Martinez-Valls, M. Vento, F.V. Pallardo, J. Sastre and J. Vinna, 2002, Xanthine oxidase is involved in free radical production in type 1 diabetes, protection by allopurinol, Diabetes, 51, 1118-2.

Di Minno, G., A.M. Cerbone, P.L. Mattioli, S. Turco, C. Iovine and M. Mancini, 1985, Functionally thrombasthenic state in normal platelets following the administration of ticlopidine, J Clin Invest, 75, 328-38.

Diacovo, T.G., S.J. Roth, J.M. Buccola, D.F. Bainton and T.A. Springer, 1996, Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18, Blood, 88, 146-57.

Dickfeld, T., E. Lengyel, A.E. May, S. Massberg, K. Brand, S. Page, C. Thielen, K. Langenbrink and M. Gawaz, 2001a, Transient interaction of activated platelets with endothelial cells induces expression of monocyte-chemoattractant protein-1 via a p38 mitogen-activated protein kinase mediated pathway. Implications for atherogenesis, Cardiovasc Res, 49, 189-99.

Dickfeld, T., A. Ruf, G. Pogatsa-Murray, I. Muller, B. Engelmann, W. Taubitz, J. Fischer, O. Meier and M. Gawaz, 2001b, Differential antiplatelet effects of various glycoprotein IIb-IIIa antagonists, Thromb Res, 101, 53-64.

Dikalov, S., B. Fink, M. Skatchkov and E. Bassenge, 1999, Comparison of glyceryl trinitrate-induced with pentaerythrityl tetranitrate-induced *in vivo* formation of superoxide radicals: effect of vitamin C, Free Radic Biol Med, 27, 170-6.

Dikalov, S., B. Fink, M. Skatchkov, O. Sommer and E. Bassenge, 1998a, Formation of Reactive Oxygen Species in Various Vascular Cells During Glyceryltrinitrate Metabolism, J Cardiovasc Pharmacol Ther, 3, 51-62.

Dikalov, S., B. Fink, M. Skatchkov, D. Stalleicken and E. Bassenge, 1998b, Formation of reactive oxygen species by pentaerithrityltetranitrate and glyceryl trinitrate *in vitro* and development of nitrate tolerance, J Pharmacol Exp Ther, 286, 938-44.

Diodati, J., P. Theroux, J.G. Latour, L. Lacoste, J.Y. Lam and D. Waters, 1990, Effects of nitroglycerin at therapeutic doses on platelet aggregation in unstable angina pectoris and acute myocardial infarction, Am J Cardiol, 66, 683-8.

Diodati, J.G., R.O. Cannon, S.E. Epstein and A.A. Quyyumi, 1992, Platelet hyperaggregability across the coronary bed in response to rapid atrial pacing in patients with stable coronary artery disease, Circulation, 86, 1186-93.

Diodati, J.G., R.O. Cannon, 3rd, N. Hussain and A.A. Quyyumi, 1995, Inhibitory effect of nitroglycerin and sodium nitroprusside on platelet activation across the coronary circulation in stable angina pectoris, Am J Cardiol, 75, 443-8.

Diodati, J.G., R.O. Cannon and A.A. Quyyumi, 1994, Platelet activation in stable coronary artery disease, Am J Cardiol, 73, 8B-11B.

Diodati, J.G., N. Dakak, D.M. Gilligan and A.A. Quyyumi, 1998, Effect of atherosclerosis on endothelium-dependent inhibition of platelet activation in humans, Circulation, 98, 17-24.

Djellas, Y., J.M. Manganello, K. Antonakis and G.C. Le Breton, 1999, Identification of Galpha13 as one of the G-proteins that couple to human platelet thromboxane A_2 receptors, J Biol Chem, 274, 14325-30.

Dockrell, M.E., B.R. Walker, J.P. Noon, G.C. Watt, B.C. Williams and D.J. Webb, 1999, Platelet aggregation in young men with contrasting predisposition to high blood pressure, Am J Hypertens, 12, 115-9.

Dogra, G., L. Rich, K. Stanton and G.F. Watts, 2001, Endothelium-dependent and independent vasodilation studies at normoglycaemia in type I diabetes mellitus with and without microalbuminuria, Diabetologia, 44, 593-601.

Dorn, G.W.d., 1989, Distinct platelet thromboxane A_2/prostaglandin H_2 receptor subtypes. A radioligand binding study of human platelets, J Clin Invest, 84, 1883-91.

Dorn, G.W.d. and A. DeJesus, 1991, Human platelet aggregation and shape change are coupled to separate thromboxane A_2-prostaglandin H_2 receptors, Am J Physiol, 260, H327-34.

Dorn, G.W.d., N. Liel, J.L. Trask, D.E. Mais, M.E. Assey and P.V. Halushka, 1990, Increased platelet thromboxane A_2/prostaglandin H_2 receptors in patients with acute myocardial infarction, Circulation, 81, 212-8.

Doussiere, J. and P.V. Vignais, 1992, Diphenylene iodonium as an inhibitor of the NADPH oxidase complex of bovine neutrophils. Factors controlling the inhibitory potency of diphenylene iodonium in a cell-free system of oxidase activation, Eur J Biochem, 208, 61-71.

Downs, J.R., M. Clearfield, S. Weis, E. Whitney, D.R. Shapiro, P.A. Beere, A. Langendorfer, E.A. Stein, W. Kruyer and A.M. Gotto, 1998, Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study, JAMA, 279, 1615-22.

Draude, G., N. Hrboticky and R.L. Lorenz, 1999, The expression of the lectin-like oxidized low-density lipoprotein receptor (LOX-1) on human vascular smooth muscle cells and monocytes and its down-regulation by lovastatin, Biochem Pharmacol, 57, 383-6.

Drexler, H., A.M. Zeiher, H. Wollschlager, T. Meinertz, H. Just and T. Bonzel, 1989, Flow-dependent coronary artery dilatation in humans, Circulation, 80, 466-74.

Droge, W., 2002, Free radicals in the physiological control of cell function, Physiol Rev, 82, 47-95.

Drummer, C., U. Valta-Seufzer, B. Karrenbrock, J.M. Heim and R. Gerzer, 1991, Comparison of anti-platelet properties of molsidomine, isosorbide-5-mononitrate and placebo in healthy volunteers, Eur Heart J, 12, 541-9.

Du, Z.Y., G.J. Dusting and O.L. Woodman, 1991, Effect of tolerance to glyceryl trinitrate on vascular responses in conscious rabbits, Clin Exp Pharmacol Physiol, 18, 439-47.

Dubois-Rande, J.L., J.Y. Artigou, J.Y. Darmon, R. Habbal, C. Manuel, I. Tayarani, A. Castaigne and Y. Grosgogeat, 1994, Oxidative stress in patients with unstable angina, Eur Heart J, 15, 179-83.

Dupuis, J., G. Lalonde, R. Lemieux and J.L. Rouleau, 1990, Tolerance to intravenous nitroglycerin in patients with congestive heart failure: role of increased intravascular volume, neurohumoral activation and lack of prevention with N-acetylcysteine, J Am Coll Cardiol, 16, 923-31.

Dupuis, J., J.C. Tardif, P. Cernacek and P. Theroux, 1999, Cholesterol reduction rapidly improves endothelial function after acute coronary syndromes. The RECIFE (reduction of cholesterol in ischemia and function of the endothelium) trial, Circulation, 99, 3227-33.

Ebright, G.E., 1914, The effects of nitroglycerine in those engaged in its manufacture, JAMA, 62, 201-9.

Egashira, K., Y. Hirooka, H. Kai, M. Sugimachi, S. Suzuki, T. Inou and A. Takeshita, 1994, Reduction in serum cholesterol with pravastatin improves endothelium-dependent coronary vasomotion in patients with hypercholesterolemia, Circulation, 89, 2519-24.

El-Tamimi, H., M. Mansour, C.J. Pepine, T.J. Wargovich and H. Chen, 1995, Circadian variation in coronary tone in patients with stable angina. Protective role of the endothelium, Circulation, 92, 3201-5.

Elkayam, U., A. Roth, B. Henriquez, L. Weber, D. Tonnemacher and S.H. Rahimtoola, 1985, Hemodynamic and hormonal effects of high-dose transdermal nitroglycerin in patients with chronic congestive heart failure, Am J Cardiol, 56, 555-9.

Elkayam, U., A. Roth, A. Mehra, E. Ostrzega, A. Shotan, D. Kulick, M. Jamison, J.V. Johnston and S.H. Rahimtoola, 1991, Randomized study to evaluate the relation between oral isosorbide dinitrate dosing interval and the development of early tolerance to its effect on left ventricular filling pressure in patients with chronic heart failure, Circulation, 84, 2040-8.
Ellie, E., C. Durrieu, P. Besse, J. Julien and G. Gbipki-Benissan, 1992, Thrombotic thrombocytopenic purpura associated with ticlopidine, Stroke, 23, 922-3.

Elliott, T.G., J.D. Barth and G.B. Mancini, 1995, Effects of vitamin E on endothelial function in men after myocardial infarction, Am J Cardiol, 76, 1188-90.

Ellis, G.R., R.A. Anderson, Y.Y. Chirkov, J. Morris-Thurgood, S.K. Jackson, M.J. Lewis, J.D. Horowitz and M.P. Frenneaux, 2001, Acute effects of vitamin C on platelet responsiveness to nitric oxide donors and endothelial function in patients with chronic heart failure, J Cardiovasc Pharmacol, 37, 564-70.

Ellis, G.R., R.A. Anderson, D. Lang, D.J. Blackman, R.H. Morris, J. Morris-Thurgood, I.F. McDowell, S.K. Jackson, M.J. Lewis and M.P. Frenneaux, 2000, Neutrophil superoxide anion-generating capacity, endothelial function and oxidative stress in chronic heart failure: effects of short- and long-term vitamin C therapy, J Am Coll Cardiol, 36, 1474-82.

Elwood, P.C., A. Beswick, J. Pickering, P. McCarron, J.R. O'Brien, S.R. Renaud and R.J. Flower, 2001, Platelet tests in the prediction of myocardial infarction and ischaemic stroke: evidence from the Caerphilly Prospective Study, Br J Haematol, 113, 514-20.

Elwood, P.C., A.D. Beswick, D.S. Sharp, J.W. Yarnell, S. Rogers and S. Renaud, 1990, Whole blood impedance platelet aggregometry and ischemic heart disease. The Caerphilly Collaborative Heart Disease Study, Arteriosclerosis, 10, 1032-6.

Elwood, P.C., S. Renaud, A.D. Beswick, J.R. O'Brien and P.M. Sweetnam, 1998, Platelet aggregation and incident ischaemic heart disease in the Caerphilly cohort, Heart, 80, 578-82.

Elwood, P.C., S. Renaud, D.S. Sharp, A.D. Beswick, J.R. O'Brien and J.W. Yarnell, 1991, Ischemic heart disease and platelet aggregation. The Caerphilly Collaborative Heart Disease Study, Circulation, 83, 38-44.

Essler, M., M. Retzer, M. Bauer, K.J. Zangl, G. Tigyi and W. Siess, 2000, Stimulation of platelets and endothelial cells by mildly oxidized LDL proceeds through activation of lysophosphatidic acid receptors and the Rho/Rho-kinase pathway. Inhibition by lovastatin, Ann N Y Acad Sci, 905, 282-6.

Eto, K., S. Takeshita, M. Ochiai, Y. Ozaki, T. Sato and T. Isshiki, 1998, Platelet aggregation in acute coronary syndromes: use of a new aggregometer with laser light scattering to assess platelet aggregability, Cardiovasc Res, 40, 223-9.

Evangelista, V., S. Manarini, S. Rotondo, N. Martelli, R. Polischuk, J.L. McGregor, G. de Gaetano and C. Cerletti, 1996, Platelet/polymorphonuclear leukocyte interaction in dynamic conditions: evidence of adhesion cascade and cross talk between P-selectin and the beta 2 integrin CD11b/CD18, Blood, 88, 4183-94.

Falk, E., 1985, Unstable angina with fatal outcome: dynamic coronary thrombosis leading to infarction and/or sudden death. Autopsy evidence of recurrent mural thrombosis with peripheral embolization culminating in total vascular occlusion, Circulation, 71, 699-708.

Falk, E., 1991, Coronary thrombosis: pathogenesis and clinical manifestations, Am J Cardiol, 68, 28B-35B.

Faraday, N., P.J. Goldschmidt-Clermont and P.F. Bray, 1997, Gender differences in platelet GPIIb-IIIa activation, Thromb Haemost, 77, 748-54.

Faraday, N., R.B. Scharpf, J.M. Dodd-o, E.A. Martinez, B.A. Rosenfeld and T. Dorman, 2001, Leukocytes can enhance platelet-mediated aggregation and thromboxane release via interaction of P-selectin glycoprotein ligand 1 with P-selectin, Anesthesiology, 94, 145-51.

Fard, A., C.H. Tuck, J.A. Donis, R. Sciacca, M.R. Di Tullio, H.D. Wu, T.A. Bryant, N.T. Chen, M. Torres-Tamayo, R. Ramasamy, L. Berglund, H.N. Ginsberg, S. Homma and P.J. Cannon, 2000, Acute elevations of plasma asymmetric dimethylarginine and impaired endothelial function in response to a high-fat meal in patients with type 2 diabetes, Arterioscler Thromb Vasc Biol, 20, 2039-44.

Faulds, D. and E.M. Sorkin, 1994, Abciximab (c7E3 Fab). A review of its pharmacology and therapeutic potential in ischaemic heart disease, Drugs, 48, 583-98.

Faulkner, K. and I. Fridovich, 1993, Luminol and lucigenin as detectors for O_2, Free Radic Biol Med, 15, 447-51.

Favaloro, E.J., G. Kershaw, M. Bukuya, M. Hertzberg and J. Koutts, 2001, Laboratory diagnosis of von Willebrand disorder (vWD) and monitoring of DDAVP therapy: efficacy of the PFA-100(R) and vWF:CBA as combined diagnostic strategies, Haemophilia, 7, 180-9.

Feelisch, M., P. Kotsonis, J. Siebe, B. Clement and H.H. Schmidt, 1999, The soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a] quinoxalin-1-one is a nonselective heme protein inhibitor of nitric oxide synthase and other cytochrome P_{450} enzymes involved in nitric oxide donor bioactivation, Mol Pharmacol, 56, 243-53.

Feinstein, M.B., J.J. Egan, R.I. Sha'afi and J. White, 1983, The cytoplasmic concentration of free calcium in platelets is controlled by stimulators of cyclic AMP production (PGD_2, PGE_1, forskolin), Biochem Biophys Res Commun, 113, 598-604.

Feldman, R.L., J.D. Marx, C.J. Pepine and C.R. Conti, 1982, Analysis of coronary responses to various doses of intracoronary nitroglycerin, Circulation, 66, 321-7.

Feldman, R.L., C.J. Pepine, R.C. Curry, Jr. and C.R. Conti, 1979, Coronary arterial responses to graded doses of nitroglycerin, Am J Cardiol, 43, 91-7.

Feng, Q., X. Lu, A.J. Fortin, A. Pettersson, T. Hedner, R.L. Kline and J.M. Arnold, 1998, Elevation of an endogenous inhibitor of nitric oxide synthesis in experimental congestive heart failure, Cardiovasc Res, 37, 667-75.

Fennell, J.P., M.J. Brosnan, A.J. Frater, C.A. Hamilton, M.Y. Alexander, S.A. Nicklin, D.D. Heistad, A.H. Baker and A.F. Dominiczak, 2002, Adenovirus-mediated overexpression of extracellular superoxide dismutase improves endothelial dysfunction in a rat model of hypertension, Gene Ther, 9, 110-7.

Fernandez, P.C., J. Machado, Jr., V.T. Heussler, C. Botteron, G.H. Palmer and D.A. Dobbelaere, 1999, The inhibition of NF-kappaB activation pathways and the induction of apoptosis by dithiocarbamates in T cells are blocked by the glutathione precursor N-acetyl-L-cysteine, Biol Chem, 380, 1383-94.

Feron, O., C. Dessy, J.P. Desager and J.L. Balligand, 2001, Hydroxy-methylglutaryl-coenzyme A reductase inhibition promotes endothelial nitric oxide synthase activation through a decrease in caveolin abundance, Circulation, 103, 113-8.

Ferrari, R., L. Agnoletti, L. Comini, G. Gaia, T. Bachetti, A. Cargnoni, C. Ceconi, S. Curello and O. Visioli, 1998, Oxidative stress during myocardial ischaemia and heart failure, Eur Heart J, 19 Suppl B, B2-11.

Ferratini, M., S. Pirelli, P. Merlini, P. Silva and G. Pollavini, 1989, Intermittent transdermal nitroglycerin monotherapy in stable exercise-induced angina: a comparison with a continuous schedule, Eur Heart J, 10, 998-1002.

Festa, A., R. D'Agostino, G. Howard, L. Mykkanen, R.P. Tracy and S.M. Haffner, 2000, Inflammation and microalbuminuria in nondiabetic and type 2 diabetic subjects: The Insulin Resistance Atherosclerosis Study, Kidney Int, 58, 1703-10.

Fickling, S.A., D. Williams, P. Vallance, S.S. Nussey and G.S. Whitley, 1993, Plasma concentrations of endogenous inhibitor of nitric oxide synthesis in normal pregnancy and pre-eclampsia, Lancet, 342 (8865), 242-3.

Figueras, J., R. Lidon and J. Cortadellas, 1991, Rebound myocardial ischaemia following abrupt interruption of intravenous nitroglycerin infusion in patients with unstable angina at rest, Eur Heart J, 12, 405-11.

Fink, B. and E. Bassenge, 1997, Unexpected, tolerance-devoid vasomotor and platelet actions of pentaerythrityl tetranitrate, J Cardiovasc Pharmacol, 30, 831-6.

Fink, B., M. Schwemmer, N. Fink and E. Bassenge, 1999, Tolerance to nitrates with enhanced radical formation suppressed by carvedilol, J Cardiovasc Pharmacol, 34, 800-5.

Finta, K.M., M.J. Fischer, L. Lee, D. Gordon, B. Pitt and R.C. Webb, 1993, Ramipril prevents impaired endothelium-dependent relaxation in arteries from rabbits fed an atherogenic diet, Atherosclerosis, 100, 149-56.

Fisslthaler, B., S. Dimmeler, C. Hermann, R. Busse and I. Fleming, 2000, Phosphorylation and activation of the endothelial nitric oxide synthase by fluid shear stress, Acta Physiol Scand, 168, 81-8.

Fitzgerald, D.J., F. Catella, L. Roy and G.A. FitzGerald, 1988, Marked platelet activation *in vivo* after intravenous streptokinase in patients with acute myocardial infarction, Circulation, 77, 142-50.

Fitzgerald, D.J., L. Roy, F. Catella and G.A. FitzGerald, 1986, Platelet activation in unstable coronary disease, N Engl J Med, 315, 983-9.

Fitzgerald, D.J., L. Roy, R.M. Robertson and G.A. FitzGerald, 1984, The effects of organic nitrates on prostacyclin biosynthesis and platelet function in humans, Circulation, 70, 297-302.

FitzGerald, G.A., 1991, Mechanisms of platelet activation: thromboxane A_2 as an amplifying signal for other agonists, Am J Cardiol, 68, 11B-15B.

FitzGerald, G.A., J.A. Oates, J. Hawiger, R.L. Maas, L.J.d. Roberts, J.A. Lawson and A.R. Brash, 1983, Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man, J Clin Invest, 71, 676-88.

Fitzsimmons, C., D. Proudfoot and D.E. Bowyer, 1999, Monocyte prostaglandins inhibit procollagen secretion by human vascular smooth muscle cells: implications for plaque stability, Atherosclerosis, 142, 287-93.

Fleckenstein-Grun, G., A. Fleckenstein, Y.K. Byon and K.W. Kem, 1978, Mechanism of action of Ca^{2+} antagonists in the treatment of coronary disease, with special reference to perhexiline maleate. (Excerpta Medica, Amsterdam) p. 1-22.

Fleming, I., U.R. Michaelis, D. Bredenkotter, B. Fisslthaler, F. Dehghani, R.P. Brandes and R. Busse, 2001, Endothelium-derived hyperpolarizing factor synthase (Cytochrome P_{450} 2C9) is a functionally significant source of reactive oxygen species in coronary arteries, Circ Res, 88, 44-51.

Folts, J., 1991a, An *in vivo* model of experimental arterial stenosis, intimal damage, and periodic thrombosis, Circulation, 83, IV3-14.

Folts, J.D., 1991b, Inhibition of platelet function *in vivo* or *in vitro* by organic nitrates, J Am Coll Cardiol, 18, 1537-8.

Folts, J.D., K. Gallagher and G.G. Rowe, 1982, Blood flow reductions in stenosed canine coronary arteries: vasospasm or platelet aggregation?, Circulation, 65, 248-55.

Folts, J.D., J. Stamler and J. Loscalzo, 1991, Intravenous nitroglycerin infusion inhibits cyclic blood flow responses caused by periodic platelet thrombus formation in stenosed canine coronary arteries, Circulation, 83, 2122-7.

Forstermann, U., E.I. Closs, J.S. Pollock, M. Nakane, P. Schwarz, I. Gath and H. Kleinert, 1994, Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions, Hypertension, 23, 1121-31.

Forstermann, U., A. Mugge, U. Alheid, A. Haverich and J.C. Frolich, 1988, Selective attenuation of endothelium-mediated vasodilation in atherosclerotic human coronary arteries, Circ Res, 62, 185-90.

Forstermann, U., J.S. Pollock, H.H. Schmidt, M. Heller and F. Murad, 1991, Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells, Proc Natl Acad Sci U S A, 88, 1788-92.

Frangogiannis, N.G., C.W. Smith and M.L. Entman, 2002, The inflammatory response in myocardial infarction, Cardiovasc Res, 53, 31-47.

Frater-Schroder, M., G. Muller, W. Birchmeier and P. Bohlen, 1986, Transforming growth factor-beta inhibits endothelial cell proliferation, Biochem Biophys Res Commun, 137, 295-302.

Fredholm, B.B., M.P. Abbracchio, G. Burnstock, J.W. Daly, T.K. Harden, K.A. Jacobson, P. Leff and M. Williams, 1994, Nomenclature and classification of purinoceptors, Pharmacol Rev, 46, 143-56.

Freedman, J.E. and J.F. Keaney, Jr., 1999, Nitric oxide and superoxide detection in human platelets, Methods Enzymol, 301, 61-70.

Freedman, J.E., J. Loscalzo, M.R. Barnard, C. Alpert, J.F. Keaney and A.D. Michelson, 1997, Nitric oxide released from activated platelets inhibits platelet recruitment, J Clin Invest, 100, 350-6.

Freedman, J.E., J. Loscalzo, S.E. Benoit, C.R. Valeri, M.R. Barnard and A.D. Michelson, 1996, Decreased platelet inhibition by nitric oxide in two brothers with a history of arterial thrombosis, J Clin Invest, 97, 979-87.

Freedman, J.E., B. Ting, B. Hankin, J. Loscalzo, J.F. Keaney, Jr. and J.A. Vita, 1998, Impaired platelet production of nitric oxide predicts presence of acute coronary syndromes, Circulation, 98, 1481-6.

Frelinger, A.L., 3rd and R.S. Hillman, 1998, Novel methods for assessing platelet function, Am Heart J, 135, S184-6.

Fridovich, I., 1995, Superoxide radical and superoxide dismutases, Annu Rev Biochem, 64, 97-112.

Frishman, W.H., 1992, Tolerance, rebound, and time-zero effect of nitrate therapy, Am J Cardiol, 70, 43G-47G.

Fromenty, B., C. Fisch, G. Labbe, C. Degott, D. Deschamps, A. Berson, P. Letteron and D. Pessayre, 1990, Amiodarone inhibits the mitochondrial beta-oxidation of fatty acids and produces microvesicular steatosis of the liver in mice, J Pharmacol Exp Ther, 255, 1371-6.

Fujimoto, Y., S. Tagano, K. Ogawa, S. Sakuma and T. Fujita, 1998, Comparison of the effects of nitric oxide and peroxynitrite on the 12-lipoxygenase and cyclooxygenase metabolism of arachidonic acid in rabbit platelets, Prostaglandins Leukot Essent Fatty Acids, 59, 95-100.

Fukai, T., M.R. Siegfried, M. Ushio-Fukai, K.K. Griendling and D.G. Harrison, 1999, Modulation of extracellular superoxide dismutase expression by angiotensin II and hypertension, Circ Res, 85, 23-8.

Fukui, T., N. Ishizaka, S. Rajagopalan, J.B. Laursen, Q.t. Capers, W.R. Taylor, D.G. Harrison, H. de Leon, J.N. Wilcox and K.K. Griendling, 1997, $p22^{phox}$ mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats, Circ Res, 80, 45-51.

Fukui, T., B. Lassegue, H. Kai, R.W. Alexander and K.K. Griendling, 1995, Cytochrome $b\text{-}_{558}$ alpha-subunit cloning and expression in rat aortic smooth muscle cells, Biochim Biophys Acta, 1231, 215-9.

Fukumoto, Y., P. Libby, E. Rabkin, C.C. Hill, M. Enomoto, Y. Hirouchi, M. Shiomi and M. Aikawa, 2001, Statins alter smooth muscle cell accumulation and collagen content in established atheroma of watanabe heritable hyperlipidemic rabbits, Circulation, 103, 993-9.

Fukuto, J.M., K. Chiang, R. Hszieh, P. Wong and G. Chaudhuri, 1992, The pharmacological activity of nitroxyl: a potent vasodilator with activity similar to nitric oxide and/or endothelium-derived relaxing factor, J Pharmacol Exp Ther, 263, 546-51.

Fung, H.L., 1992, Do nitrates differ?, Br J Clin Pharmacol, 34 Suppl 1, 5S-9S.

Fung, H.L., S. Chong, E. Kowaluk, K. Hough and M. Kakemi, 1988, Mechanisms for the pharmacologic interaction of organic nitrates with thiols. Existence of an extracellular pathway for the reversal of nitrate vascular tolerance by N-acetylcysteine, J Pharmacol Exp Ther, 245, 524-30.

Fung, H.L. and R. Poliszczuk, 1986, Nitrosothiol and nitrate tolerance, Z Kardiol, 75 Suppl 3, 25-7.

Furberg, C.D., B.M. Psaty and J.V. Meyer, 1995, Nifedipine. Dose-related increase in mortality in patients with coronary heart disease, Circulation, 92, 1326-31.

Furchgott, R.F. and J.V. Zawadzki, 1980, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine, Nature, 288, 373-6.

Furman, M.I., S.E. Benoit, M.R. Barnard, C.R. Valeri, M.L. Borbone, R.C. Becker, H.B. Hechtman and A.D. Michelson, 1998a, Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease, J Am Coll Cardiol, 31, 352-8.

Furman, M.I., L.A. Krueger, A.L. Frelinger, 3rd, M.R. Barnard, M.A. Mascelli, M.T. Nakada and A.D. Michelson, 2000, GPIIb-IIIa antagonist-induced reduction in platelet surface factor V/Va binding and phosphatidylserine expression in whole blood, Thromb Haemost, 84, 492-8.

Furman, M.I., L. Liu, S.E. Benoit, R.C. Becker, M.R. Barnard and A.D. Michelson, 1998b, The cleaved peptide of the thrombin receptor is a strong platelet agonist, Proc Natl Acad Sci U S A, 95, 3082-7.

Fusegawa, Y., S. Goto, S. Handa, T. Kawada and Y. Ando, 1999, Platelet spontaneous aggregation in platelet-rich plasma is increased in habitual smokers, Thromb Res, 93, 271-8.

Fusegawa, Y. and S. Handa, 2000, Platelet aggregation induced by ADP or epinephrine is enhanced in habitual smokers, Thromb Res, 97, 287-95.

Fuster, V., L. Badimon, J.J. Badimon and J.H. Chesebro, 1992, The pathogenesis of coronary artery disease and the acute coronary syndromes (2), N Engl J Med, 326, 310-8.

Gaarder, A., L. Jonsen, S. Laland, A. Hellem and P.A. Owren, 1961, Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets, Nature, 192, 531-532.

Gachet, C., 2001, ADP receptors of platelets and their inhibition, Thromb Haemost, 86, 222-32.

Gachet, C., M. Cattaneo, P. Ohlmann, B. Hechler, A. Lecchi, J. Chevalier, D. Cassel, P.M. Mannucci and J.P. Cazenave, 1995, Purinoceptors on blood platelets: further pharmacological and clinical evidence to suggest the presence of two ADP receptors, Br J Haematol, 91, 434-44.

Gachet, C., B. Hechler, C. Leon, C. Vial, C. Leray, P. Ohlmann and J.P. Cazenave, 1997, Activation of ADP receptors and platelet function, Thromb Haemost, 78, 271-5.

Gage, J.E., O.M. Hess, T. Murakami, M. Ritter, J. Grimm and H.P. Krayenbuehl, 1986, Vasoconstriction of stenotic coronary arteries during dynamic exercise in patients with classic angina pectoris: reversibility by nitroglycerin, Circulation, 73, 865-76.

Galis, Z.S., G.K. Sukhova, R. Kranzhofer, S. Clark and P. Libby, 1995, Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases, Proc Natl Acad Sci U S A, 92, 402-6.

Gartner, T.K. and J.S. Bennett, 1985, The tetrapeptide analogue of the cell attachment site of fibronectin inhibits platelet aggregation and fibrinogen binding to activated platelets, J Biol Chem, 260, 11891-4.

Gaston, B., 1999, Nitric oxide and thiol groups, Biochim Biophys Acta, 1411, 323-33.

Gatzka, C.D., J.D. Cameron, B.A. Kingwell and A.M. Dart, 1998, Relation between coronary artery disease, aortic stiffness, and left ventricular structure in a population sample, Hypertension, 32, 575-8.

Gawaz, M., K. Brand, T. Dickfeld, G. Pogatsa-Murray, S. Page, C. Bogner, W. Koch, A. Schomig and F. Neumann, 2000, Platelets induce alterations of chemotactic and adhesive properties of endothelial cells mediated through an interleukin-1-dependent mechanism. Implications for atherogenesis, Atherosclerosis, 148, 75-85.

Geiger, J., C. Nolte, E. Butt, S.O. Sage and U. Walter, 1992, Role of cGMP and cGMP-dependent protein kinase in nitrovasodilator inhibition of agonist-evoked calcium elevation in human platelets, Proc Natl Acad Sci U S A, 89, 1031-5.

George, J.N., 2000, Platelets, Lancet, 355 (9214), 1531-9.

Giannessi, D., S. Del Ry and R.L. Vitale, 2001, The role of endothelins and their receptors in heart failure, Pharmacol Res, 43, 111-26.

Gibson, R.S., W.E. Boden, P. Theroux, H.D. Strauss, C.M. Pratt, M. Gheorghiade, R.J. Capone, M.H. Crawford, R.C. Schlant and R.E. Kleiger, 1986, Diltiazem and reinfarction in patients with non-Q-wave myocardial infarction. Results of a double-blind, randomized, multicenter trial, N Engl J Med, 315, 423-9.

Gilligan, D.M., M.N. Sack, V. Guetta, P.R. Casino, A.A. Quyyumi, D.J. Rader, J.A. Panza and R.O. Cannon, 3rd, 1994, Effect of antioxidant vitamins on low density lipoprotein oxidation and impaired endothelium-dependent vasodilation in patients with hypercholesterolemia, J Am Coll Cardiol, 24, 1611-7.

GISSI-3., 1994, Effects of lisinopril and transdermal glyceryl trinitrate singly and together on 6-week mortality and ventricular function after acute myocardial infarction. Gruppo Italiano per lo Studio della Sopravvivenza nell'infarto Miocardico, Lancet, 343 (8916), 1115-22.

Giugliano, D., R. Marfella, G. Verrazzo, R. Acampora, C. Donzella, A. Quatraro, L. Coppola and F. D'Onofrio, 1995, Abnormal rheologic effects of glyceryl trinitrate in patients with non-insulin-dependent diabetes mellitus and reversal by antioxidants, Ann Intern Med, 123, 338-43.

Glasser, S.P., 1999, Prospects for therapy of nitrate tolerance, Lancet, 353 (9164), 1545-6.

Gobel, E.J., R.W. Hautvast, W.H. van Gilst, J.N. Spanjaard, H.L. Hillege, M.J. DeJongste, G.P. Molhoek and K.I. Lie, 1995, Randomised, double-blind trial of intravenous diltiazem versus glyceryl trinitrate for unstable angina pectoris, Lancet, 346 (8991-8992), 1653-7.

Gobel, E.J., W.H. van Gilst, P.J. de Kam, M.G. ter Napel, G.P. Molhoek and K.I. Lie, 1998, Long-term follow-up after early intervention with intravenous diltiazem or intravenous nitroglycerin for unstable angina pectoris, Eur Heart J, 19, 1208-13.

Gogia, H., A. Mehra, S. Parikh, M. Raman, J. Ajit-Uppal, J.V. Johnson and U. Elkayam, 1995, Prevention of tolerance to hemodynamic effects of nitrates with concomitant use of hydralazine in patients with chronic heart failure, J Am Coll Cardiol, 26, 1575-80.

Gokce, N., J.F. Keaney, Jr., B. Frei, M. Holbrook, M. Olesiak, B.J. Zachariah, C. Leeuwenburgh, J.W. Heinecke and J.A. Vita, 1999, Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease, Circulation, 99, 3234-40.

Gokce, N., J.F. Keaney, Jr., L.M. Hunter, M.T. Watkins, J.O. Menzoian and J.A. Vita, 2002, Risk Stratification for Postoperative Cardiovascular Events via Noninvasive Assessment of Endothelial Function: A Prospective Study, Circulation, 105, 1567-72.

Goldbourt, U., S. Behar, H. Reicher-Reiss, M. Zion, L. Mandelzweig and E. Kaplinsky, 1993, Early administration of nifedipine in suspected acute myocardial infarction. The Secondary Prevention Reinfarction Israel Nifedipine Trial 2 Study, Arch Intern Med, 153, 345-53.

Golino, P., L.M. Buja, S.K. Yao, J. McNatt and J.T. Willerson, 1990, Failure of nitroglycerin and diltiazem to reduce platelet-mediated vasoconstriction in dogs with coronary artery stenosis and endothelial injury: further evidence for thromboxane A_2 and serotonin as mediators of coronary artery vasoconstriction *in vivo*, J Am Coll Cardiol, 15, 718-26.

Gori, T., J.M. Burstein, S. Ahmed, S.E. Miner, A. Al-Hesayen, S. Kelly and J.D. Parker, 2001, Folic acid prevents nitroglycerin-induced nitric oxide synthase dysfunction and nitrate tolerance: a human *in vivo* study, Circulation, 104, 1119-23.

Gorlach, A., R.P. Brandes, K. Nguyen, M. Amidi, F. Dehghani and R. Busse, 2000, A $gp91^{phox}$ containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall, Circ Res, 87, 26-32.

Gorman, R.R., F.A. Fitzpatrick and O.V. Miller, 1977, A selective thromboxane synthetase inhibitor blocks the cAMP lowering activity of PGH_2, Biochem Biophys Res Commun, 79, 305-13.

Goto, S., N. Tamura, K. Eto, Y. Ikeda and S. Handa, 2002, Functional significance of adenosine 5'-diphosphate receptor ($P2Y_{12}$) in platelet activation initiated by binding of von Willebrand factor to platelet GPIb-alpha induced by conditions of high shear rate., Circulation, 105, 2531-2533.

Gow, I.F., A.D. Flapan, M. Morris, E. Davies, B.C. Williams, P.L. Padfield, T.R. Shaw and C.R. Edwards, 1991, A lack of effect of captopril on platelet aggregation in patients with congestive heart failure, Eur J Clin Pharmacol, 41, 47-9.

Gralnick, H.R., W.S. Kramer, L.P. McKeown, L. Garfinkel, A. Pinot, S.B. Williams and H. Krutzsch, 1996, Platelet adhesion at high shear rates: the roles of von Willebrand factor/GPIb and the beta 1 integrin alpha 2 beta 1, Thromb Res, 81, 113-9.

Grande, P., A.M. Grauholt and J.K. Madsen, 1990, Unstable angina pectoris. Platelet behavior and prognosis in progressive angina and intermediate coronary syndrome, Circulation, 81, I16-9.

Green, L.H., E. Seroppian and R.I. Handin, 1980, Platelet activation during exercise-induced myocardial ischemia, N Engl J Med, 302, 193-7.

Greenlees, C., C.L. Wainwright and R.M. Wadsworth, 1996, Vasorelaxant and antiaggregatory properties of the endothelium: a comparative study in normocholesterolaemic and hereditary and dietary hypercholesterolaemic rabbits, Br J Pharmacol, 119, 1470-6.

Griendling, K.K., C.A. Minieri, J.D. Ollerenshaw and R.W. Alexander, 1994, Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells, Circ Res, 74, 1141-8.

Griendling, K.K., D. Sorescu and M. Ushio-Fukai, 2000, NAD(P)H oxidase: role in cardiovascular biology and disease, Circ Res, 86, 494-501.

Griendling, K.K. and M. Ushio-Fukai, 1998, Redox control of vascular smooth muscle proliferation, J Lab Clin Med, 132, 9-15.

Gries, A., C. Bode, K. Peter, A. Herr, H. Bohrer, J. Motsch and E. Martin, 1998, Inhaled nitric oxide inhibits human platelet aggregation, P-selectin expression, and fibrinogen binding *in vitro* and *in vivo*, Circulation, 97, 1481-7.

Griesshammer, M., H. Beneke, B. Nussbaumer, M. Grunewald, M. Bangerter and L. Bergmann, 1999, Increased platelet surface expression of P-selectin and thrombospondin as markers of platelet activation in essential thrombocythaemia, Thromb Res, 96, 191-6.

Griffith, O.W. and D.J. Stuehr, 1995, Nitric oxide synthases: properties and catalytic mechanism, Annu Rev Physiol, 57, 707-36.

Griscavage, J.M., A.J. Hobbs and L.J. Ignarro, 1995, Negative modulation of nitric oxide synthase by nitric oxide and nitroso compounds, Adv Pharmacol, 34, 215-34.

Gross, G.J., D.C. Warltier, H.F. Hardman and K.A. Lamping, 1985, Enhanced subendocardial perfusion distal to a flow-limiting coronary artery stenosis in dogs: comparative effects of nicorandil, a potential new antianginal agent, and nitroglycerin, J Cardiovasc Pharmacol, 7, 977-82.

Gross, S.S. and M.S. Wolin, 1995, Nitric oxide: pathophysiological mechanisms, Annu Rev Physiol, 57, 737-69.

Groves, P., S. Kurz, H. Just and H. Drexler, 1995, Role of endogenous bradykinin in human coronary vasomotor control, Circulation, 92, 3424-30.

Gruetter, D.Y., C.A. Gruetter, B.K. Barry, W.H. Baricos, A.L. Hyman, P.J. Kadowitz and L.J. Ignarro, 1980, Activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside, and nitrosoguanidine-inhibition by calcium, lanthanum, and other cations, enhancement by thiols, Biochem Pharmacol, 29, 2943-50.

Gryglewski, R.J., R.M. Palmer and S. Moncada, 1986, Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor, Nature, 320, 454-6.

Gudmundsson, G.S., C.A. Sinkey, C.A. Chenard, P.J. Stumbo and W.G. Haynes, 2000, Resistance vessel endothelial function in healthy humans during transient postprandial hypertriglyceridemia, Am J Cardiol, 85, 381-5.

Guerra-Cuesta, J.I., M. Monton, J.A. Rodriguez-Feo, A.M. Jimenez, F. Gonzalez-Fernandez, L.A. Rico, R. Garcia, J. Gomez, J. Farre, S. Casado and A. Lopez-Farre, 1999, Effect of losartan on human platelet activation, J Hypertens, 17, 447-52.

Gupta, R.K., S.E. Kjeldsen, E. Motley, A.B. Weder, A.J. Zweifler and S. Julius, 1991, Platelet function during antihypertensive treatment with quinapril, a novel angiotensin converting enzyme inhibitor, J Cardiovasc Pharmacol, 17, 13-9.

Gutierrez, J.A., S.G. Clark, A.D. Giulumian and L.C. Fuchs, 1997, Superoxide anions contribute to impaired regulation of blood pressure by nitric oxide during the development of cardiomyopathy, J Pharmacol Exp Ther, 282, 1643-9.

Gutstein, D.E. and V. Fuster, 1999, Pathophysiology and clinical significance of atherosclerotic plaque rupture, Cardiovasc Res, 41, 323-33.

Gutteridge, J.M., 1986, Aspects to consider when detecting and measuring lipid peroxidation, Free Radic Res Commun, 1, 173-84.

Guzik, T.J., N.E. West, E. Black, D. McDonald, C. Ratnatunga, R. Pillai and K.M. Channon, 2000a, Functional effect of the C242T polymorphism in the NAD(P)H oxidase $p22^{phox}$ gene on vascular superoxide production in atherosclerosis, Circulation, 102, 1744-7.

Guzik, T.J., N.E. West, E. Black, D. McDonald, C. Ratnatunga, R. Pillai and K.M. Channon, 2000b, UltraRapid communications : vascular superoxide production by NAD(P)H OxidaseAssociation with endothelial dysfunction and clinical risk factors, Circ Res, 86, 1008.

Guzik, T.J., N.E. West, E. Black, D. McDonald, C. Ratnatunga, R. Pillai and K.M. Channon, 2000c, Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors, Circ Res, 86, E85-90.

Gyllenhammar, H., 1987, Lucigenin chemiluminescence in the assessment of neutrophil superoxide production, J Immunol Methods, 97, 209-13.

Habib, A., G.A. FitzGerald and J. Maclouf, 1999, Phosphorylation of the thromboxane receptor alpha, the predominant isoform expressed in human platelets, J Biol Chem, 274, 2645-51.

Habib, F., D. Dutka, D. Crossman, C.M. Oakley and J.G. Cleland, 1994, Enhanced basal nitric oxide production in heart failure: another failed counter-regulatory vasodilator mechanism?, Lancet, 344 (8919), 371-3.

Haffner, S.M., S. Lehto, T. Ronnemaa, K. Pyorala and M. Laakso, 1998, Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction, N Engl J Med, 339, 229-34.

Hagberg, I.A. and T. Lyberg, 2000, Blood platelet activation evaluated by flow cytometry: optimised methods for clinical studies, Platelets, 11, 137-50.

Haines, A.P., D. Howarth, W.R. North, E. Goldenberg, Y. Stirling, T.W. Meade, E.B. Raftery and M.W. Millar Craig, 1983, Haemostatic variables and the outcome of myocardial infarction, Thromb Haemost, 50, 800-3.

Haj-Yehia, A.I., T. Nassar, P. Assaf, H. Nassar and E.E. Anggard, 1999, Effects of the superoxide dismutase-mimic compound TEMPOL on oxidant stress-mediated endothelial dysfunction, Antioxid Redox Signal, 1, 221-32.

Hakkinen, T., K. Karkola and S. Yla-Herttuala, 2000, Macrophages, smooth muscle cells, endothelial cells, and T-cells express CD40 and CD40L in fatty streaks and more advanced human atherosclerotic lesions. Colocalization with epitopes of oxidized low-density lipoprotein, scavenger receptor, and CD16 (Fc gammaRIII), Virchows Arch, 437, 396-405.

Hall, D.A. and S.M. Hourani, 1994, Effects of suramin on increases in cytosolic calcium and on inhibition of adenylate cyclase induced by adenosine 5'-diphosphate in human platelets, Biochem Pharmacol, 47, 1013-8.

Hallam, T.J. and T.J. Rink, 1985, Responses to adenosine diphosphate in human platelets loaded with the fluorescent calcium indicator quin2, J Physiol, 368, 131-46.

Halliwell, B., 1989, Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis, Br J Exp Pathol, 70, 737-57.

Halliwell, B. and S. Chirico, 1993, Lipid peroxidation: its mechanism, measurement, and significance, Am J Clin Nutr, 57, 715S-724S.

Hamabe, A., B. Takase, A. Uehata, A. Kurita, F. Ohsuzu and S. Tamai, 2001, Impaired endothelium-dependent vasodilation in the brachial artery in variant angina pectoris and the effect of intravenous administration of vitamin C, Am J Cardiol, 87, 1154-9.

Hamberg, M., J. Svensson and B. Samuelsson, 1975, Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides, Proc Natl Acad Sci U S A, 72, 2994-8.

Hambrecht, R., L. Hilbrich, S. Erbs, S. Gielen, E. Fiehn, N. Schoene and G. Schuler, 2000, Correction of endothelial dysfunction in chronic heart failure: additional effects of exercise training and oral L-arginine supplementation, J Am Coll Cardiol, 35, 706-13.

Hamm, C.W., R.L. Lorenz, W. Bleifeld, W. Kupper, W. Wober and P.C. Weber, 1987, Biochemical evidence of platelet activation in patients with persistent unstable angina, J Am Coll Cardiol, 10, 998-1006.

Hampton, M.B., A.J. Kettle and C.C. Winterbourn, 1998, Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing, Blood, 92, 3007-17.

Han, P., C. Boatwright and N.G. Ardlie, 1983, Verapamil and collagen-induced platelet reactions-evidence for a role for intracellular calcium in platelet activation, Thromb Haemost, 50, 537-40.

Haque, S.F., H. Matsubayashi, S. Izumi, T. Sugi, T. Arai, A. Kondo and T. Makino, 2001, Sex difference in platelet aggregation detected by new aggregometry using light scattering, Endocr J, 48, 33-41.

Haramaki, N., H. Ikeda, Y. Takajo, A. Katoh, S. Kanaya, S. Shintani, R. Haramaki, T. Murohara and T. Imaizumi, 2001, Long-term smoking causes nitroglycerin resistance in platelets by depletion of intraplatelet glutathione, Arterioscler Thromb Vasc Biol, 21, 1852-6.

Hardisty, R.M., M.J. Powling and T.J. Nokes, 1990, The action of ticlopidine on human platelets. Studies on aggregation, secretion, calcium mobilization and membrane glycoproteins, Thromb Haemost, 64, 150-5.

Harrington, R.A., N.S. Kleiman, K. Kottke-Marchant, A.M. Lincoff, J.E. Tcheng, K.N. Sigmon, D. Joseph, G. Rios, K. Trainor and D. Rose, 1995, Immediate and reversible platelet inhibition after intravenous administration of a peptide glycoprotein IIb/IIIa inhibitor during percutaneous coronary intervention, Am J Cardiol, 76, 1222-7.

Harrison, D.G., 1997, Endothelial function and oxidant stress, Clin Cardiol, 20, II-11-7.

Harrison, D.G., M.L. Armstrong, P.C. Freiman and D.D. Heistad, 1987, Restoration of endothelium-dependent relaxation by dietary treatment of atherosclerosis, J Clin Invest, 80, 1808-11.

Harrison, D.G. and J.N. Bates, 1993, The nitrovasodilators. New ideas about old drugs, Circulation, 87, 1461-7.

Harrison, P., 2000, Progress in the assessment of platelet function, Br J Haematol, 111, 733-44.

Hass, W.K., J.D. Easton, H.P. Adams, Jr., W. Pryse-Phillips, B.A. Molony, S. Anderson and B. Kamm, 1989, A randomized trial comparing ticlopidine hydrochloride with aspirin for the prevention of stroke in high-risk patients. Ticlopidine Aspirin Stroke Study Group, N Engl J Med, 321, 501-7.

Hathaway, C.A., D.D. Heistad, D.J. Piegors and F.J. Miller, Jr., 2002, Regression of atherosclerosis in monkeys reduces vascular superoxide levels, Circ Res, 90, 277-83.

Hauser, W., K.P. Knobeloch, M. Eigenthaler, S. Gambaryan, V. Krenn, J. Geiger, M. Glazova, E. Rohde, I. Horak, U. Walter and M. Zimmer, 1999, Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice, Proc Natl Acad Sci U S A, 96, 8120-5.

Haverkate, F., S.G. Thompson, S.D. Pyke, J.R. Gallimore and M.B. Pepys, 1997, Production of C-reactive protein and risk of coronary events in stable and unstable angina. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group, Lancet, 349 (9050), 462-6.

Haverstick, D.M., J.F. Cowan, K.M. Yamada and S.A. Santoro, 1985, Inhibition of platelet adhesion to fibronectin, fibrinogen, and von Willebrand factor substrates by a synthetic tetrapeptide derived from the cell-binding domain of fibronectin, Blood, 66, 946-52.

Hebert, D. and J.Y. Lam, 2000, Nitroglycerin rebound associated with vascular, rather than platelet, hypersensitivity, J Am Coll Cardiol, 36, 2311-6.

Hebert, D., J.X. Xiang and J.Y. Lam, 1997, Persistent inhibition of platelets during continuous nitroglycerin therapy despite hemodynamic tolerance, Circulation, 95, 1308-13.

Hecker, M., M. Cattaruzza and A.H. Wagner, 1999, Regulation of inducible nitric oxide synthase gene expression in vascular smooth muscle cells, Gen Pharmacol, 32, 9-16.

Heeschen, C., C.W. Hamm, U. Laufs, S. Snapinn, M. Bohm and H.D. White, 2002, Withdrawal of statins increases event rates in patients with acute coronary syndromes, Circulation, 105, 1446-52.

Hegde, L.G., P. Srivastava, R. Kumari and M. Dikshit, 1998, Alterations in the vasoreactivity of hypertensive rat aortic rings: role of nitric oxide and superoxide radicals, Clin Exp Hypertens, 20, 885-901.

Heimark, R.L., D.R. Twardzik and S.M. Schwartz, 1986, Inhibition of endothelial regeneration by type-beta transforming growth factor from platelets, Science, 233, 1078-80.

Heinzel, B., M. John, P. Klatt, E. Bohme and B. Mayer, 1992, Ca^{2+}/calmodulin-dependent formation of hydrogen peroxide by brain nitric oxide synthase, Biochem J, 281, 627-30.

Heinzel, B., P. Schmidt, N. Maurin, R. Kirsten, K. Nelson, D. Wieland and H.G. Sieberth, 1994, Endothelin-1 potentiates ADP-induced platelet aggregation in chronic renal failure, Ren Fail, 16, 481-9.

Heitzer, T., C. Brockhoff, B. Mayer, A. Warnholtz, H. Mollnau, S. Henne, T. Meinertz and T. Munzel, 2000, Tetrahydrobiopterin improves endothelium-dependent vasodilation in chronic smokers : evidence for a dysfunctional nitric oxide synthase, Circ Res, 86, E36-41.

Heller, R., F. Munscher-Paulig, R. Grabner and U. Till, 1999a, L-Ascorbic acid potentiates nitric oxide synthesis in endothelial cells, J Biol Chem, 274, 8254-60.

Heller, R., T. Polack, R. Grabner and U. Till, 1999b, Nitric oxide inhibits proliferation of human endothelial cells via a mechanism independent of cGMP, Atherosclerosis, 144, 49-57.

Henn, V., J.R. Slupsky, M. Grafe, I. Anagnostopoulos, R. Forster, G. Muller-Berghaus and R.A. Kroczek, 1998, CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells, Nature, 391, 591-4.

Hennekens, C.H., J.E. Buring, P. Sandercock, R. Collins and R. Peto, 1989, Aspirin and other antiplatelet agents in the secondary and primary prevention of cardiovascular disease, Circulation, 80, 749-56.

Hennekens, C.H., R. Peto, G.B. Hutchison and R. Doll, 1988, An overview of the British and American aspirin studies, N Engl J Med, 318, 923-4.

Hermann, A., B.H. Rauch, M. Braun, K. Schror and A.A. Weber, 2001, Platelet CD40 ligand (CD40L)-subcellular localization, regulation of expression, and inhibition by clopidogrel, Platelets, 12, 74-82.

Hernandez-Perera, O., D. Perez-Sala, J. Navarro-Antolin, R. Sanchez-Pascuala, G. Hernandez, C. Diaz and S. Lamas, 1998, Effects of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, atorvastatin and simvastatin, on the expression of endothelin-1 and endothelial nitric oxide synthase in vascular endothelial cells, J Clin Invest, 101, 2711-9.

Hines, R. and P.G. Barash, 1989, Infusion of sodium nitroprusside induces platelet dysfunction *in vitro*, Anesthesiology, 70, 611-5.

Hink, U., H. Li, H. Mollnau, M. Oelze, E. Matheis, M. Hartmann, M. Skatchkov, F. Thaiss, R.A. Stahl, A. Warnholtz, T. Meinertz, K. Griendling, D.G. Harrison, U. Forstermann and T. Munzel, 2001, Mechanisms underlying endothelial dysfunction in diabetes mellitus, Circ Res, 88, E14-22.

Hinz, B. and H. Schroder, 1999, The nitric oxide donor SIN-1 is free of tolerance and maintains its cyclic GMP stimulatory potency in nitrate-tolerant LLC-PK1 cells, Pharm Res, 16, 633-6.

Hirai, T., S. Sasayama, T. Kawasaki and S. Yagi, 1989, Stiffness of systemic arteries in patients with myocardial infarction. A noninvasive method to predict severity of coronary atherosclerosis, Circulation, 80, 78-86.

Hiramatsu, K., H. Nozaki and S. Arimori, 1987, Reduction of platelet aggregation induced by euglycaemic insulin clamp, Diabetologia, 30, 310-3.

Hirashima, O., H. Kawano, T. Motoyama, N. Hirai, M. Ohgushi, K. Kugiyama, H. Ogawa and H. Yasue, 2000, Improvement of endothelial function and insulin sensitivity with vitamin C in patients with coronary spastic angina: possible role of reactive oxygen species, J Am Coll Cardiol, 35, 1860-6.

Hirata, K., N. Miki, Y. Kuroda, T. Sakoda, S. Kawashima and M. Yokoyama, 1995, Low concentration of oxidized low-density lipoprotein and lysophosphatidylcholine upregulate constitutive nitric oxide synthase mRNA expression in bovine aortic endothelial cells, Circ Res, 76, 958-62.

Hirata, T., F. Ushikubi, A. Kakizuka, M. Okuma and S. Narumiya, 1996, Two thromboxane A_2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation, J Clin Invest, 97, 949-56.

Hirsh, P.D., L.D. Hillis, W.B. Campbell, B.G. Firth and J.T. Willerson, 1981, Release of prostaglandins and thromboxane into the coronary circulation in patients with ischemic heart disease, N Engl J Med, 304, 685-91.

Hoberg, E., R. Dietz, U. Frees, H.A. Katus, B. Rauch, A. Schomig, G. Schuler, F. Schwarz, H. Tillmanns and J. Niebauer, 1994, Verapamil treatment after coronary angioplasty in patients at high risk of recurrent stenosis, Br Heart J, 71, 254-60.

Hoeffner, U., C. Boulanger and P.M. Vanhoutte, 1989, Proximal and distal dog coronary arteries respond differently to basal EDRF but not to NO, Am J Physiol, 256, H828-31.

Hogan, J.C., M.J. Lewis and A.H. Henderson, 1989, Glyceryl trinitrate and platelet aggregation: effects of N-acetyl-cysteine, Br J Clin Pharmacol, 27, 617-9.

Hogg, N., 2000, Biological chemistry and clinical potential of S-nitrosothiols, Free Radic Biol Med, 28, 1478-86.

Holland, J.A., J.W. Meyer, M.M. Chang, R.W. O'Donnell, D.K. Johnson and L.M. Ziegler, 1998, Thrombin stimulated reactive oxygen species production in cultured human endothelial cells, Endothelium, 6, 113-21.

Holmes, M.B., S.S. Kabbani, C.M. Terrien, M.W. Watkins, B.E. Sobel and D.J. Schneider, 2001, Quantification by flow cytometry of the efficacy of and interindividual variation of platelet inhibition induced by treatment with tirofiban and abciximab, Coron Artery Dis, 12, 245-53.

Holmes, M.B., B.E. Sobel, C.P. Cannon and D.J. Schneider, 2000, Increased platelet reactivity in patients given orbofiban after an acute coronary syndrome: an OPUS-TIMI 16 substudy. Orbofiban in Patients with Unstable coronary syndromes. Thrombolysis In Myocardial Infarction, Am J Cardiol, 85, 491-3, A10.

Holmes, M.B., B.E. Sobel, D.B. Howard and D.J. Schneider, 1999, Differences between activation thresholds for platelet P-selectin glycoprotein IIb-IIIa expression and their clinical implications, Thromb Res, 95, 75-82.

Holmsen, H., 1989, Physiological functions of platelets, Ann Med, 21, 23-30.

Homoncik, M., A.D. Blann, U. Hollenstein, T. Pernerstorfer, H.G. Eichler and B. Jilma, 2000, Systemic inflammation increases shear stress-induced platelet plug formation measured by the PFA-100, Br J Haematol, 111, 1250-2.

Hornig, B., U. Landmesser, C. Kohler, D.Ahlersmann, S. Spiekermann, A. Christoph, H. Tatge and H. Drexler, 2001, Comparative effect of ACE inhibition and angiotensin II type I receptor antagonism on bioavailability of nitric oxide in patients with coronary artery disease: role of superoxide dismutase, Circulation, 103, 799-805.

Horowitz, J.D., 2000, Nitrovasodilators, Vol. 4 (Humana Press Inc, Totowa).

Horowitz, J.D., E.M. Antman, B.H. Lorell, W.H. Barry and T.W. Smith, 1983, Potentiation of the cardiovascular effects of nitroglycerin by N-acetylcysteine, Circulation, 68, 1247-53.

Horowitz, J.D., C.A. Henry, M.L. Syrjanen, W.J. Louis, R.D. Fish, T.W. Smith and E.M. Antman, 1988, Combined use of nitroglycerin and N-acetylcysteine in the management of unstable angina pectoris, Circulation, 77, 787-94.

Horowitz, J.D. and M.L. Mashford, 1979, Perhexiline maleate in the treatment of severe angina pectoris, Med J Aust, 1, 485-8.

Horstrup, K., B. Jablonka, P. Honig-Liedl, M. Just, K. Kochsiek and U. Walter, 1994, Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition, Eur J Biochem, 225, 21-7.

Hourani, S.M. and N.J. Cusack, 1991, Pharmacological receptors on blood platelets, Pharmacol Rev, 43, 243-98.

Hourani, S.M. and D.A. Hall, 1994, Receptors for ADP on human blood platelets, Trends Pharmacol Sci, 15, 103-8.

Howard, C.M., D.J. Sexton and B. Mutus, 1998, S-nitrosoglutathione/glutathione disulphide/Cu^{2+}-dependent stimulation of L-arginine transport in human platelets, Thromb Res, 91, 113-20.

Hoylaerts, M.F., C. Oury, E. Toth-Zsamboki and J. Vermylen, 2000, ADP receptors in platelet activation and aggregation, Platelets, 11, 307-9.

Hrboticky, N., G. Draude, G. Hapfelmeier, R. Lorenz and P.C. Weber, 1999, Lovastatin decreases the receptor-mediated degradation of acetylated and oxidized LDLs in human blood monocytes during the early stage of differentiation into macrophages, Arterioscler Thromb Vasc Biol, 19, 1267-75.

Huang, A. and A. Koller, 1996, Both nitric oxide and prostaglandin-mediated responses are impaired in skeletal muscle arterioles of hypertensive rats, J Hypertens, 14, 887-95.

Huang, A., D. Sun, G. Kaley and A. Koller, 1998, Superoxide released to high intra-arteriolar pressure reduces nitric oxide-mediated shear stress- and agonist-induced dilations, Circ Res, 83, 960-5.

Huang, E.M. and T.C. Detwiler, 1981, Characteristics of the synergistic actions of platelet agonists, Blood, 57, 685-91.

Huggins, G.S., R.C. Pasternak, N.M. Alpert, A.J. Fischman and H. Gewirtz, 1998, Effects of short-term treatment of hyperlipidemia on coronary vasodilator function and myocardial perfusion in regions having substantial impairment of baseline dilator reverse, Circulation, 98, 1291-6.

Hughes, A., S. Daunt, G. Vass and J. Wickes, 1982, *In vivo* platelet activation following myocardial infarction and acute coronary ischaemia, Thromb Haemost, 48, 133-5.

Hughes, M.N., 1999, Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite, Biochim Biophys Acta, 1411, 263-72.

Humbert, M., P. Nurden, C. Bihour, J.M. Pasquet, J. Winckler, E. Heilmann, P. Savi, J.M. Herbert, T.J. Kunicki and A.T. Nurden, 1996, Ultrastructural studies of platelet aggregates from human subjects receiving clopidogrel and from a patient with an inherited defect of an ADP-dependent pathway of platelet activation, Arterioscler Thromb Vasc Biol, 16, 1532-43.

Hussain, A.S., J.F. Brien, G.S. Marks and K. Nakatsu, 1996, Superoxide does not inhibit glyceryl trinitrate-rabbit aortic strip-mediated relaxation of rabbit Taenia coli. Evidence against a role for nitric oxide itself as the smooth muscle active drug metabolite?, Drug Metab Dispos, 24, 780-5.

Huvers, F.C., P.W. De Leeuw, A.J. Houben, C.H. De Haan, K. Hamulyak, H. Schouten, B.H. Wolffenbuttel and N.C. Schaper, 1999, Endothelium-dependent vasodilatation, plasma markers of endothelial function, and adrenergic vasoconstrictor responses in type 1 diabetes under near-normoglycemic conditions, Diabetes, 48, 1300-7.

Hwang, S.J., C.M. Ballantyne, A.R. Sharrett, L.C. Smith, C.E. Davis, A.M. Gotto, Jr. and E. Boerwinkle, 1997, Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study, Circulation, 96, 4219-25.

Ide, T., H. Tsutsui, S. Kinugawa, H. Utsumi and A. Takeshita, 1999, Amiodarone protects cardiac myocytes against oxidative injury by its free radical scavenging action, Circulation, 100, 690-2.

Ignarro, L.J., G.M. Buga, K.S. Wood, R.E. Byrns and G. Chaudhuri, 1987a, Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide, Proc Natl Acad Sci U S A, 84, 9265-9.

Ignarro, L.J., R.E. Byrns, G.M. Buga and K.S. Wood, 1987b, Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical, Circ Res, 61, 866-79.

Ignarro, L.J. and C.A. Gruetter, 1980, Requirement of thiols for activation of coronary arterial guanylate cyclase by glyceryl trinitrate and sodium nitrite: possible involvement of S-nitrosothiols, Biochim Biophys Acta, 631, 221-31.

Ignarro, L.J., C. Napoli and J. Loscalzo, 2002, Nitric oxide donors and cardiovascular agents modulating the bioactivity of nitric oxide: an overview, Circ Res, 90, 21-8.

Iida, N., R. Iida, N. Takeyama and T. Tanaka, 1993, Increased platelet aggregation and fatty acid oxidation in diabetic rats, Biochem Mol Biol Int, 30, 177-85.

Ikeda, H., Y. Koga, T. Oda, K. Kuwano, H. Nakayama, T. Ueno, H. Toshima, L.H. Michael and M.L. Entman, 1994, Free oxygen radicals contribute to platelet aggregation and cyclic flow variations in stenosed and endothelium-injured canine coronary arteries, J Am Coll Cardiol, 24, 1749-56.

Ikeda, Y., M. Handa, K. Kawano, T. Kamata, M. Murata, Y. Araki, H. Anbo, Y. Kawai, K. Watanabe and I. Itagaki, 1991, The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress, J Clin Invest, 87, 1234-40.

Ikeda, Y., K. Matsumoto, K. Dohi, H. Jimbo, K. Sasaki and K. Satoh, 2001, Direct superoxide scavenging activity of nonsteroidal anti-inflammatory drugs: determination by electron spin resonance using the spin trap method, Headache, 41, 138-41.

IMPACT-II., 1997, Randomised placebo-controlled trial of effect of eptifibatide on complications of percutaneous coronary intervention. Integrilin to Minimise Platelet Aggregation and Coronary Thrombosis-II, Lancet, 349 (9063), 1422-8.

Indik, J.H., S. Goldman and M.A. Gaballa, 2001, Oxidative stress contributes to vascular endothelial dysfunction in heart failure, Am J Physiol Heart Circ Physiol, 281, H1767-70.

Inoue, N., R.C. Venema, H.S. Sayegh, Y. Ohara, T.J. Murphy and D.G. Harrison, 1995, Molecular regulation of the bovine endothelial cell nitric oxide synthase by transforming growth factor-beta 1, Arterioscler Thromb Vasc Biol, 15, 1255-61.

Inoue, T., M. Hayashi, T. Uchida, K. Takayanagi, T. Hayashi and S. Morooka, 2001, Significance of platelet aggregability immediately after blood sampling and effect of cigarette smoking, Platelets, 12, 415-8.

Islim, I.F., D.G. Beevers and D. Bareford, 1992, The effect of antihypertensive drugs on *in vivo* platelet activity in essential hypertension, J Hypertens, 10, 379-83.

ISIS-2 (Second International Study of Infarct Survival) Collaborative Group, 1988, Randomized trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2, J Am Coll Cardiol, 12, 3A-13A.

ISIS-4 (Fourth International Study of Infarct Survival) Collaborative Group., 1995, A randomised factorial trial assessing early oral captopril, oral mononitrate, and intravenous magnesium sulphate in 58,050 patients with suspected acute myocardial infarction: ISIS-4, Lancet, 345 (8951), 669-85.

Israels, S.J., J.M. Gerrard, Y.V. Jacques, A. McNicol, B. Cham, M. Nishibori and D.F. Bainton, 1992, Platelet dense granule membranes contain both granulophysin and P-selectin (GMP-140), Blood, 80, 143-52.

Iuliano, L., A.R. Colavita, R. Leo, D. Pratico and F. Violi, 1997, Oxygen free radicals and platelet activation, Free Radic Biol Med, 22, 999-1006.

Iuliano, L., J.Z. Pedersen, D. Pratico, G. Rotilio and F. Violi, 1994, Role of hydroxyl radicals in the activation of human platelets, Eur J Biochem, 221, 695-704.

Iuliano, L., D. Pratico, A. Ghiselli, M.S. Bonavita and F. Violi, 1991, Superoxide dismutase triggers activation of "primed" platelets, Arch Biochem Biophys, 289, 180-3.

Iuliano, L., D. Pratico, C. Greco, E. Mangieri, G. Scibilia, G.A. FitzGerald and F. Violi, 2001, Angioplasty increases coronary sinus F_2-isoprostane formation: evidence for *in vivo* oxidative stress during PTCA., J Am Coll Cardiol, 37, 76-80.

Ivanova, K., M. Schaefer, C. Drummer and R. Gerzer, 1993, Effects of nitric oxide-containing compounds on increases in cytosolic ionized Ca^{2+} and on aggregation of human platelets, Eur J Pharmacol, 244, 37-47.

Iwase, E., M. Tawata, K. Aida, Y. Ozaki, S. Kume, K. Satoh, R. Qi and T. Onaya, 1998, A cross-sectional evaluation of spontaneous platelet aggregation in relation to complications in patients with type II diabetes mellitus, Metabolism, 47, 699-705.

Jackson, S.K., K.J. Liu, M. Liu and G.S. Timmins, 2002, Detection and removal of contaminating hydroxylamines from the spin trap DEPMPO, and re-evaluation of its use to indicate nitrone radical cation formation and S(N)1 reactions, Free Radic Biol Med, 32, 228-32.

Jackson, T.S., A. Xu, J.A. Vita and J.F. Keaney, Jr., 1998, Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations, Circ Res, 83, 916-22.

Jafri, S.M., T. Ozawa, E. Mammen, T.B. Levine, C. Johnson and S. Goldstein, 1993, Platelet function, thrombin and fibrinolytic activity in patients with heart failure, Eur Heart J, 14, 205-12.

Jager, A., V.W. van Hinsbergh, P.J. Kostense, J.J. Emeis, J.S. Yudkin, G. Nijpels, J.M. Dekker, R.J. Heine, L.M. Bouter and C.D. Stehouwer, 1999, von Willebrand factor, C-reactive protein, and 5-year mortality in diabetic and nondiabetic subjects: the Hoorn Study, Arterioscler Thromb Vasc Biol, 19, 3071-8.

Jansson, J.H., T.K. Nilsson and O. Johnson, 1991, von Willebrand factor in plasma: a novel risk factor for recurrent myocardial infarction and death, Br Heart J, 66, 351-5.

Jarvis, B. and K. Simpson, 2000, Clopidogrel: a review of its use in the prevention of atherothrombosis, Drugs, 60, 347-77.

Jay, M.T., S. Chirico, R.C. Siow, K.R. Bruckdorfer, M. Jacobs, D.S. Leake, J.D. Pearson and G.E. Mann, 1997, Modulation of vascular tone by low density lipoproteins: effects on L-arginine transport and nitric oxide synthesis, Exp Physiol, 82, 349-60.

Jia, L., C. Bonaventura, J. Bonaventura and J.S. Stamler, 1996, S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control, Nature, 380, 221-6.

Jialal, I., D. Stein, D. Balis, S.M. Grundy, B. Adams-Huet and S. Devaraj, 2001, Effect of hydroxymethyl glutaryl coenzyme a reductase inhibitor therapy on high sensitive C-reactive protein levels, Circulation, 103, 1933-5.

Jiang, J., G. Valen, S. Tokuno, P. Thoren and J. Pernow, 2000, Endothelial dysfunction in atherosclerotic mice: improved relaxation by combined supplementation with L-arginine-tetrahydrobiopterin and enhanced vasoconstriction by endothelin, Br J Pharmacol, 131, 1255-61.

Jimi, S., K. Saku, H. Kusaba, H. Itabe, N. Koga and S. Takebayashi, 1998, Deposition of oxidized low-density lipoprotein and collagenosis occur coincidentally in human coronary stenosis: an immunohistochemical study of atherectomy, Coron Artery Dis, 9, 551-7.

Jin, J., J.L. Daniel and S.P. Kunapuli, 1998, Molecular basis for ADP-induced platelet activation. II. The $P2Y_1$ receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets, J Biol Chem, 273, 2030-4.

Jin, J. and S.P. Kunapuli, 1998, Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation, Proc Natl Acad Sci U S A, 95, 8070-4.

Johnson, G.J., L.A. Leis and G.S. Francis, 1986, Disparate effects of the calcium-channel blockers, nifedipine and verapamil, on alpha 2-adrenergic receptors and thromboxane A_2-induced aggregation of human platelets, Circulation, 73, 847-54.

Jones, R.D., J.T. Hancock and A.H. Morice, 2000, NADPH oxidase: a universal oxygen sensor?, Free Radic Biol Med, 29, 416-24.

Jones, S.A., V.B. O'Donnell, J.D. Wood, J.P. Broughton, E.J. Hughes and O.T. Jones, 1996, Expression of phagocyte NADPH oxidase components in human endothelial cells, Am J Physiol, 271, H1626-34.

Jordan, R.A., L. Seth, P. Casebolt, M.J. Hayes, M.M. Wilen and J. Franciosa, 1986, Rapidly developing tolerance to transdermal nitroglycerin in congestive heart failure, Ann Intern Med, 104, 295-8.

Jugdutt, B.I., B.L. Schwarz-Michorowski, W.J. Tymchak and J.R. Burton, 1997, Prompt improvement of left ventricular function and preservation of topography with combined reperfusion and intravenous nitroglycerin in acute myocardial infarction, Cardiol, 88, 170-9.

Jukema, J.W., A.V. Bruschke, A.J. van Boven, J.H. Reiber, E.T. Bal, A.H. Zwinderman, H. Jansen, G.J. Boerma, F.M. van Rappard and K.I. Lie, 1995, Effects of lipid lowering by pravastatin on progression and regression of coronary artery disease in symptomatic men with normal to moderately elevated serum cholesterol levels. The Regression Growth Evaluation Statin Study (REGRESS), Circulation, 91, 2528-40.

Jurt, U., T. Gori, A. Ravandi, S. Babaei, P. Zeman and J.D. Parker, 2001, Differential effects of pentaerythritol tetranitrate and nitroglycerin on the development of tolerance and evidence of lipid peroxidation: a human *in vivo* study, J Am Coll Cardiol, 38, 854-9.

Juul-Moller, S., N. Edvardsson, B. Jahnmatz, A. Rosen, S. Sorensen and R. Omblus, 1992, Double-blind trial of aspirin in primary prevention of myocardial infarction in patients with stable chronic angina pectoris. The Swedish Angina Pectoris Aspirin Trial (SAPAT) Group, Lancet, 340 (8833), 1421-5.

Kaesemeyer, W.H., R.B. Caldwell, J. Huang and R.W. Caldwell, 1999, Pravastatin sodium activates endothelial nitric oxide synthase independent of its cholesterol-lowering actions, J Am Coll Cardiol, 33, 234-41.

Kaesemeyer, W.H., A.A. Ogonowski, L. Jin, R.B. Caldwell and R.W. Caldwell, 2000, Endothelial nitric oxide synthase is a site of superoxide synthesis in endothelial cells treated with glyceryl trinitrate, Br J Pharmacol, 131, 1019-23.

Kahn, M.L., M. Nakanishi-Matsui, M.J. Shapiro, H. Ishihara and S.R. Coughlin, 1999, Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin, J Clin Invest, 103, 879-87.

Kahn, M.L., Y.W. Zheng, W. Huang, V. Bigornia, D. Zeng, S. Moff, R.V. Farese, Jr., C. Tam and S.R. Coughlin, 1998, A dual thrombin receptor system for platelet activation, Nature, 394, 690-4.

Kahn, N.N., M.A. Najeeb, M. Ishaq, A. Rahim and A.K. Sinha, 1992, Normalization of impaired response of platelets to prostaglandin E_1/I_2 and synthesis of prostacyclin by insulin in unstable angina pectoris and in acute myocardial infarction, Am J Cardiol, 70, 582-6.

Kalinowski, L., L.W. Dobrucki, V. Brovkovych and T. Malinski, 2002, Increased nitric oxide bioavailability in endothelial cells contributes to the pleiotrophic effects of cerivastatin, Circulation, 105, 933-8.

Kallmann, R., H.K. Nieuwenhuis, P.G. de Groot, J. van Gijn and J.J. Sixma, 1987, Effects of low doses of aspirin, 10 mg and 30 mg daily, on bleeding time, thromboxane production and 6-keto-PGF_1 alpha excretion in healthy subjects, Thromb Res, 45, 355-61.

Kanani, P.M., C.A. Sinkey, R.L. Browning, M. Allaman, H.R. Knapp and W.G. Haynes, 1999, Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans, Circulation, 100, 1161-8.

Karamanoglu, M., M.F. O'Rourke, A.P. Avolio and R.P. Kelly, 1993, An analysis of the relationship between central aortic and peripheral upper limb pressure waves in man, Eur Heart J, 14, 160-7.

Karlberg, K.E., K. Torfgard, J. Ahlner and C. Sylven, 1992, Dose-dependent effect of intravenous nitroglycerin on platelet aggregation, and correlation with plasma glyceryl dinitrate concentration in healthy men, Am J Cardiol, 69, 802-5.

Karmann, K., C.C. Hughes, J. Schechner, W.C. Fanslow and J.S. Pober, 1995, CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression, Proc Natl Acad Sci U S A, 92, 4342-6.

Kato, M., N. Shiode, T. Yamagata, H. Matsuura and G. Kajiyama, 1997, Coronary segmental responses to acetylcholine and bradykinin in patients with atherosclerotic risk factors, Am J Cardiol, 80, 751-5.

Katz, R.J., W.S. Levy, L. Buff and A.G. Wasserman, 1991, Prevention of nitrate tolerance with angiotension converting enzyme inhibitors, Circulation, 83, 1271-7.

Katz, S.D., L. Biasucci, C. Sabba, J.A. Strom, G. Jondeau, M. Galvao, S. Solomon, S.D. Nikolic, R. Forman and T.H. LeJemtel, 1992, Impaired endothelium-mediated vasodilation in the peripheral vasculature of patients with congestive heart failure, J Am Coll Cardiol, 19, 918-25.

Katz, S.D., M. Schwarz, J. Yuen and T.H. LeJemtel, 1993, Impaired acetylcholine-mediated vasodilation in patients with congestive heart failure. Role of endothelium-derived vasodilating and vasoconstricting factors, Circulation, 88, 55-61.

Katzman, P.L., R. Bose, S. Henry, D.L. McLean, S. Walker, C. Fyfe, Y. Perry, D. Mymin and P. Bolli, 1994, Serum lipid profile determines platelet reactivity to native and modified LDL-cholesterol in humans, Thromb Haemost, 71, 627-32.

Kaye, D.M., B.A. Ahlers, D.J. Autelitano and J.P. Chin-Dusting, 2000, *In vivo* and *in vitro* evidence for impaired arginine transport in human heart failure, Circulation, 102, 2707-2712.

Keaney, J.F., Jr., J.M. Gaziano, A. Xu, B. Frei, J. Curran-Celentano, G.T. Shwaery, J. Loscalzo and J.A. Vita, 1993, Dietary antioxidants preserve endothelium-dependent vessel relaxation in cholesterol-fed rabbits, Proc Natl Acad Sci U S A, 90, 11880-4.

Keaney, J.F., Jr., A. Xu, D. Cunningham, T. Jackson, B. Frei and J.A. Vita, 1995, Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular oxidative stress and superoxide generation, J Clin Invest, 95, 2520-9.

Keegan, A., H. Walbank, M.A. Cotter and N.E. Cameron, 1995, Chronic vitamin E treatment prevents defective endothelium-dependent relaxation in diabetic rat aorta, Diabetologia, 38, 1475-8.

Kehrer, J.P., 2000, The Haber-Weiss reaction and mechanisms of toxicity, Toxicology, 149, 43-50.

Kelly, R., C. Hayward, A. Avolio and M. O'Rourke, 1989, Noninvasive determination of age-related changes in the human arterial pulse, Circulation, 80, 1652-9.

Kelly, R.P., S.C. Millasseau, J.M. Ritter and P.J. Chowienczyk, 2001, Vasoactive drugs influence aortic augmentation index independently of pulse-wave velocity in healthy men, Hypertension, 37, 1429-33.

Kenet, G., A. Lubetsky, B. Shenkman, I. Tamarin, R. Dardik, G. Rechavi, A. Barzilai, U. Martinowitz, N. Savion and D. Varon, 1998, Cone and platelet analyser (CPA): a new test for the prediction of bleeding among thrombocytopenic patients, Br J Haematol, 101, 255-9.

Kenkare, S.R., C. Han and L.Z. Benet, 1994, Correlation of the response to nitroglycerin in rabbit aorta with the activity of the mu class glutathione S-transferase, Biochem Pharmacol, 48, 2231-5.

Kennedy, J.A., A.J. Kiosoglous, G.A. Murphy, M.A. Pelle and J.D. Horowitz, 2000, Effect of perhexiline and oxfenicine on myocardial function and metabolism during low-flow ischemia/reperfusion in the isolated rat heart, J Cardiovasc Pharmacol, 36, 794-801.

Kennedy, J.A., P. Mohan, M.A. Pelle, S.R. Wade and J.D. Horowitz, 1999, The effects of perhexiline on the rat coronary vasculature, Eur J Pharmacol, 370, 263-70.

Kennedy, J.A., S.A. Unger and J.D. Horowitz, 1996, Inhibition of carnitine palmitoyltransferase-1 in rat heart and liver by perhexiline and amiodarone, Biochem Pharmacol, 52, 273-80.

Kereiakes, D.J., N.S. Kleiman, J. Ambrose, M. Cohen, S. Rodriguez, T. Palabrica, H.C. Herrmann, J.M. Sutton, W.D. Weaver, D.B. McKee, V. Fitzpatrick and F.L. Sax, 1996, Randomized, double-blind, placebo-controlled dose-ranging study of tirofiban (MK-383) platelet IIb/IIIa blockade in high risk patients undergoing coronary angioplasty, J Am Coll Cardiol, 27, 536-42.

Kerins, D.M., L. Roy, G.A. FitzGerald and D.J. Fitzgerald, 1989, Platelet and vascular function during coronary thrombolysis with tissue-type plasminogen activator, Circulation, 80, 1718-25.

Kerr, S., M.J. Brosnan, M. McIntyre, J.L. Reid, A.F. Dominiczak and C.A. Hamilton, 1999, Superoxide anion production is increased in a model of genetic hypertension: role of the endothelium, Hypertension, 33, 1353-8.

Kestin, A.S., P.A. Ellis, M.R. Barnard, A. Errichetti, B.A. Rosner and A.D. Michelson, 1993, Effect of strenuous exercise on platelet activation state and reactivity, Circulation, 88, 1502-11.

Khalil, M.E., A.W. Basher, E.J. Brown, Jr. and I.A. Alhaddad, 2001, A remarkable medical story: benefits of angiotensin-converting enzyme inhibitors in cardiac patients, J Am Coll Cardiol, 37, 1757-64.

Khan, A.U., D. Kovacic, A. Kolbanovskiy, M. Desai, K. Frenkel and N.E. Geacintov, 2000, The decomposition of peroxynitrite to nitroxyl anion (NO-) and singlet oxygen in aqueous solution, Proc Natl Acad Sci U S A, 97, 2984-9.

Kikuta, K., T. Sawamura, S. Miwa, N. Hashimoto and T. Masaki, 1998, High-affinity arginine transport of bovine aortic endothelial cells is impaired by lysophosphatidylcholine, Circ Res, 83, 1088-96.

Kim, D., S.D. Rybalkin, X. Pi, Y. Wang, C. Zhang, T. Munzel, J.A. Beavo, B.C. Berk and C. Yan, 2001, Upregulation of phosphodiesterase $1A_1$ expression is associated with the development of nitrate tolerance, Circulation, 104, 2338-43.

Kim, H., J.Y. Seo, K.H. Roh, J.W. Lim and K.H. Kim, 2000, Suppression of NF-kappaB activation and cytokine production by N-acetylcysteine in pancreatic acinar cells, Free Radic Biol Med, 29, 674-83.

Kinlough-Rathbone, R.L., M.A. Packham and J.F. Mustard, 1983, Platelet aggregation (Churchil Livingstone, London) p. 64-91.

Kinsella, B.T., D.J. O'Mahony and G.A. Fitzgerald, 1997, The human thromboxane A_2 receptor alpha isoform (TP alpha) functionally couples to the G proteins Gq and G11 *in vivo* and is activated by the isoprostane 8-epi prostaglandin F_2 alpha, J Pharmacol Exp Ther, 281, 957-64.

Kirk, E.A., M.C. Dinauer, H. Rosen, A. Chait, J.W. Heinecke and R.C. LeBoeuf, 2000, Impaired superoxide production due to a deficiency in phagocyte NADPH oxidase fails to inhibit atherosclerosis in mice, Arterioscler Thromb Vasc Biol, 20, 1529-35.

Kishi, Y., T. Ashikaga and F. Numano, 1992, Inhibition of platelet aggregation by prostacyclin is attenuated after exercise in patients with angina pectoris, Am Heart J, 123, 291-7.

Kjekshus, J. and T.R. Pedersen, 1995, Reducing the risk of coronary events: evidence from the Scandinavian Simvastatin Survival Study (4S), Am J Cardiol, 76, 64C-68C.

Kjekshus, J., K. Swedberg and S. Snapinn, 1992, Effects of enalapril on long-term mortality in severe congestive heart failure. CONSENSUS Trial Group, Am J Cardiol, 69, 103-7.

Kjeldsen, S.E., K. Lande, K. Gjesdal, A. Westheim, O.P. Foss, P. Leren and I.K. Eide, 1987, Increased platelet release reaction in 50-year-old men with essential hypertension: correlation with atherogenic cholesterol fractions, Am Heart J, 113, 151-5.

Kjeldsen, S.E., R.E. Kolloch, G. Leonetti, J.M. Mallion, A. Zanchetti, D. Elmfeldt, I. Warnold, and L. Hansson, 2000, Influence of gender and age on preventing cardiovascular disease by antihypertensive treatment and acetylsalicylic acid. The HOT study, J Hypertens, 18, 629-42.

Kleiman, N.S., K.B. Schechtman, P.M. Young, D.A. Goodman, W.E. Boden, C.M. Pratt and R. Roberts, 1990, Lack of diurnal variation in the onset of non-Q wave infarction, Circulation, 81, 548-55.

Klemsdal, T.O., T.L. Andersson, J. Matz, G.A. Ferns, K. Gjesdal and E.E. Anggard, 1994, Vitamin E restores endothelium dependent vasodilatation in cholesterol fed rabbits: *in vivo* measurements by photoplethysmography, Cardiovasc Res, 28, 1397-402.

Klipstein-Grobusch, K., J.M. Geleijnse, J.H. den Breeijen, H. Boeing, A. Hofman, D.E. Grobbee and J.C. Witteman, 1999, Dietary antioxidants and risk of myocardial infarction in the elderly: the Rotterdam Study, Am J Clin Nutr, 69, 261-6.

Klockenbusch, W. and K. Schror, 1993, Prostacyclin contributes to nitroglycerin-mediated relaxation of human umbilical arteries, J Cardiovasc Pharmacol, 22, 510-1.

Knight, D.E. and M.C. Scrutton, 1984, Cyclic nucleotides control a system which regulates Ca^{2+} sensitivity of platelet secretion, Nature, 309, 66-8.

Knobler, H., N. Savion, B. Shenkman, S. Kotev-Emeth and D. Varon, 1998, Shear-induced platelet adhesion and aggregation on subendothelium are increased in diabetic patients, Thromb Res, 90, 181-90.

Knofler, R., T. Urano, J. Malyszko, Y. Takada and A. Takada, 1995, *In vitro* effect of endothelin-1 on collagen and ADP-induced aggregation in human whole blood and platelet rich plasma, Thromb Res, 77, 69-78.

Koenig, W., M. Sund, M. Frohlich, H.G. Fischer, H. Lowel, A. Doring, W.L. Hutchinson and M.B. Pepys, 1999, C-Reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: results from the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) Augsburg Cohort Study, 1984 to 1992, Circulation, 99, 237-42.

Koh, K.K., 2000, Effects of statins on vascular wall: vasomotor function, inflammation, and plaque stability, Cardiovasc Res, 47, 648-57.

Kolodgie, F.D., R. Virmani, H.E. Rice and W.J. Mergner, 1990, Vascular reactivity during the progression of atherosclerotic plaque. A study in Watanabe heritable hyperlipidemic rabbits, Circ Res, 66, 1112-26.

Koltai, M.Z., P. Hadhazy, I. Posa, E. Kocsis, G. Winkler, P. Rosen and G. Pogatsa, 1997, Characteristics of coronary endothelial dysfunction in experimental diabetes, Cardiovasc Res, 34, 157-63.

Komatsu, M., T. Kawagishi, M. Emoto, T. Shoji, A. Yamada, K. Sato, M. Hosoi and Y Nishizawa, 2002, ecNOS gene polymorphism is associated with endothelium-dependent vasodilation in Type 2 diabetes, Am J Physiol Circ Physiol, 283(2), H557-61.

Korbut, R., E. Marcinkiewicz, K. Cieslik and R.J. Gryglewski, 1995, The effect of nitric oxide donors on the release of plasminogen activator inhibitor (PAI) from rabbit platelets *in vitro*, J Physiol Pharmacol, 46, 37-44.

Kostner, K., S. Hornykewycz, P. Yang, T. Neunteufl, D. Glogar, F. Weidinger, G. Maurer and K. Huber, 1997, Is oxidative stress causally linked to unstable angina pectoris? A study in 100 CAD patients and matched controls, Cardiovasc Res, 36, 330-6.

Kosugi, H., T. Kato and K. Kikugawa, 1987, Formation of yellow, orange, and red pigments in the reaction of alk-2-enals with 2-thiobarbituric acid, Anal Biochem, 165, 456-64.

Kotsonis, P., L.G. Frohlich, Z.V. Shutenko, R. Horejsi, W. Pfleiderer and H.H. Schmidt, 2000, Allosteric regulation of neuronal nitric oxide synthase by tetrahydrobiopterin and suppression of auto-damaging superoxide, Biochem J, 346 Pt 3, 767-76.

Kowaluk, E.A. and H.L. Fung, 1990, Dissociation of nitrovasodilator-induced relaxation from cyclic GMP levels during *in vitro* nitrate tolerance, Eur J Pharmacol, 176, 91-5.

Kroll, M.H., T.S. Harris, J.L. Moake, R.I. Handin and A.I. Schafer, 1991, von Willebrand factor binding to platelet GPIb initiates signals for platelet activation, J Clin Invest, 88, 1568-73.

Kroll, M.H., J.D. Hellums, L.V. McIntire, A.I. Schafer and J.L. Moake, 1996, Platelets and shear stress, Blood, 88, 1525-41.

Kroll, M.H. and A.I. Schafer, 1989, Biochemical mechanisms of platelet activation, Blood, 74, 1181-95.

Kugiyama, K., M. Ohgushi, T. Motoyama, O. Hirashima, H. Soejima, K. Misumi, M. Yoshimura, H. Ogawa, S. Sugiyama and H. Yasue, 1998, Intracoronary infusion of reduced glutathione improves endothelial vasomotor response to acetylcholine in human coronary circulation, Circulation, 97, 2299-301.

Kulick, D., A. Roth, N. McIntosh, S.H. Rahimtoola and U. Elkayam, 1988, Resistance to isosorbide dinitrate in patients with severe chronic heart failure: incidence and attempt at hemodynamic prediction, J Am Coll Cardiol, 12, 1023-8.

Kunsch, C. and R.M. Medford, 1999, Oxidative stress as a regulator of gene expression in the vasculature, Circ Res, 85, 753-66.

Kupfer, Y. and S. Tessler, 1997, Ticlopidine and thrombotic thrombocytopenic purpura, N Engl J Med, 337, 1245.

Kurata, T., M. Kurata and T. Okada, 2001, Cerivastatin induces carotid artery plaque stabilization independently of cholesterol lowering in patients with hypercholesterolaemia, J Int Med Res, 29, 329-34.

Kurz, M.A., T.D. Boyer, R. Whalen, T.E. Peterson and D.G. Harrison, 1993, Nitroglycerin metabolism in vascular tissue: role of glutathione S-transferases and relationship between $NO^{\cdot}$ and NO_2^- formation, Biochem J, 292, 545-50.

Kurz, S., U. Hink, G. Nickenig, A.B. Borthayre, D.G. Harrison and T. Munzel, 1999, Evidence for a causal role of the renin-angiotensin system in nitrate tolerance, Circulation, 99, 3181-7.

Kusterer, K., T. Pohl, H.P. Fortmeyer, W. Marz, H. Scharnagl, A. Oldenburg, S. Angermuller, I. Fleming, K.H. Usadel and R. Busse, 1999, Chronic selective hypertriglyceridemia impairs endothelium-dependent vasodilatation in rats, Cardiovasc Res, 42, 783-93.

Kuvin, J.T., A.R. Patel and R.H. Karas, 2001, Need for standardization of noninvasive assessment of vascular endothelial function, Am Heart J, 141, 327-8.

Lacoste, L., J.Y. Lam, J. Hung and D. Waters, 1994, Oral verapamil inhibits platelet thrombus formation in humans, Circulation, 89, 630-4.

Lacoste, L., J.Y. Lam, J. Hung, G. Letchacovski, C.B. Solymoss and D. Waters, 1995, Hyperlipidemia and coronary disease. Correction of the increased thrombogenic potential with cholesterol reduction, Circulation, 92, 3172-7.

Lacoste, L.L., P. Theroux, R.M. Lidon, R. Colucci and J.Y. Lam, 1994, Antithrombotic properties of transdermal nitroglycerin in stable angina pectoris, Am J Cardiol, 73, 1058-62.

Laight, D.W., M.J. Carrier and E.E. Anggard, 1997, Investigation of role for oxidant stress in vascular tolerance development to glyceryl trinitrate *in vitro*, Br J Pharmacol, 120, 1477-82.

Lam, J.Y., J.H. Chesebro and V. Fuster, 1988, Platelets, vasoconstriction, and nitroglycerin during arterial wall injury. A new antithrombotic role for an old drug, Circulation, 78, 712-6.

Lam, J.Y., J.G. Latour, J. Lesperance and D. Waters, 1994, Platelet aggregation, coronary artery disease progression and future coronary events, Am J Cardiol, 73, 333-8.

Laman, J.D., B.J. de Smet, A. Schoneveld and M. van Meurs, 1997, CD40-CD40L interactions in atherosclerosis, Immunol Today, 18, 272-7.

Lambeth, J.D., G. Cheng, R.S. Arnold and W.A. Edens, 2000, Novel homologs of gp91^{phox}, Trends Biochem Sci, 25, 459-61.

Lande, K., S.E. Kjeldsen, I. Os, A. Westheim, I. Hjermann, I. Eide and K. Gjesdal, 1988, Increased platelet and vascular smooth muscle reactivity to low-dose adrenaline infusion in mild essential hypertension, J Hypertens, 6, 219-25.

Lande, K., I. Os, S.E. Kjeldsen, A. Westheim, I. Hjermann, I. Eide and K. Gjesdal, 1987, Increased platelet size and release reaction in essential hypertension, J Hypertens, 5, 401-6.

Lange, R.L., M.S. Reid, D.D. Tresch, M.H. Keelan, V.M. Bernhard and G. Coolidge, 1972, Nonatheromatous ischemic heart disease following withdrawal from chronic industrial nitroglycerin exposure, Circulation, 46, 666-78.

Langford, E.J., A.S. Brown, R.J. Wainwright, A.J. de Belder, M.R. Thomas, R.E. Smith, M.W. Radomski, J.F. Martin and S. Moncada, 1994, Inhibition of platelet activity by S-nitrosoglutathione during coronary angioplasty, Lancet, 344 (8935), 1458-60.

Langford, E.J., R.J. Wainwright and J.F. Martin, 1996, Platelet activation in acute myocardial infarction and unstable angina is inhibited by nitric oxide donors, Arterioscler Thromb Vasc Biol, 16, 51-5.

Larsen, A.I., L. Goransson, T. Aarsland, J.F. Tamby and K. Dickstein, 1997, Comparison of the degree of hemodynamic tolerance during intravenous infusion of nitroglycerin versus nicorandil in patients with congestive heart failure, Am Heart J, 134, 435-41.

Lassegue, B., D. Sorescu, K. Szocs, Q. Yin, M. Akers, Y. Zhang, S.L. Grant, J.D. Lambeth and K.K. Griendling, 2001, Novel gp91^{phox} homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways, Circ Res, 88, 888-94.

Laufs, U., K. Gertz, P. Huang, G. Nickenig, M. Bohm, U. Dirnagl and M. Endres, 2000, Atorvastatin upregulates type III nitric oxide synthase in thrombocytes, decreases platelet activation, and protects from cerebral ischemia in normocholesterolemic mice, Stroke, 31, 2442-9.

Laufs, U., V. La Fata, J. Plutzky and J.K. Liao, 1998, Upregulation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors, Circulation, 97, 1129-35.

Laursen, J.B., S. Boesgaard, H.E. Poulsen and J. Aldershvile, 1996, Nitrate tolerance impairs nitric oxide-mediated vasodilation *in vivo*, Cardiovasc Res, 31, 814-9.

Lawson, D.L., W.W. Nichols, P. Mehta and J.L. Mehta, 1991, Captopril-induced reversal of nitroglycerin tolerance: role of sulfhydryl group vs. ACE-inhibitory activity, J Cardiovasc Pharmacol, 17, 411-8.

Lechi, C., E. Arosio, P. Minuz, P. Guzzo, F. Paluani, D. Sinigaglia and A. Lechi, 1989, Increased platelet aggregation and intracellular calcium in hypertensive patients: effects of cyclo-oxygenase blockade, J Hypertens Suppl, 7, S160-1.

Lee, Y., W.H. Lee, S.C. Lee, K.J. Ahn, Y.H. Choi, S.W. Park, J.D. Seo and J.E. Park, 1999, CD40L activation in circulating platelets in patients with acute coronary syndrome, Cardiology, 92, 11-6.

Lehmann, E.D., R.G. Gosling and P.H. Sonksen, 1992, Arterial wall compliance in diabetes, Diabet Med, 9, 114-9.

Lenda, D.M., B.A. Sauls and M.A. Boegehold, 2000, Reactive oxygen species may contribute to reduced endothelium-dependent dilation in rats fed high salt, Am J Physiol Heart Circ Physiol, 279, H7-H14.

Leo, R., D. Pratico, L. Iuliano, F.M. Pulcinelli, A. Ghiselli, P. Pignatelli, A.R. Colavita, G.A. FitzGerald and F. Violi, 1997, Platelet activation by superoxide anion and hydroxyl radicals intrinsically generated by platelets that had undergone anoxia and then reoxygenated, Circulation, 95, 885-91.

Leon, M.B., D.S. Baim, J.J. Popma, P.C. Gordon, D.E. Cutlip, K.K. Ho, A. Giambartolomei, D.J. Diver, D.M. Lasorda, D.O. Williams, S.J. Pocock and R.E. Kuntz, 1998, A clinical trial comparing three antithrombotic-drug regimens after coronary-artery stenting. Stent Anticoagulation Restenosis Study Investigators, N Engl J Med, 339, 1665-71.

Leoncini, G., M. Maresca and C. Colao, 1991, Oxidative metabolism of human platelets, Biochem Int, 25, 647-55.

Lerea, K.M., J.A. Glomset and E.G. Krebs, 1987, Agents that elevate cAMP levels in platelets decrease thrombin binding, J Biol Chem, 262, 282-8.

Leung, W.H., C.P. Lau and C.K. Wong, 1993, Beneficial effect of cholesterol-lowering therapy on coronary endothelium-dependent relaxation in hypercholesterolaemic patients, Lancet, 341 (8859), 1496-500.

Levine, G.N., B. Frei, S.N. Koulouris, M.D. Gerhard, J.F. Keaney, Jr. and J.A. Vita, 1996, Ascorbic acid reverses endothelial vasomotor dysfunction in patients with coronary artery disease, Circulation, 93, 1107-13.

Levine, P.H., D.G. Sladdin and N.I. Krinsky, 1981, Superoxide, xanthine oxidase and platelet reactions: further studies on mechanisms by which oxidants influence platelets, Thromb Haemost, 45, 290-3.

Lewis, H.D., Jr., J.W. Davis, D.G. Archibald, W.E. Steinke, T.C. Smitherman, J.E.d. Doherty, H.W. Schnaper, M.M. LeWinter, E. Linares, J.M. Pouget, S.C. Sabharwal, E. Chesler and H. DeMots, 1983, Protective effects of aspirin against acute myocardial infarction and death in men with unstable angina. Results of a Veterans Administration Cooperative Study, N Engl J Med, 309, 396-403.

Li, A., A. Prasad, R. Mincemoyer, C. Satorius, N. Epstein, T. Finkel and A.A. Quyyumi, 1999a, Relationship of the C242T $p22^{phox}$ gene polymorphism to angiographic coronary artery disease and endothelial function, Am J Med Genet, 86, 57-61.

Li, C.G., J. Karagiannis and M.J. Rand, 1999b, Comparison of the redox forms of nitrogen monoxide with the nitrergic transmitter in the rat anococcygeus muscle, Br J Pharmacol, 127, 826-34.

Li, J.M., A.M. Mullen, S. Yun, F. Wientjes, G.Y. Brouns, A.J. Thrasher and A.M. Shah, 2002, Essential role of the NADPH oxidase subunit $p47^{phox}$ in endothelial cell superoxide production in response to phorbol ester and tumor necrosis factor-alpha, Circ Res, 90, 143-50.

Li, N., N.H. Wallen and P. Hjemdahl, 1999c, Evidence for prothrombotic effects of exercise and limited protection by aspirin, Circulation, 100, 1374-9.

Li, Y., K.H. Stansbury, H. Zhu and M.A. Trush, 1999d, Biochemical characterization of lucigenin (Bis-N-methylacridinium) as a chemiluminescent probe for detecting intra-mitochondrial superoxide anion radical production, Biochem Biophys Res Commun, 262, 80-7.

Liao, J.K., W.S. Shin, W.Y. Lee and S.L. Clark, 1995, Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase, J Biol Chem, 270, 319-24.

Libby, P., 1995, Molecular bases of the acute coronary syndromes, Circulation, 91, 2844-50.

Libby, P., P.M. Ridker and A. Maseri, 2002, Inflammation and atherosclerosis, Circulation, 105, 1135-43.

Lincoff, A.M., 2000, Potent complementary clinical benefit of abciximab and stenting during percutaneous coronary revascularization in patients with diabetes mellitus: results of the EPISTENT trial, Am Heart J, 139, S46-52.

Lincoff, A.M., R.M. Califf, K.M. Anderson, H.F. Weisman, F.V. Aguirre, N.S. Kleiman, R.A. Harrington and E.J. Topol, 1997, Evidence for prevention of death and myocardial infarction with platelet membrane glycoprotein IIb/IIIa receptor blockade by abciximab (c7E3 Fab) among patients with unstable angina undergoing percutaneous coronary revascularization. EPIC Investigators. Evaluation of 7E3 in Preventing Ischemic Complications, J Am Coll Cardiol, 30, 149-56.

Lincoff, A.M., R.M. Califf, D.J. Moliterno, S.G. Ellis, J. Ducas, J.H. Kramer, N.S. Kleiman, E.A. Cohen, J.E. Booth, S.K. Sapp, C.F. Cabot and E.J. Topol, 1999a, Complementary clinical benefits of coronary-artery stenting and blockade of platelet glycoprotein IIb/IIIa receptors. Evaluation of Platelet IIb/IIIa Inhibition in Stenting Investigators, N Engl J Med, 341, 319-27.

Lincoff, A.M., J.E. Tcheng, R.M. Califf, D.J. Kereiakes, T.A. Kelly, G.C. Timmis, N.S. Kleiman, J.E. Booth, C. Balog, C.F. Cabot, K.M. Anderson, H.F. Weisman and E.J. Topol, 1999b, Sustained suppression of ischemic complications of coronary intervention by platelet GP IIb/IIIa blockade with abciximab: one-year outcome in the EPILOG trial. Evaluation in PTCA to Improve Long-term Outcome with abciximab GP IIb/IIIa blockade, Circulation, 99, 1951-8.

Lind, L., J. Hall, A. Larsson, M. Annuk, B. Fellstrom and H. Lithell, 2000, Evaluation of endothelium-dependent vasodilation in the human peripheral circulation, Clin Physiol, 20, 440-8.

Liochev, S.I. and I. Fridovich, 1997, Lucigenin luminescence as a measure of intracellular superoxide dismutase activity in Escherichia coli, Proc Natl Acad Sci U S A, 94, 2891-6.

Liochev, S.I. and I. Fridovich, 1998, Lucigenin as mediator of superoxide production: revisited, Free Radic Biol Med, 25, 926-8.

Lip, G.Y. and A. Blann, 1997, von Willebrand factor: a marker of endothelial dysfunction in vascular disorders?, Cardiovasc Res, 34, 255-65.

Liuzzo, G., L.M. Baisucci, J.R. Gallimore, G. Caligiuri, A. Buffon, A.G. Rebuzzi, M.B. Pepys and A. Maseri, 1999, Enhanced inflammatory response in patients with preinfarction unstable angina, J Am Coll Cardiol, 34, 1696-703.

Lloyd-Jones, D.M. and K.D. Bloch, 1996, The vascular biology of nitric oxide and its role in atherogenesis, Annu Rev Med, 47, 365-75.

London, G.M., S.J. Marchais, M.E. Safar, A.F. Genest, A.P. Guerin, F. Metivier, K. Chedid and A.M. London, 1990, Aortic and large artery compliance in end-stage renal failure, Kidney Int, 37, 137-42.

Lonn, E.M., S. Yusuf, P. Jha, T.J. Montague, K.K. Teo, C.R. Benedict and B. Pitt, 1994, Emerging role of angiotensin-converting enzyme inhibitors in cardiac and vascular protection, Circulation, 90, 2056-69.

Lopez, J.A., R.K. Andrews, V. Afshar-Kharghan and M.C. Berndt, 1998, Bernard-Soulier syndrome, Blood, 91, 4397-418.

Lopez-Lopez, J.G., F. Perez-Vizcaino, A.L. Cogolludo, M. Ibarra, F. Zaragoza-Arnaez and J. Tamargo, 2001, Nitric oxide-and nitric oxide donors-induced relaxation and its modulation by oxidative stress in piglet pulmonary arteries, Br J Pharmacol, 133, 615-24.

Loscalzo, J., 1985, N-Acetylcysteine potentiates inhibition of platelet aggregation by nitroglycerin, J Clin Invest, 76, 703-8.

Loscalzo, J., 1992, Antiplatelet and antithrombotic effects of organic nitrates, Am J Cardiol, 70, 18B-22B.

Loscalzo, J., 2001, Nitric oxide insufficiency, platelet activation, and arterial thrombosis, Circ Res, 88, 756-62.

Loscalzo, J. and G. Welch, 1995, Nitric oxide and its role in the cardiovascular system, Prog Cardiovasc Dis, 38, 87-104.

Lu, F.J., J.T. Lin, H.P. Wang and W.C. Huang, 1996, A simple, sensitive, non-stimulated photon counting system for detection of superoxide anion in whole blood, Experientia, 52, 141-4.

Ludmer, P.L., A.P. Selwyn, T.L. Shook, R.R. Wayne, G.H. Mudge, R.W. Alexander and P. Ganz, 1986, Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries, N Engl J Med, 315, 1046-51.

Lundman, P., M. Eriksson, K. Schenck-Gustafsson, F. Karpe and P. Tornvall, 1997, Transient triglyceridemia decreases vascular reactivity in young, healthy men without risk factors for coronary heart disease, Circulation, 96, 3266-8.

Lusis, A.J., 2000, Atherosclerosis, Nature, 407, 233-41.

Lynch, J.J., Jr., J.J. Cook, G.R. Sitko, M.A. Holahan, D.R. Ramjit, M.J. Mellott, M.T. Stranieri, Stabilito, II, G. Zhang and R.J. Lynch, 1995, Nonpeptide glycoprotein IIb/IIIa inhibitors. 5. Antithrombotic effects of MK-0383, J Pharmacol Exp Ther, 272, 20-32.

Lynch, S.M., B. Frei, J.D. Morrow, L.J. Roberts, 2nd, A. Xu, T. Jackson, R. Reyna, L.M. Klevay, J.A. Vita and J.F. Keaney, Jr., 1997, Vascular superoxide dismutase deficiency impairs endothelial vasodilator function through direct inactivation of nitric oxide and increased lipid peroxidation, Arterioscler Thromb Vasc Biol, 17, 2975-81.

MacCarthy, P.A., D.J. Grieve, J.M. Li, C. Dunster, F.J. Kelly and A.M. Shah, 2001, Impaired endothelial regulation of ventricular relaxation in cardiac hypertrophy: role of reactive oxygen species and NADPH oxidase, Circulation, 104, 2967-74.

Mach, F., U. Schonbeck, J.Y. Bonnefoy, J.S. Pober and P. Libby, 1997a, Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of CD40: induction of collagenase, stromelysin, and tissue factor, Circulation, 96, 396-9.

Mach, F., U. Schonbeck, G.K. Sukhova, E. Atkinson and P. Libby, 1998, Reduction of atherosclerosis in mice by inhibition of CD40 signalling, Nature, 394, 200-3.

Mach, F., U. Schonbeck, G.K. Sukhova, T. Bourcier, J.Y. Bonnefoy, J.S. Pober and P. Libby, 1997b, Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40-CD40 ligand signaling in atherosclerosis, Proc Natl Acad Sci U S A, 94, 1931-6.

MacKenzie, A.B., M.P. Mahaut-Smith and S.O. Sage, 1996, Activation of receptor-operated cation channels via $P2X_1$ not P2T purinoceptors in human platelets, J Biol Chem, 271, 2879-81.

Mackie, I.J., R. Jones and S.J. Machin, 1984, Platelet impedance aggregation in whole blood and its inhibition by antiplatelet drugs, J Clin Pathol, 37, 874-8.

Madan, M., S.D. Berkowitz, D.J. Christie, L.K. Jennings, A.C. Smit, K.N. Sigmon, S. Glazer and J.E. Tcheng, 2001, Rapid assessment of glycoprotein IIb/IIIa blockade with the platelet function analyzer (PFA-100) during percutaneous coronary intervention, Am Heart J, 141, 226-33.

Magrini, F. and A.P. Niarchos, 1980, Ineffectiveness of sublingual nitroglycerin in acute left ventricular failure in the presence of massive peripheral edema, Am J Cardiol, 45, 841-7.

Maier, W., F. Cosentino, R. Lutolf, M. Fleisch, C. Seiler, O.M. Hess and B. Meier, 1998, Tetrahydrobiopterin improves endothelial function in patients with coronary artery disease, J Am Coll Cardiol, 178A.

Makhoul, N., N. Dakak, M.Y. Flugelman, A. Merdler, A. Shefer, A. Schneeweiss, D.A. Halon and B.S. Lewis, 1990, Nitrate tolerance in heart failure: differential venous, pulmonary and systemic arterial effects, Am J Cardiol, 65, 28J-31J.

Makino, K., T. Hagiwara, A. Hagi, M. Nishi and A. Murakami, 1990, Cautionary note for DMPO spin trapping in the presence of iron ion, Biochem Biophys Res Commun, 172, 1073-80.

Mancini, G.B., 2000, Long-term use of angiotensin-converting enzyme inhibitors to modify endothelial dysfunction: a review of clinical investigations, Clin Invest Med, 23, 144-61.

Mancini, G.B., G.C. Henry, C. Macaya, B.J. O'Neill, A.L. Pucillo, R.G. Carere, T.J. Wargovich, H. Mudra, T.F. Luscher, M.I. Klibaner, H.E. Haber, A.C. Uprichard, C.J. Pepine and B. Pitt, 1996, Angiotensin-converting enzyme inhibition with quinapril improves endothelial vasomotor dysfunction in patients with coronary artery disease. The TREND (Trial on Reversing ENdothelial Dysfunction) Study, Circulation, 94, 258-65.

Mandal, S., R. Sarode, S. Dash and R.J. Dash, 1993, Hyperaggregation of platelets detected by whole blood platelet aggregometry in newly diagnosed noninsulin-dependent diabetes mellitus, Am J Clin Pathol, 100, 103-7.

Manganello, J.M., Y. Djellas, C. Borg, K. Antonakis and G.C. Le Breton, 1999, Cyclic AMP-dependent phosphorylation of thromboxane A_2 receptor-associated Galpha(13), J Biol Chem, 274, 28003-10.

Marcus, A.J., 1994, Thrombosis and inflammation as multicellular processes: significance of cell-cell interactions, Semin Hematol, 31, 261-9.

Marcus, A.J., S.T. Silk, L.B. Safier and H.L. Ullman, 1977, Superoxide production and reducing activity in human platelets, J Clin Invest, 59, 149-58.

Marso, S.P., A.M. Lincoff, S.G. Ellis, D.L. Bhatt, J.F. Tanguay, N.S. Kleiman, T. Hammoud, J.E. Booth, S.K. Sapp and E.J. Topol, 1999, Optimizing the percutaneous interventional outcomes for patients with diabetes mellitus: results of the EPISTENT (Evaluation of platelet IIb/IIIa inhibitor for stenting trial) diabetic substudy, Circulation, 100, 2477-84.

Martinez, A., E. Salas, A. Radomski and M.W. Radomski, 2001, Matrix metalloproteinase-2 in platelet adhesion to fibrinogen: interactions with nitric oxide, Med Sci Monit, 7, 646-51.

Martinez, G.R., P. Di Mascio, M.G. Bonini, O. Augusto, K. Briviba, H. Sies, P. Maurer, U. Rothlisberger, S. Herold and W.H. Koppenol, 2000, Peroxynitrite does not decompose to singlet oxygen ((1)Delta (g)O(2)) and nitroxyl (NO(-)), Proc Natl Acad Sci U S A, 97, 10307-12.

Martinez-Nieves, B. and J.C. Dunbar, 1999, Vascular dilatatory responses to sodium nitroprusside (SNP) and alpha-adrenergic antagonism in female and male normal and diabetic rats, Proc Soc Exp Biol Med, 222, 90-8.

Marumo, T., V.B. Schini-Kerth, B. Fisslthaler and R. Busse, 1997, Platelet-derived growth factor-stimulated superoxide anion production modulates activation of transcription factor NF-kappaB and expression of monocyte chemoattractant protein 1 in human aortic smooth muscle cells, Circulation, 96, 2361-7.

Mascelli, M.A., S. Worley, N.J. Veriabo, E.T. Lance, S. Mack, T. Schaible, H.F. Weisman and R.E. Jordan, 1997, Rapid assessment of platelet function with a modified whole-blood aggregometer in percutaneous transluminal coronary angioplasty patients receiving anti-GP IIb/IIIa therapy, Circulation, 96, 3860-6.

Maseri, A., 1997, Inflammation, atherosclerosis, and ischemic events - exploring the hidden side of the moon, N Engl J Med, 336, 1014-6.

Massberg, S., M. Sausbier, P. Klatt, M. Bauer, A. Pfeifer, W. Siess, R. Fassler, P. Ruth, F. Krombach and F. Hofmann, 1999, Increased adhesion and aggregation of platelets lacking cyclic guanosine 3',5'-monophosphate kinase I, J Exp Med, 189, 1255-64.

Mathis, P.C., H. Wohl, S.R. Wallach and R.L. Engler, 1981, Lack of release of platelet factor 4 during exercise-induced myocardial ischemia, N Engl J Med, 304, 1275-8.

Matsuoka, H., S. Itoh, M. Kimoto, K. Kohno, O. Tamai, Y. Wada, H. Yasukawa, G. Iwami, S. Okuda and T. Imaizumi, 1997, Asymmetrical dimethylarginine, an endogenous nitric oxide synthase inhibitor, in experimental hypertension, Hypertension, 29, 242-7.

Maurice, D.H. and R.J. Haslam, 1990, Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP, Mol Pharmacol, 37, 671-81.

May, D.C., J.J. Popma, W.H. Black, S. Schaefer, H.R. Lee, B.D. Levine and L.D. Hillis, 1987, *In vivo* induction and reversal of nitroglycerin tolerance in human coronary arteries, N Engl J Med, 317, 805-9.

May, J.M., 2000, How does ascorbic acid prevent endothelial dysfunction?, Free Radic Biol Med, 28, 1421-9.

Mayer, B., K. Schmidt, P. Humbert and E. Bohme, 1989, Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca^{2+}-dependently converts L-arginine into an activator of soluble guanylyl cyclase, Biochem Biophys Res Commun, 164, 678-85.

Mayhan, W.G., 1997, Superoxide dismutase partially restores impaired dilatation of the basilar artery during diabetes mellitus, Brain Res, 760, 204-9.

McDonald, B.J. and B.M. Bennett, 1993, Biotransformation of glyceryl trinitrate by rat aortic cytochrome P_{450}, Biochem Pharmacol, 45, 268-70.

McGarrity, S.T., T.M. Hyers and R.O. Webster, 1988a, Inhibition of neutrophil functions by platelets and platelet-derived products: description of multiple inhibitory properties, J Leukoc Biol, 44, 93-100.

McGarrity, S.T., A.H. Stephenson, T.M. Hyers and R.O. Webster, 1988b, Inhibition of neutrophil superoxide anion generation by platelet products: role of adenine nucleotides, J Leukoc Biol, 44, 411-21.

McGuire, J.J., D.J. Anderson and B.M. Bennett, 1994, Inhibition of the biotransformation and pharmacological actions of glyceryl trinitrate by the flavoprotein inhibitor, diphenyleneiodonium sulfate, J Pharmacol Exp Ther, 271, 708-14.

McKay, R.G. and W.E. Boden, 2001, Small peptide GP IIb/IIIa receptor inhibitors as upstream therapy in non-ST-segment elevation acute coronary syndromes: results of the PURSUIT, PRISM, PRISM-PLUS, TACTICS, and PARAGON trials, Curr Opin Cardiol, 16, 364-9.

McMurray, J., M. Chopra, I. Abdullah, W.E. Smith and H.J. Dargie, 1992, Evidence for oxidative stress in unstable angina, Br Heart J, 68, 454-7.

McVeigh, G.E., P. Hamilton, M. Wilson, C. Hanratty, W. J. Leahey, A.B. Devine, D.G. Morgan, L.J. Dixon, and L.T. McGrath, 2002, Platelet nitric oxide and superoxide release during the development of nitrate tolerance. Effect of supplemental ascorbate, Circuation, 106, 208-13.

Meade, T.W., M.V. Vickers, S.G. Thompson, Y. Stirling, A.P. Haines and G.J. Miller, 1985, Epidemiological characteristics of platelet aggregability, Br Med J (Clin Res Ed), 290, 428-32.

Meagher, E.A. and G.A. FitzGerald, 2000, Indices of lipid peroxidation *in vivo*: strengths and limitations, Free Radic Biol Med, 28, 1745-50.

Mehra, A., A. Shotan, E. Ostrzega, W. Hsueh, J. Vasquez-Johnson and U. Elkayam, 1994, Potentiation of isosorbide dinitrate effects with N-acetylcysteine in patients with chronic heart failure, Circulation, 89, 2595-600.

Mehrabi, M.R., C. Ekmekcioglu, F. Tatzber, A. Oguogho, R. Ullrich, A. Morgan, F. Tamaddon, M. Grimm, H.D. Glogar and H. Sinzinger, 1999, The isoprostane, 8-epi-PGF_2 alpha, is accumulated in coronary arteries isolated from patients with coronary heart disease, Cardiovasc Res, 43, 492-9.

Mehta, J. and P. Mehta, 1979, Platelet function studies in heart disease. VI. Enhanced platelet aggregate formation activity in congestive heart failure: inhibition by sodium nitroprusside, Circulation, 60, 497-503.

Mehta, J. and P. Mehta, 1980, Comparative effects of nitroprusside and nitroglycerin on platelet aggregation in patients with heart failure, J Cardiovasc Pharmacol, 2, 25-33.

Mehta, J. and P. Mehta, 1982, Comparison of platelet function during exercise in normal subjects and coronary artery disease patients: Potential role of platelet activation in myocardial ischemia, Am Heart J, 103, 49-53.

Mehta, J., P. Mehta, N. Ostrowski and F. Crews, 1983, Effects of verapamil on platelet aggregation, ATP release and thromboxane generation, Thromb Res, 30, 469-75.

Mehta, J.L., L.Y. Chen, B.C. Kone, P. Mehta and P. Turner, 1995, Identification of constitutive and inducible forms of nitric oxide synthase in human platelets, J Lab Clin Med, 125, 370-7.

Meier, B., A.J. Jesaitis, A. Emmendorffer, J. Roesler and M.T. Quinn, 1993, The cytochrome b-558 molecules involved in the fibroblast and polymorphonuclear leucocyte superoxide-generating NADPH oxidase systems are structurally and genetically distinct, Biochem J, 289, 481-6.

Mellion, B.T., L.J. Ignarro, C.B. Myers, E.H. Ohlstein, B.A. Ballot, A.L. Hyman and P.J. Kadowitz, 1983, Inhibition of human platelet aggregation by S-nitrosothiols. Heme-dependent activation of soluble guanylate cyclase and stimulation of cyclic GMP accumulation, Mol Pharmacol, 23, 653-64.

Mellion, B.T., L.J. Ignarro, E.H. Ohlstein, E.G. Pontecorvo, A.L. Hyman and P.J. Kadowitz, 1981, Evidence for the inhibitory role of guanosine 3', 5'-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators, Blood, 57, 946-55.

Mendelsohn, M.E., S. O'Neill, D. George and J. Loscalzo, 1990, Inhibition of fibrinogen binding to human platelets by S-nitroso-N-acetylcysteine, J Biol Chem, 265, 19028-34.

Meredith, I.T., J.F. Alison, F.M. Zhang, J.D. Horowitz and R.W. Harper, 1993, Captopril potentiates the effects of nitroglycerin in the coronary vascular bed, J Am Coll Cardiol, 22, 581-7.

Merenyi, G., J. Lind, G. Czapski and S. Goldstein, 2000, The decomposition of peroxynitrite does not yield nitroxyl anion and singlet oxygen, Proc Natl Acad Sci U S A, 97, 8216-8.

Meyer, J.W. and M.E. Schmitt, 2000, A central role for the endothelial NADPH oxidase in atherosclerosis, FEBS Lett, 472, 1-4.

Michelson, A.D., M.R. Barnard, H.B. Hechtman, H. MacGregor, R.J. Connolly, J. Loscalzo and C.R. Valeri, 1996a, *In vivo* tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function, Proc Natl Acad Sci U S A, 93, 11877-82.

Michelson, A.D., S.E. Benoit, M.I. Furman, W.L. Breckwoldt, M.J. Rohrer, M.R. Barnard and J. Loscalzo, 1996b, Effects of nitric oxide/EDRF on platelet surface glycoproteins, Am J Physiol, 270, H1640-8.

Mickelson, J.K., P.J. Simpson and B.R. Lucchesi, 1989, Antiplatelet monoclonal $F(ab')_2$ antibody directed against the platelet GPIIb/IIIa receptor complex prevents coronary artery thrombosis in the canine heart, J Mol Cell Cardiol, 21, 393-405.

Mihm, M.J., C.M. Coyle, L. Jing and J.A. Bauer, 1999, Vascular peroxynitrite formation during organic nitrate tolerance, J Pharmacol Exp Ther, 291, 194-8.

Millar-Craig, M.W., C.N. Bishop and E.B. Raftery, 1978, Circadian variation of blood-pressure, Lancet, 1 (8068), 795-7.

Miller, F.J., Jr., D.D. Gutterman, C.D. Rios, D.D. Heistad and B.L. Davidson, 1998, Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis, Circ Res, 82, 1298-305.

Miller, R.T., P. Martasek, L.J. Roman, J.S. Nishimura and B.S. Masters, 1997, Involvement of the reductase domain of neuronal nitric oxide synthase in superoxide anion production, Biochemistry, 36, 15277-84.

Milone, S.D., E.R. Azevedo, C. Forster and J.D. Parker, 1999a, The angiotensin II-receptor antagonist losartan does not prevent hemodynamic or vascular tolerance to nitroglycerin, J Cardiovasc Pharmacol, 34, 645-50.

Milone, S.D., C.R. Pace-Asciak, D. Reynaud, E.R. Azevedo, G.E. Newton and J.D. Parker, 1999b, Biochemical, hemodynamic, and vascular evidence concerning the free radical hypothesis of nitrate tolerance, J Cardiovasc Pharmacol, 33, 685-90.

Minamiyama, Y., S. Imaoka, S. Takemura, S. Okada, M. Inoue and Y. Funae, 2001, Escape from tolerance of organic nitrate by induction of cytochrome P_{450}, Free Radic Biol Med, 31, 1498-508.

Minuz, P., G. Andrioli, M. Degan, S. Gaino, R. Ortolani, R. Tommasoli, V. Zuliani, A. Lechi and C. Lechi, 1998, The F_2-isoprostane 8-epiprostaglandin F_2alpha increases platelet adhesion and reduces the antiadhesive and antiaggregatory effects of NO, Arterioscler Thromb Vasc Biol, 18, 1248-56.

Mital, S., X. Zhang, G. Zhao, R.D. Bernstein, C.J. Smith, D.L. Fulton, W.C. Sessa, J.K. Liao and T.H. Hintze, 2000, Simvastatin upregulates coronary vascular endothelial nitric oxide production in conscious dogs, Am J Physiol Heart Circ Physiol, 279, H2649-57.

Moake, J.L., N.A. Turner, N.A. Stathopoulos, L. Nolasco and J.D. Hellums, 1988, Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin, Blood, 71, 1366-74.

Mohanty, N., A.G. Wasserman, P. Walker and R.J. Katz, 1995, Prevention of nitroglycerin tolerance with diuretics, Am Heart J, 130, 522-7.

Mollnau, H., M. Wendt, K. Szocs, B. Lassegue, E. Schulz, M. Oelze, H. Li, M. Bodenschatz, M. August, A.L. Kleschyov, N. Tsilimingas, U. Walter, U. Forstermann, T. Meinertz, K. Griendling and T. Munzel, 2002, Effects of angiotensin II infusion on the expression and function of NAD(P)H oxidase and components of nitric oxide/cGMP signaling, Circ Res, 90, E58-65.

Moncada, S., R. Gryglewski, S. Bunting and J.R. Vane, 1976, An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation, Nature, 263, 663-5.

Moncada, S. and E.A. Higgs, 1991, Endogenous nitric oxide: physiology, pathology and clinical relevance, Eur J Clin Invest, 21, 361-74.

Mondoro, T.H., B.C. Shafer and J.G. Vostal, 1997, Peroxynitrite-induced tyrosine nitration and phosphorylation in human platelets, Free Radic Biol Med, 22, 1055-63.

Montalescot, G., A. Ankri, E. Vicaut, G. Drobinski, Y. Grosgogeat and D. Thomas, 1995, Fibrinogen after coronary angioplasty as a risk factor for restenosis, Circulation, 92, 31-8.

Moon, D.G., H. van der Zee, L.K. Weston, P.W. Gudewicz, J.W.d. Fenton and J.E. Kaplan, 1990, Platelet modulation of neutrophil superoxide anion production, Thromb Haemost, 63, 91-6.

Moore, K.P., V. Darley-Usmar, J. Morrow and L.J. Roberts, 1995, Formation of F_2-isoprostanes during oxidation of human low-density lipoprotein and plasma by peroxynitrite, Circ Res, 77, 335-41.

Moriwaki, Y., T. Yamamoto and K. Higashino, 1999, Enzymes involved in purine metabolism-a review of histochemical localization and functional implications, Histol Histopathol, 14, 1321-40.

Moro, M.A., V.M. Darley-Usmar, D.A. Goodwin, N.G. Read, R. Zamora-Pino, M. Feelisch, M.W. Radomski and S. Moncada, 1994, Paradoxical fate and biological action of peroxynitrite on human platelets, Proc Natl Acad Sci U S A, 91, 6702-6.

Moro, M.A., V.M. Darley-Usmar, I. Lizasoain, Y. Su, R.G. Knowles, M.W. Radomski and S. Moncada, 1995, The formation of nitric oxide donors from peroxynitrite, Br J Pharmacol, 116, 1999-2004.

Moro, M.A., R.J. Russel, S. Cellek, I. Lizasoain, Y. Su, V.M. Darley-Usmar, M.W. Radomski and S. Moncada, 1996, cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase, Proc Natl Acad Sci U S A, 93, 1480-5.

Moroi, M. and S.M. Jung, 1997, Platelet receptors for collagen, Thromb Haemost, 78, 439-44.

Morris, D.L., S.B. Kritchevsky and C.E. Davis, 1994, Serum carotenoids and coronary heart disease. The Lipid Research Clinics Coronary Primary Prevention Trial and Follow-up Study, JAMA, 272, 1439-41.

Moser, L., K.S. Callahan, A.K. Cheung, G.J. Stoddard and M.A. Munger, 1997, ACE inhibitor effects on platelet function in stages I-II hypertension, J Cardiovasc Pharmacol, 30, 461-7.

Moshfegh, K., M. Redondo, F. Julmy, W.A. Wuillemin, M.U. Gebauer, A. Haeberli and B.J. Meyer, 2000, Antiplatelet effects of clopidogrel compared with aspirin after myocardial infarction: enhanced inhibitory effects of combination therapy, J Am Coll Cardiol, 36, 699-705.

Motoyama, T., H. Kawano, K. Kugiyama, O. Hirashima, M. Ohgushi, R. Tsunoda, Y. Moriyama, Y. Miyao, M. Yoshimura, H. Ogawa and H. Yasue, 1998, Vitamin E administration improves impairment of endothelium-dependent vasodilation in patients with coronary spastic angina, J Am Coll Cardiol, 32, 1672-9.

Mueck, A.O., H. Seeger and D. Wallwiener, 2001, Further evidence for direct vascular actions of statins: effect on endothelial nitric oxide synthase and adhesion molecules, Exp Clin Endocrinol Diabetes, 109, 181-3.

Mugge, A., R.P. Brandes, R.H. Boger, A. Dwenger, S. Bode-Boger, S. Kienke, J.C. Frolich and P.R. Lichtlen, 1994, Vascular release of superoxide radicals is enhanced in hypercholesterolemic rabbits, J Cardiovasc Pharmacol, 24, 994-8.

Mugge, A., J.H. Elwell, T.E. Peterson, T.G. Hofmeyer, D.D. Heistad and D.G. Harrison, 1991, Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits, Circ Res, 69, 1293-300.

Muiesan, G., E. Agabiti-Rosei, L. Muiesan, G. Romanelli, P. Pollavini, C. Pasotti, G. Fiori, L. Muratori, A.M. Zuarini and C. Pastorini, 1986, A multicenter trial of transdermal nitroglycerin in exercise-induced angina: individual antianginal response after repeated administration, Am Heart J, 112, 233-8.

Muikku, O., A. Kuitunen and M. Hynynen, 1995, Effects of organic nitrate vasodilators on platelet function before and after cardiopulmonary bypass, Acta Anaesthesiol Scand, 39, 618-23.

Mullen, M.J., P. Clarkson, A.E. Donald, H. Thomson, S.A. Thorne, A.J. Powe, T. Furuno, T. Bull and J.E. Deanfield, 1998a, Effect of enalapril on endothelial function in young insulin-dependent diabetic patients: a randomized, double-blind study, J Am Coll Cardiol, 31, 1330-5.

Mullen, M.J., A.E. Donald, H. Thomson, G. O'Connor, S. Thorne, D.J. Wright and J.E. Deanfield, 1998b, Atorvastatin but not L-arginine improves endothelial function in young subjects with insulin dependent diabetes mellitus, J Am Coll Cardio, 178A.

Mullenheim, J., S. Muller, U. Laber, V. Thamer, W. Meyer, E. Bassenge, B. Fink and G. Kojda, 2001, The effect of high-dose pentaerythritol tetranitrate on the development of nitrate tolerance in rabbits, Naunyn Schmiedebergs Arch Pharmacol, 364, 269-75.

Mulsch, A., E. Bassenge and R. Busse, 1989, Nitric oxide synthesis in endothelial cytosol: evidence for a calcium-dependent and a calcium-independent mechanism, Naunyn Schmiedebergs Arch Pharmacol, 340, 767-70.

Mulsch, A., M. Oelze, S. Kloss, H. Mollnau, A. Topfer, A. Smolenski, U. Walter, J.P. Stasch, A. Warnholtz, U. Hink, T. Meinertz and T. Munzel, 2001, Effects of *in vivo* nitroglycerin treatment on activity and expression of the guanylyl cyclase and cGMP-dependent protein kinase and their downstream target vasodilator-stimulated phosphoprotein in aorta, Circulation, 103, 2188-94.

Munzel, T. and E. Bassenge, 1996, Long-term angiotensin-converting enzyme inhibition with high-dose enalapril retards nitrate tolerance in large epicardial arteries and prevents rebound coronary vasoconstriction *in vivo*, Circulation, 93, 2052-8.

Munzel, T., A. Giaid, S. Kurz, D.J. Stewart and D.G. Harrison, 1995a, Evidence for a role of endothelin 1 and protein kinase C in nitroglycerin tolerance, Proc Natl Acad Sci U S A, 92, 5244-8.

Munzel, T., U. Hink, H. Yigit, R. Macharzina, D.G. Harrison and A. Mulsch, 1999, Role of superoxide dismutase in *in vivo* and *in vitro* nitrate tolerance, Br J Pharmacol, 127, 1224-30.

Munzel, T., J. Holtz, A. Mulsch, D.J. Stewart and E. Bassenge, 1989, Nitrate tolerance in epicardial arteries or in the venous system is not reversed by N-acetylcysteine *in vivo*, but tolerance-independent interactions exist, Circulation, 79, 188-97.

Munzel, T., S. Kurz, T. Heitzer and D.G. Harrison, 1996a, New insights into mechanisms underlying nitrate tolerance, Am J Cardiol, 77, 24C-30C.

Munzel, T., S. Kurz, S. Rajagopalan, M. Thoenes, W.R. Berrington, J.A. Thompson, B.A. Freeman and D.G. Harrison, 1996b, Hydralazine prevents nitroglycerin tolerance by inhibiting activation of a membrane-bound NADH oxidase. A new action for an old drug, J Clin Invest, 98, 1465-70.

Munzel, T., H. Li, H. Mollnau, U. Hink, E. Matheis, M. Hartmann, M. Oelze, M. Skatchkov, A. Warnholtz, L. Duncker, T. Meinertz and U. Forstermann, 2000a, Effects of long-term nitroglycerin treatment on endothelial nitric oxide synthase (NOS III) gene expression, NOS III-mediated superoxide production, and vascular NO bioavailability, Circ Res, 86, E7-E12.

Munzel, T., H. Mollnau, M. Hartmann, C. Geiger, M. Oelze, A. Warnholtz, A.H. Yehia, U. Forstermann and T. Meinertz, 2000b, Effects of a nitrate-free interval on tolerance, vasoconstrictor sensitivity and vascular superoxide production, J Am Coll Cardiol, 36, 628-34.

Munzel, T., H. Sayegh, B.A. Freeman, M.M. Tarpey and D.G. Harrison, 1995b, Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance, J Clin Invest, 95, 187-94.

Murgo, J.P., N. Westerhof, J.P. Giolma and S.A. Altobelli, 1980, Aortic input impedance in normal man: relationship to pressure wave forms, Circulation, 62, 105-16.

Murrel, W., 1879, Nitro-glycerine as a remedy for angina pectoris, Lancet, 1, 80-81,113-115,151-152,226-227.

Murthy, M.S. and S.V. Pande, 1987, Malonyl-CoA binding site and the overt carnitine palmitoyltransferase activity reside on the opposite sides of the outer mitochondrial membrane, Proc Natl Acad Sci U S A, 84, 378-82.

Myers, P.R., R.L. Minor, R. Guerra, J.N. Bates and D.G. Harrison, 1990, Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide, Nature, 345, 161-3.

Nagata, K., T. Tsuji, N. Todoroki, Y. Katagiri, K. Tanoue, H. Yamazaki, N. Hanai and T. Irimura, 1993, Activated platelets induce superoxide anion release by monocytes and neutrophils through P-selectin (CD62), J Immunol, 151, 3267-73.

Nakano, M., 1998, Detection of active oxygen species in biological systems, Cell Mol Neurobiol, 18, 565-79.

Napoli, C., C. Cicala, F.P. D'Armiento, F. Roviezzo, P. Somma, F. de Nigris, P. Zuliani, M. Bucci, L. Aleotti, A. Casini, F. Franconi and G. Cirino, 1999, Beneficial effects of ACE-inhibition with zofenopril on plaque formation and low-density lipoprotein oxidation in watanabe heritable hyperlipidemic rabbits, Gen Pharmacol, 33, 467-77.

Napoli, C., F.P. D'Armiento, F.P. Mancini, A. Postiglione, J.L. Witztum, G. Palumbo and W. Palinski, 1997, Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions, J Clin Invest, 100, 2680-90.

Nassar, T., B. Kadery, C. Lotan, N. Da'as, Y. Kleinman and A. Haj-Yehia, 2002, Effects of the superoxide dismutase-mimetic compound tempol on endothelial dysfunction in streptozotocin-induced diabetic rats, Eur J Pharmacol, 436, 111-8.

Natali, A., A.M. Sironi, E. Toschi, S. Camastra, G. Sanna, A. Perissinotto, S. Taddei and E. Ferrannini, 2000, Effect of vitamin C on forearm blood flow and glucose metabolism in essential hypertension, Arterioscler Thromb Vasc Biol, 20, 2401-6.

Navab, M., J.A. Berliner, A.D. Watson, S.Y. Hama, M.C. Territo, A.J. Lusis, D.M. Shih, B.J. Van Lenten, J.S. Frank, L.L. Demer, P.A. Edwards and A.M. Fogelman, 1996, The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture, Arterioscler Thromb Vasc Biol, 16, 831-42.

Needleman, P. and E.M. Johnson, Jr., 1973, Mechanism of tolerance development to organic nitrates, J Pharmacol Exp Ther, 184, 709-15.

Nelli, S., M. Hillen, K. Buyukafsar and W. Martin, 2000, Oxidation of nitroxyl anion to nitric oxide by copper ions, Br J Pharmacol, 131, 356-62.

Nelli, S., L. McIntosh and W. Martin, 2001, Role of copper ions and cytochrome P_{450} in the vasodilator actions of the nitroxyl anion generator, Angeli's salt, on rat aorta, Eur J Pharmacol, 412, 281-9.

Neunteufl, T., U. Priglinger, S. Heher, M. Zehetgruber, G. Soregi, S. Lehr, K. Huber, G. Maurer, F. Weidinger and K. Kostner, 2000, Effects of vitamin E on chronic and acute endothelial dysfunction in smokers, J Am Coll Cardiol, 35, 277-83.

Nguyen, B.L., M. Saitoh and J.A. Ware, 1991, Interaction of nitric oxide and cGMP with signal transduction in activated platelets, Am J Physiol, 261, H1043-52.

Nguyen-Khoa, T., Z.A. Massy, V. Witko-Sarsat, S. Canteloup, M. Kebede, B. Lacour, T. Drueke and B. Descamps-Latscha, 1999, Oxidized low-density lipoprotein induces macrophage respiratory burst via its protein moiety: A novel pathway in atherogenesis?, Biochem Biophys Res Commun, 263, 804-9.

Nidorf, S.M., M. Sturm, J. Strophair, P.J. Kendrew and R.R. Taylor, 1989, Whole blood aggregation, thromboxane release and the lyso derivative of platelet activating factor in myocardial infarction and unstable angina, Cardiovasc Res, 23, 273-8.

Nigam, R., D.J. Anderson, S.F. Lee and B.M. Bennett, 1996, Isoform-specific biotransformation of glyceryl trinitrate by rat aortic glutathione S-transferases, J Pharmacol Exp Ther, 279, 1527-34.

Nikol, S., J.M. Isner, J.G. Pickering, M. Kearney, G. Leclerc and L. Weir, 1992, Expression of transforming growth factor-beta 1 is increased in human vascular restenosis lesions, J Clin Invest, 90, 1582-92.

Nishida, K., D.G. Harrison, J.P. Navas, A.A. Fisher, S.P. Dockery, M. Uematsu, R.M. Nerem, R.W. Alexander and T.J. Murphy, 1992, Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase, J Clin Invest, 90, 2092-6.

Noack, E. and M. Feelisch, 1991, Molecular mechanisms of nitrovasodilator bioactivation, Basic Res Cardiol, 86 Suppl 2, 37-50.

Nomura, S., T. Nakamura, J. Cone, N.N. Tandon and J. Kambayashi, 2000, Cytometric analysis of high shear-induced platelet microparticles and effect of cytokines on microparticle generation, Cytometry, 40, 173-81.

Nong, Z., M. Hoylaerts, N. Van Pelt, D. Collen and S. Janssens, 1997, Nitric oxide inhalation inhibits platelet aggregation and platelet-mediated pulmonary thrombosis in rats, Circ Res, 81, 865-9.

Notarbartolo, A., G. Davi, M. Averna, C.M. Barbagallo, A. Ganci, C. Giammarresi, F.P. La Placa and C. Patrono, 1995, Inhibition of thromboxane biosynthesis and platelet function by simvastatin in type IIa hypercholesterolemia, Arterioscler Thromb Vasc Biol, 15, 247-51.

Nurden, P., P. Savi, E. Heilmann, C. Bihour, J.M. Herbert, J.P. Maffrand and A. Nurden, 1995, An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb-IIIa complex function, J Clin Invest, 95, 1612-22.

O'Brien, J.R., 1968, Effects of salicylates on human platelets, Lancet, 1 (7578), 779-83.

O'Driscoll, G., D. Green, J. Rankin, K. Stanton and R. Taylor, 1997a, Improvement in endothelial function by angiotensin converting enzyme inhibition in insulin-dependent diabetes mellitus, J Clin Invest, 100, 678-84.

O'Driscoll, G., D. Green and R.R. Taylor, 1997b, Simvastatin, an HMG-coenzyme A reductase inhibitor, improves endothelial function within 1 month, Circulation, 95, 1126-31.

O'Driscoll, J.G., D.J. Green, J.M. Rankin and R.R. Taylor, 1999, Nitric oxide-dependent endothelial function is unaffected by allopurinol in hypercholesterolaemic subjects, Clin Exp Pharmacol Physiol, 26, 779-83.

O'Keefe, J.H., M. Wetzel, R.R. Moe, K. Bronsnahan and C.J. Lavie, 2001, Should an angiotensin-converting enzyme inhibitor be standard therapy for patients with atherosclerotic disease?, J Am Coll Cardiol, 37, 1-8.

O'Neill, W.W., P. Serruys, M. Knudtson, G.A. van Es, G.C. Timmis, C. van der Zwaan, J. Kleiman, J. Gong, E.B. Roecker, R. Dreiling, J. Alexander and R. Anders, 2000, Long-term treatment with a platelet glycoprotein-receptor antagonist after percutaneous coronary revascularization. EXCITE Trial Investigators. Evaluation of Oral Xemilofiban in Controlling Thrombotic Events, N Engl J Med, 342, 1316-24.

O'Rourke, F.A., S.P. Halenda, G.B. Zavoico and M.B. Feinstein, 1985, Inositol 1,4,5-trisphosphate releases Ca^{2+} from a Ca^{2+}-transporting membrane vesicle fraction derived from human platelets, J Biol Chem, 260, 956-62.

O'Rourke, M.F. and G. Mancia, 1999, Arterial stiffness, J Hypertens, 17, 1-4.

O'Rourke, M.F., A. Pauca and X.J. Jiang, 2001, Pulse wave analysis, Br J Clin Pharmacol, 51, 507-22.

Ohara, Y., T.E. Peterson and D.G. Harrison, 1993, Hypercholesterolemia increases endothelial superoxide anion production, J Clin Invest, 91, 2546-51.

Ohman, E.M., N.S. Kleiman, G. Gacioch, S.J. Worley, F.I. Navetta, J.D. Talley, H.V. Anderson, S.G. Ellis, M.D. Cohen, D. Spriggs, M. Miller, D. Kereiakes, S. Yakubov, M.M. Kitt, K.N. Sigmon, R.M. Califf, M.W. Krucoff and E.J. Topol, 1997, Combined accelerated tissue-plasminogen activator and platelet glycoprotein IIb/IIIa integrin receptor blockade with Integrilin in acute myocardial infarction. Results of a randomized, placebo-controlled, dose-ranging trial. IMPACT-AMI Investigators, Circulation, 95, 846-54.

Ohno, A., M. Fujita, K. Miwa, M. Ejiri, H. Asanoi and S. Sasayama, 1991, Importance of coronary collateral circulation for increased treadmill exercise capacity by nitrates in patients with stable effort angina pectoris, Cardiology, 78, 323-8.

Ohyashiki, T., M. Kobayashi and K. Matsui, 1991, Oxygen-radical-mediated lipid peroxidation and inhibition of ADP-induced platelet aggregation, Arch Biochem Biophys, 288, 282-6.

Oliva, D. and S. Nicosia, 1987, PGI_2-receptors and molecular mechanisms in platelets and vasculature: state of the art, Pharmacol Res Commun, 19, 735-65.

Oliva, D.W., P. Maderna, M.R. Accomazzo, S. Nicosia and E. Tremoli, 1989, Iloprost binding and inhibition of aggregation in platelet rich plasma. Differences between normal and type IIa hypercholesterolemic subjects, Biochem Pharmacol, 38, 39-45.

Olsson, G., J. Allgen, O. Amtorp, G. Nyberg and J.O. Parker, 1992, Absence of pre-dose rebound phenomena with once daily 5-ISMN in a controlled-release formulation, Eur Heart J, 13, 814-7.

Omura, T., T. Matsumoto, I. Nakae, M. Takahashi and M. Kinoshita, 2001, Two possible mechanisms underlying nitrate tolerance in monkey coronary arteries, Clin Exp Pharmacol Physiol, 28, 259-65.

Ono, H. and M. Kimura, 1981, Effect of Ca^{2+}-antagonistic vasodilators, diltiazem, nifedipine, perhexiline and verapamil, on platelet aggregation *in vitro*, Arzneimittelforschung, 31, 1131-4.

Osende, J.I., V. Fuster, E.I. Lev, D. Shimbo, U. Rauch, J.D. Marmur, M. Richard, D. Varon and J.J. Badimon, 2001, Testing platelet activation with a shear-dependent platelet function test versus aggregation-based tests: relevance for monitoring long-term glycoprotein IIb/IIIa inhibition, Circulation, 103, 1488-91.

Oskarsson, H.J. and T.G. Hofmeyer, 1996, Platelets from patients with diabetes mellitus have impaired ability to mediate vasodilation, J Am Coll Cardiol, 27, 1464-70.

Osmialowska, Z., M. Nartowicz-Sloniewska, J.M. Slominski and B. Krupa-Wojciechowska, 1990, Effect of nifedipine monotherapy on platelet aggregation in patients with untreated essential hypertension, Eur J Clin Pharmacol, 39, 403-4.

Ott, I., F.J. Neumann, M. Gawaz, M. Schmitt and A. Schomig, 1996, Increased neutrophil-platelet adhesion in patients with unstable angina, Circulation, 94, 1239-46.

Ozaki, Y., K. Satoh, Y. Yatomi, T. Yamamoto, Y. Shirasawa and S. Kume, 1994, Detection of platelet aggregates with a particle counting method using light scattering, Anal Biochem, 218, 284-94.

Packer, M., W.H. Lee, P.D. Kessler, S.S. Gottlieb, N. Medina and M. Yushak, 1987, Prevention and reversal of nitrate tolerance in patients with congestive heart failure, N Engl J Med, 317, 799-804.

Packer, M., N. Medina, M. Yushak and W.H. Lee, 1986, Hemodynamic factors limiting the response to transdermal nitroglycerin in severe chronic congestive heart failure, Am J Cardiol, 57, 260-7.

Pagano, P.J., J.K. Clark, M.E. Cifuentes-Pagano, S.M. Clark, G.M. Callis and M.T. Quinn, 1997, Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II, Proc Natl Acad Sci U S A, 94, 14483-8.

Page, Y., B. Tardy, F. Zeni, C. Comtet, R. Terrana and J.C. Bertrand, 1991, Thrombotic thrombocytopenic purpura related to ticlopidine, Lancet, 337 (8744), 774-6.

Palmer, R.M., D.S. Ashton and S. Moncada, 1988a, Vascular endothelial cells synthesize nitric oxide from L-arginine, Nature, 333, 664-6.

Palmer, R.M., A.G. Ferrige and S. Moncada, 1987, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor, Nature, 327, 524-6.

Palmer, R.M., D.D. Rees, D.S. Ashton and S. Moncada, 1988b, L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation, Biochem Biophys Res Commun, 153, 1251-6.

Pannocchia, A. and R.M. Hardisty, 1985, Cyclic AMP inhibits platelet activation independently of its effect on cytosolic free calcium, Biochem Biophys Res Commun, 127, 339-45.

Parise, L.V., 1999, Integrin alpha(IIb)beta(3) signaling in platelet adhesion and aggregation, Curr Opin Cell Biol, 11, 597-601.

Parker, J.D., B. Farrell, T. Fenton, M. Cohanim and J.O. Parker, 1991, Counter-regulatory responses to continuous and intermittent therapy with nitroglycerin, Circulation, 84, 2336-45.

Parker, J.D., B. Farrell, T. Fenton and J.O. Parker, 1992, Effects of diuretic therapy on the development of tolerance during continuous therapy with nitroglycerin, J Am Coll Cardiol, 20, 616-22.

Parker, J.D., A.B. Parker, B. Farrell and J.O. Parker, 1996, Effects of diuretic therapy on the development of tolerance to nitroglycerin and exercise capacity in patients with chronic stable angina, Circulation, 93, 691-6.

Parker, J.D. and J.O. Parker, 1993, Effect of therapy with an angiotensin-converting enzyme inhibitor on hemodynamic and counterregulatory responses during continuous therapy with nitroglycerin, J Am Coll Cardiol, 21, 1445-53.

Parker, J.D. and J.O. Parker, 1998, Nitrate therapy for stable angina pectoris, N Engl J Med, 338, 520-31.

Parker, J.O., 1992, Update on nitrate tolerance, Br J Clin Pharmacol, 34 Suppl 1, 11S-14S.

Parker, J.O., 1993, Eccentric dosing with isosorbide-5-mononitrate in angina pectoris, Am J Cardiol, 72, 871-6.

Parker, J.O., M.H. Amies, R.W. Hawkinson, J.M. Heilman, A.J. Hougham, M.C. Vollmer and R.R. Wilson, 1995, Intermittent transdermal nitroglycerin therapy in angina pectoris. Clinically effective without tolerance or rebound. Minitran Efficacy Study Group, Circulation, 91, 1368-74.

Parker, J.O., B. Farrell, K.A. Lahey and B.F. Rose, 1987, Nitrate tolerance: the lack of effect of N-acetylcysteine, Circulation, 76, 572-6.

Parker, J.O. and H.L. Fung, 1984, Transdermal nitroglycerin in angina pectoris, Am J Cardiol, 54, 471-6.

Parker, J.O., J.D. Parker, R.W. Caldwell, B. Farrell and W.H. Kaesemeyer, 2002, The effect of supplemental L-arginine on tolerance development during continuous transdermal nitroglycerin therapy, J Am Coll Cardiol, 39, 1199-203.

Parker, R.I. and H.R. Gralnick, 1986, Identification of platelet glycoprotein IIb/IIIa as the major binding site for released platelet-von Willebrand factor, Blood, 68, 732-6.

Patrignani, P., P. Filabozzi and C. Patrono, 1982, Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects, J Clin Invest, 69, 1366-72.

Patrignani, P., M.R. Panara, S. Tacconelli, F. Seta, T. Bucciarelli, G. Ciabattoni, P. Alessandrini, A. Mezzetti, G. Santini, M.G. Sciulli, F. Cipollone, G. Davi, P. Gallina, G.B. Bon and C. Patrono, 2000, Effects of vitamin E supplementation on F_2-isoprostane and thromboxane biosynthesis in healthy cigarette smokers, Circulation, 102, 539-45.

Patrono, C., G. Ciabattoni, E. Pinca, F. Pugliese, G. Castrucci, A. De Salvo, M.A. Satta and B.A. Peskar, 1980, Low dose aspirin and inhibition of thromboxane B_2 production in healthy subjects, Thromb Res, 17, 317-27.

Patrono, C. and G.A. FitzGerald, 1997, Isoprostanes: potential markers of oxidant stress in atherothrombotic disease, Arterioscler Thromb Vasc Biol, 17, 2309-15.

Pawloski, J.R., R.V. Swaminathan and J.S. Stamler, 1998, Cell-free and erythrocytic S-nitrosohemoglobin inhibits human platelet aggregation, Circulation, 97, 263-7.

PDAY., 1993, Natural history of aortic and coronary atherosclerotic lesions in youth. Findings from the PDAY Study. Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group, Arterioscler Thromb, 13, 1291-8.

Peerlinck, K., I. De Lepeleire, M. Goldberg, D. Farrell, J. Barrett, E. Hand, D. Panebianco, H. Deckmyn, J. Vermylen and J. Arnout, 1993, MK-383 (L-700,462), a selective nonpeptide platelet glycoprotein IIb/IIIa antagonist, is active in man, Circulation, 88, 1512-7.

Pepine, C.J., G. Faich and R. Makuch, 1998, Verapamil use in patients with cardiovascular disease: an overview of randomized trials, Clin Cardiol, 21, 633-41.

Peto, R., R. Gray, R. Collins, K. Wheatley, C. Hennekens, K. Jamrozik, C. Warlow, B. Hafner, E. Thompson and S. Norton, 1988, Randomised trial of prophylactic daily aspirin in British male doctors, Br Med J (Clin Res Ed), 296, 313-6.

Pfeffer, M.A., E. Braunwald, L.A. Moye, L. Basta, E.J. Brown, Jr., T.E. Cuddy, B.R. Davis, E.M. Geltman, S. Goldman and G.C. Flaker, 1992, Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction. Results of the survival and ventricular enlargement trial. The SAVE Investigators, N Engl J Med, 327, 669-77.

Pfeffer, M.A., S.C. Greaves, J.M. Arnold, R.J. Glynn, F.S. LaMotte, R.T. Lee, F.J. Menapace, Jr., E. Rapaport, P.M. Ridker, J.L. Rouleau, S.D. Solomon and C.H. Hennekens, 1997, Early versus delayed angiotensin-converting enzyme inhibition therapy in acute myocardial infarction. The healing and early afterload reducing therapy trial, Circulation, 95, 2643-51.

Pigazzi, A., S. Heydrick, F. Folli, S. Benoit, A. Michelson and J. Loscalzo, 1999, Nitric oxide inhibits thrombin receptor-activating peptide-induced phosphoinositide 3-kinase activity in human platelets, J Biol Chem, 274, 14368-75.

Piret, A., G. Niset, E. Depiesse, W. Wyns, J.M. Boeynaems, J. Poortmans and S. Degre, 1990, Increased platelet aggregability and prostacyclin biosynthesis induced by intense physical exercise, Thromb Res, 57, 685-95.

Pizzulli, L., A. Hagendorff, M. Zirbes, W. Fehske, S. Ewig, W. Jung and B. Luderitz, 1996, Influence of captopril on nitroglycerin-mediated vasodilation and development of nitrate tolerance in arterial and venous circulation, Am Heart J, 131, 342-9.

Pizzulli, L., A. Hagendorff, M. Zirbes, W. Jung and B. Luderitz, 1997, N-acetylcysteine attenuates nitroglycerin tolerance in patients with angina pectoris and normal left ventricular function, Am J Cardiol, 79, 28-33.

Plow, E.F., R.P. McEver, B.S. Coller, V.L. Woods, Jr., G.A. Marguerie and M.H. Ginsberg, 1985, Related binding mechanisms for fibrinogen, fibronectin, von Willebrand factor, and thrombospondin on thrombin-stimulated human platelets, Blood, 66, 724-7.

Plutzky, J., 2001, Inflammatory pathways in atherosclerosis and acute coronary syndromes, Am J Cardiol, 88, 10K-15K.

Poley, S. and W. Mempel, 2001, Laboratory diagnosis of heparin-induced thrombocytopenia: advantages of a functional flow cytometric test in comparison to the heparin-induced platelet-activation test, Eur J Haematol, 66, 253-62.

Pollock, J.S., U. Forstermann, J.A. Mitchell, T.D. Warner, H.H. Schmidt, M. Nakane and F. Murad, 1991, Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells, Proc Natl Acad Sci U S A, 88, 10480-4.

Pollock, J.S., M. Nakane, L.D. Buttery, A. Martinez, D. Springall, J.M. Polak, U. Forstermann and F. Murad, 1993, Characterization and localization of endothelial nitric oxide synthase using specific monoclonal antibodies, Am J Physiol, 265, C1379-87.

Prasad, A., N.P. Andrews, F.A. Padder, M. Husain and A.A. Quyyumi, 1999, Glutathione reverses endothelial dysfunction and improves nitric oxide bioavailability, J Am Coll Cardiol, 34, 507-14.

Prasad, A., T. Tupas-Habib, W.H. Schenke, R. Mincemoyer, J.A. Panza, M.A. Waclawin, S. Ellahham and A.A. Quyyumi, 2000, Acute and chronic angiotensin-1 receptor antagonism reverses endothelial dysfunction in atherosclerosis, Circulation, 101, 2349-54.

Pratico, D., 1999, F_2-isoprostanes: sensitive and specific non-invasive indices of lipid peroxidation *in vivo*, Atherosclerosis, 147, 1-10.

Pratico, D., L. Iuliano, C. Alessandri, C. Camastra and F. Violi, 1993, Polymorphonuclear leukocyte-derived O_2^- reactive species activate primed platelets in human whole blood, Am J Physiol, 264, H1582-7.

Pratico, D., L. Iuliano, A. Ghiselli, C. Alessandri and F. Violi, 1991, Hydrogen peroxide as trigger of platelet aggregation, Haemostasis, 21, 169-74.

Pratico, D., L. Iuliano, F.M. Pulcinelli, M.S. Bonavita, P.P. Gazzaniga and F. Violi, 1992, Hydrogen peroxide triggers activation of human platelets selectively exposed to nonaggregating concentrations of arachidonic acid and collagen, J Lab Clin Med, 119, 364-70.

Pratico, D., J.A. Lawson, J. Rokach and G.A. FitzGerald, 2001, The isoprostanes in biology and medicine, Trends Endocrinol Metab, 12, 243-7.

Pratico, D., E.M. Smyth, F. Violi and G.A. FitzGerald, 1996, Local amplification of platelet function by 8-Epi prostaglandin F_2alpha is not mediated by thromboxane receptor isoforms, J Biol Chem, 271, 14916-24.

Preik, M., M. Kelm, M. Feelisch and B.E. Strauer, 1996, Impaired effectiveness of nitric oxide-donors in resistance arteries of patients with arterial hypertension, J Hypertens, 14, 903-8.

PRISM investigators., 1998, A comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina. Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM), N Engl J Med, 338, 1498-505.

PRISM-PLUS Study Investigators., 1998, Inhibition of the platelet glycoprotein IIb/IIIa receptor with tirofiban in unstable angina and non-Q-wave myocardial infarction. Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited by Unstable Signs and Symptoms (PRISM-PLUS), N Engl J Med, 338, 1488-97.

Pufahl, R.A., J.S. Wishnok and M.A. Marletta, 1995, Hydrogen peroxide-supported oxidation of N^G-hydroxy-L-arginine by nitric oxide synthase, Biochemistry, 34, 1930-41.

Pumphrey, C.W., V. Fuster, M.K. Dewanjee, J.H. Chesebro, R.E. Vlietstra and M.P. Kaye, 1983, Comparison of the antithrombotic action of calcium antagonist drugs with dipyridamole in dogs, Am J Cardiol, 51, 591-5.

Pytela, R., M.D. Pierschbacher, M.H. Ginsberg, E.F. Plow and E. Ruoslahti, 1986, Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors, Science, 231, 1559-62.

Quinn, M.J. and D.J. Fitzgerald, 1999, Ticlopidine and clopidogrel, Circulation, 100, 1667-72.

Quyyumi, A.A., R.O. Cannon, J.A. Panza, J.G. Diodati and S.E. Epstein, 1992a, Endothelial dysfunction in patients with chest pain and normal coronary arteries, Circulation, 86, 1864-71.

Quyyumi, A.A., J.A. Panza, J.G. Diodati, E. Lakatos and S.E. Epstein, 1992b, Circadian variation in ischemic threshold. A mechanism underlying the circadian variation in ischemic events, Circulation, 86, 22-8.

Rader, D.J., 2000, Inflammatory markers of coronary risk, N Engl J Med, 343, 1179-82.

Radomski, M.W., R.M. Palmer and S. Moncada, 1987a, The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide, Br J Pharmacol, 92, 639-46.

Radomski, M.W., R.M. Palmer and S. Moncada, 1987b, Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets, Br J Pharmacol, 92, 181-7.

Radomski, M.W., R.M. Palmer and S. Moncada, 1987c, Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium, Lancet, 2 (8567), 1057-8.

Radomski, M.W., R.M. Palmer and S. Moncada, 1987d, The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium, Biochem Biophys Res Commun, 148, 1482-9.

Radomski, M.W., R.M. Palmer and S. Moncada, 1990, An L-arginine/nitric oxide pathway present in human platelets regulates aggregation, Proc Natl Acad Sci U S A, 87, 5193-7.

Rafflenbeul, W. and P.R. Lichtlen, 1983, Quantitative coronary angiography: evidence of a sustained increase in vascular smooth muscle tone in coronary artery stenoses, Z Kardiol, 72 Suppl 3, 87-91.

Raha, S., G.E. McEachern, A.T. Myint and B.H. Robinson, 2000, Superoxides from mitochondrial complex III: the role of manganese superoxide dismutase, Free Radic Biol Med, 29, 170-80.

Raha, S. and B.H. Robinson, 2000, Mitochondria, oxygen free radicals, disease and ageing, Trends Biochem Sci, 25, 502-8.

Raij, L., 2001, Workshop: hypertension and cardiovascular risk factors: role of the angiotensin II-nitric oxide interaction, Hypertension, 37, 767-73.

Raitakari, O.T., M.R. Adams, R.J. McCredie, K.A. Griffiths, R. Stocker and D.S. Celermajer, 2000, Oral vitamin C and endothelial function in smokers: short-term improvement, but no sustained beneficial effect, J Am Coll Cardiol, 35, 1616-21.

Raitakari, O.T. and D.S. Celermajer, 2000, Testing for endothelial dysfunction, Ann Med, 32, 293-304.

Rajagopalan, S., S. Kurz, T. Munzel, M. Tarpey, B.A. Freeman, K.K. Griendling and D.G. Harrison, 1996, Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone, J Clin Invest, 97, 1916-23.

Rao, K.M., 2000, Molecular mechanisms regulating iNOS expression in various cell types, J Toxicol Environ Health B Crit Rev, 3, 27-58.

Rapoport, R.M., S.A. Waldman, R. Ginsburg, C.R. Molina and F. Murad, 1987, Effects of glyceryl trinitrate on endothelium-dependent and -independent relaxation and cyclic GMP levels in rat aorta and human coronary artery, J Cardiovasc Pharmacol, 10, 82-9.

Rathore, S.S., Y. Wang and H. Krumholz, 2002, Sex-based differences in the effect of digoxin for the treatment of heart failure, N Engl J Med, 347:1403-11.

Ratnatunga, C.P., S.F. Edmondson, G.M. Rees and I.B. Kovacs, 1992, High-dose aspirin inhibits shear-induced platelet reaction involving thrombin generation, Circulation, 85, 1077-82.

Ratz, J.D., A.B. Fraser, K.J. Rees-Milton, M.A. Adams and B.M. Bennett, 2000a, Endothelin receptor antagonism does not prevent the development of *In vivo* glyceryl trinitrate tolerance in the Rat, J Pharmacol Exp Ther, 295, 578-85.

Ratz, J.D., J.J. McGuire, D.J. Anderson and B.M. Bennett, 2000b, Effects of the flavoprotein inhibitor, diphenyleneiodonium sulfate, on *ex vivo* organic nitrate tolerance in the rat, J Pharmacol Exp Ther, 293, 569-77.

Rauch, U., J.I. Osende, J.H. Chesebro, V. Fuster, D.A. Vorchheimer, K. Harris, P. Harris, D.A. Sandler, J.T. Fallon, S. Jayaraman and J.J. Badimon, 2000, Statins and cardiovascular diseases: the multiple effects of lipid-lowering therapy by statins, Atherosclerosis, 153, 181-9.

Reddy, K.G., R.N. Nair, H.M. Sheehan and J.M. Hodgson, 1994, Evidence that selective endothelial dysfunction may occur in the absence of angiographic or ultrasound atherosclerosis in patients with risk factors for atherosclerosis, J Am Coll Cardiol, 23, 833-43.

Reichek, N., C. Priest, D. Zimrin, T. Chandler and M.S. Sutton, 1984, Antianginal effects of nitroglycerin patches, Am J Cardiol, 54, 1-7.

Rest, R.F., 1994, Measurement of human neutrophil respiratory burst activity during phagocytosis of bacteria, Methods in Enzymology, 236, 119-137.

Retterstol, L., L. Eikvar, M. Bohn, A. Bakken, J. Erikssen and K. Berg, 2002, C-reactive protein predicts death in patients with previous premature myocardial infarction-A 10 year follow-up study, Atherosclerosis, 160, 433-40.

Richardson, P.D., M.J. Davies and G.V. Born, 1989, Influence of plaque configuration and stress distribution on fissuring of coronary atherosclerotic plaques, Lancet, 2 (8669), 941-4.

Ridker, P.M., J.E. Buring and N. Rifai, 2001a, Soluble P-selectin and the risk of future cardiovascular events, Circulation, 103, 491-5.

Ridker, P.M., J.E. Buring, J. Shih, M. Matias and C.H. Hennekens, 1998, Prospective study of C-reactive protein and the risk of future cardiovascular events among apparently healthy women, Circulation, 98, 731-3.

Ridker, P.M., M. Cushman, M.J. Stampfer, R.P. Tracy and C.H. Hennekens, 1997, Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men, N Engl J Med, 336, 973-9.

Ridker, P.M., C.H. Hennekens, J.E. Buring and N. Rifai, 2000a, C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women, N Engl J Med, 342, 836-43.

Ridker, P.M., J.E. Manson, J.E. Buring, S.Z. Goldhaber and C.H. Hennekens, 1991a, The effect of chronic platelet inhibition with low-dose aspirin on atherosclerotic progression and acute thrombosis: clinical evidence from the Physicians' Health Study, Am Heart J, 122, 1588-92.

Ridker, P.M., J.E. Manson, J.E. Buring, J.E. Muller and C.H. Hennekens, 1990, Circadian variation of acute myocardial infarction and the effect of low-dose aspirin in a randomized trial of physicians, Circulation, 82, 897-902.

Ridker, P.M., J.E. Manson, J.M. Gaziano, J.E. Buring and C.H. Hennekens, 1991b, Low-dose aspirin therapy for chronic stable angina. A randomized, placebo-controlled clinical trial, Ann Intern Med, 114, 835-9.

Ridker, P.M., N. Rifai, M. Clearfield, J.R. Downs, S.E. Weis, J.S. Miles and A.M. Gotto, Jr., 2001b, Measurement of C-reactive protein for the targeting of statin therapy in the primary prevention of acute coronary events, N Engl J Med, 344, 1959-65.

Ridker, P.M., N. Rifai, M.A. Pfeffer, F. Sacks and E. Braunwald, 1999, Long-term effects of pravastatin on plasma concentration of C-reactive protein. The Cholesterol and Recurrent Events (CARE) Investigators, Circulation, 100, 230-5.

Ridker, P.M., N. Rifai, M.J. Stampfer and C.H. Hennekens, 2000b, Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men, Circulation, 101, 1767-72.

Rinder, H.M., J.L. Bonan, C.S. Rinder, K.A. Ault and B.R. Smith, 1991a, Activated and unactivated platelet adhesion to monocytes and neutrophils, Blood, 78, 1760-9.

Rinder, H.M., J.L. Bonan, C.S. Rinder, K.A. Ault and B.R. Smith, 1991b, Dynamics of leukocyte-platelet adhesion in whole blood, Blood, 78, 1730-7.

Ringqvist, A., K. Caidahl, A.S. Petersson and A. Wennmalm, 2000, Diurnal variation of flow-mediated vasodilation in healthy premenopausal women, Am J Physiol Heart Circ Physiol, 279, H2720-5.

Ritchie, J.L., H.D. Alexander and I.M. Rea, 2000, Flow cytometry analysis of platelet P-selectin expression in whole blood-methodological considerations, Clin Lab Haematol, 22, 359-63.

Rittenhouse, S.E., 1983, Human platelets contain phospholipase C that hydrolyzes polyphosphoinositides, Proc Natl Acad Sci U S A, 80, 5417-20.

Rittenhouse-Simmons, S., 1981, Differential activation of platelet phospholipases by thrombin and ionophore A23187, J Biol Chem, 256, 4153-5.

Roberts, L.J., 2nd and J.D. Morrow, 1997, The generation and actions of isoprostanes, Biochim Biophys Acta, 1345, 121-35.

Rodvein, R., J.N. Lindon and P.H. Levine, 1976, Physiology and ultrastructure of the blood platelet following exposure to hydrogen peroxide, Br J Haematol, 33, 19-26.

Rolland, P.H., M. Bory, F. Leca, J. Sainsous, E. Gueydon, I. Juhan, A. Serradimigni and J.P. Cano, 1984, Evidence for isosorbide dinitrate (ISDN) promoting effect on prostacyclin release by the lung and prostacyclin implication in ISDN-induced inhibition of platelet aggregation in humans, Prostaglandins Leukot Med, 16, 333-46.

Ronson, R.S., M. Nakamura and J. Vinten-Johansen, 1999, The cardiovascular effects and implications of peroxynitrite, Cardiovasc Res, 44, 47-59.

Rosado, J.A. and S.O. Sage, 2000, Regulation of plasma membrane Ca^{2+}-ATPase by small GTPases and phosphoinositides in human platelets, J Biol Chem, 275, 19529-35.

Ross, R., 1993, The pathogenesis of atherosclerosis: a perspective for the 1990s, Nature, 362, 801-9.

Ross, R., 1999, Atherosclerosis-an inflammatory disease, N Engl J Med, 340, 115-26.

Rossi, E., B. Casali, G. Regolisti, S. Davoli, F. Perazzoli, A. Negro, C. Sani, B. Tumiati and D. Nicoli, 1998, Increased plasma levels of platelet-derived growth factor (PDGF-BB + PDGF-AB) in patients with never-treated mild essential hypertension, Am J Hypertens, 11, 1239-43.

Rossi, F., E. Rossi, F.I. Pareti, S. Colli, E. Tremoli and L. Gallo, 2001, *In vitro* measurement of platelet glycoprotein IIb/IIIa receptor blockade by abciximab: interindividual variation and increased platelet secretion, Haematologica, 86, 192-8.

Rostagno, C., D. Prisco, R. Paniccia, G. Costanzo, L. Poggesi, M. Boddi and R. Abbate, 1990, Effects of calcium channel blockers on platelet aggregation and thromboxane A_2 formation: an *in vivo* double blind randomized study, Thromb Res, 59, 531-9.

Roth, G.J. and P.W. Majerus, 1975, The mechanism of the effect of aspirin on human platelets. I. Acetylation of a particulate fraction protein, J Clin Invest, 56, 624-32.

Roth, G.J., N. Stanford and P.W. Majerus, 1975, Acetylation of prostaglandin synthase by aspirin, Proc Natl Acad Sci U S A, 72, 3073-6.

Rothe, G. and G. Valet, 1990, Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescin, J Leukoc Biol, 47, 440-8.

Roubaud, V., S. Sankarapandi, P. Kuppusamy, P. Tordo and J.L. Zweier, 1997, Quantitative measurement of superoxide generation using the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide, Anal Biochem, 247, 404-11.

Rovin, J.D., J.S. Stamler, J. Loscalzo and J.D. Folts, 1993, Sodium nitroprusside, an endothelium-derived relaxing factor congener, increases platelet cyclic GMP levels and inhibits epinephrine-exacerbated *in vivo* platelet thrombus formation in stenosed canine coronary arteries, J Cardiovasc Pharmacol, 22, 626-31.

Rubanyi, G.M., E.H. Ho, E.H. Cantor, W.C. Lumma and L.H. Botelho, 1991, Cytoprotective function of nitric oxide: inactivation of superoxide radicals produced by human leukocytes, Biochem Biophys Res Commun, 181, 1392-7.

Rubanyi, G.M. and P.M. Vanhoutte, 1986, Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor, Am J Physiol, 250, H822-7.

Rutherford, J.D., M.A. Pfeffer, L.A. Moye, B.R. Davis, G.C. Flaker, P.R. Kowey, G.A. Lamas, H.S. Miller, M. Packer and J.L. Rouleau, 1994, Effects of captopril on ischemic events after myocardial infarction. Results of the Survival and Ventricular Enlargement trial. SAVE Investigators, Circulation, 90, 1731-8.

Sacks, F.M., M.A. Pfeffer, L.A. Moye, J.L. Rouleau, J.D. Rutherford, T.G. Cole, L. Brown, J.W. Warnica, J.M. Arnold, C.C. Wun, B.R. Davis and E. Braunwald, 1996, The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators, N Engl J Med, 335, 1001-9.

Safar, M.E., 1999, Epidemiological aspects of pulse pressure and arterial stiffness, J Hypertens, 17 Suppl 5, S37-40.

Safar, M.E., 2000, Pulse pressure, arterial stiffness, and cardiovascular risk, Curr Opin Cardiol, 15, 258-63.

Sage, P.R., I.S. de La Lande, I. Stafford, C.L. Bennett, G. Phillipov, J. Stubberfield and J.D. Horowitz, 2000, Nitroglycerin tolerance in human vessels : evidence for impaired nitroglycerin bioconversion, Circulation, 102, 2810-5.

Sage, S.O., J.E. Merritt, T.J. Hallam and T.J. Rink, 1989, Receptor-mediated calcium entry in fura-2-loaded human platelets stimulated with ADP and thrombin. Dual-wavelengths studies with Mn^{2+}, Biochem J, 258, 923-6.

Sage, S.O., R. Reast and T.J. Rink, 1990, ADP evokes biphasic Ca^{2+} influx in fura-2-loaded human platelets. Evidence for Ca^{2+} entry regulated by the intracellular Ca^{2+} store, Biochem J, 265, 675-80.

Sakita, S., Y. Kishi and F. Numano, 1997, Acute vigorous exercise attenuates sensitivity of platelets to nitric oxide, Thromb Res, 87, 461-71.

Salvemini, D. and R. Botting, 1993, Modulation of platelet function by free radicals and free-radical scavengers, Trends Pharmacol Sci, 14, 36-42.

Salvemini, D., M.G. Currie and V. Mollace, 1996, Nitric oxide-mediated cyclooxygenase activation. A key event in the antiplatelet effects of nitrovasodilators, J Clin Invest, 97, 2562-8.

Salvemini, D., G. de Nucci, R.J. Gryglewski and J.R. Vane, 1989a, Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor, Proc Natl Acad Sci U S A, 86, 6328-32.

Salvemini, D., G. de Nucci, J.M. Sneddon and J.R. Vane, 1989b, Superoxide anions enhance platelet adhesion and aggregation, Br J Pharmacol, 97, 1145-50.

Salvemini, D., A. Pistelli and E. Anggard, 1993, Vascular and anti-platelet actions of 1,2- and 1,3-glyceryl dinitrate, Br J Pharmacol, 110, 937-42.

Salvemini, D., W. Radziszewski, V. Mollace, A. Moore, D. Willoughby and J. Vane, 1991, Diphenylene iodonium, an inhibitor of free radical formation, inhibits platelet aggregation, Eur J Pharmacol, 199, 15-8.

Sambrano, G.R., W. Huang, T. Faruqi, S. Mahrus, C. Craik and S.R. Coughlin, 2000, Cathepsin G activates protease-activated receptor-4 in human platelets, J Biol Chem, 275, 6819-23.

Sanders, S.A., R. Eisenthal and R. Harrison, 1997, NADH oxidase activity of human xanthine oxidoreductase-generation of superoxide anion, Eur J Biochem, 245, 541-8.

Sandirasegarane, L. and J. Diamond, 1999, The nitric oxide donors, SNAP and DEA/NO, exert a negative inotropic effect in rat cardiomyocytes which is independent of cyclic GMP elevation, J Mol Cell Cardiol, 31, 799-808.

Sase, K. and T. Michel, 1995, Expression of constitutive endothelial nitric oxide synthase in human blood platelets, Life Sci, 57, 2049-55.

Sato, M., D.K. Das and R.M. Engelman, 1999, Interaction of bradykinin with angiotensin, prostacyclin, and nitric oxide in myocardial preservation, Ann N Y Acad Sci, 874, 286-94.

Saussy, D.L., Jr., D.E. Mais, R.M. Burch and P.V. Halushka, 1986, Identification of a putative thromboxane A_2/prostaglandin H_2 receptor in human platelet membranes, J Biol Chem, 261, 3025-9.

Savage, B., S.J. Shattil and Z.M. Ruggeri, 1992, Modulation of platelet function through adhesion receptors. A dual role for glycoprotein IIb-IIIa (integrin alpha IIb beta 3) mediated by fibrinogen and glycoprotein Ib-von Willebrand factor, J Biol Chem, 267, 11300-6.

Savi, P., J. Combalbert, C. Gaich, M.C. Rouchon, J.P. Maffrand, Y. Berger and J.M. Herbert, 1994, The antiaggregating activity of clopidogrel is due to a metabolic activation by the hepatic cytochrome P_{450}-1A, Thromb Haemost, 72, 313-7.

Saxon, A. and H.E. Kattlove, 1976, Platelet inhibiton by sodium nitroprusside, a smooth muscle inhibitor, Blood, 47, 957-61.

Scarborough, R.M., 1999, Development of eptifibatide, Am Heart J, 138, 1093-104.

Scandinavian Simvastatin Survival Study (4S)., 1994, Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease, Lancet, 344 (8934), 1383-9.

Scandinavian Simvastatin Survival Study (4S)., 1995, Baseline serum cholesterol and treatment effect in the Scandinavian Simvastatin Survival Study (4S), Lancet, 345 (8934), 1274-5.

Schachinger, V., M.B. Britten, S. Dimmeler and A.M. Zeiher, 2001, NADH/NADPH oxidase $p22^{phox}$ gene polymorphism is associated with improved coronary endothelial vasodilator function, Eur Heart J, 22, 96-101.

Schachinger, V., M.B. Britten and A.M. Zeiher, 2000, Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease, Circulation, 101, 1899-906.

Schachinger, V. and A.M. Zeiher, 1995, Quantitative assessment of coronary vasoreactivity in humans *in vivo*. Importance of baseline vasomotor tone in atherosclerosis, Circulation, 92, 2087-94.

Schachinger, V. and A.M. Zeiher, 2000, Atherosclerosis-associated endothelial dysfunction, Z Kardiol, 89 Suppl 9, IX/70-4.

Schafer, A.I., R.W. Alexander and R.I. Handin, 1980, Inhibition of platelet function by organic nitrate vasodilators, Blood, 55, 649-54.

Schattner, M.A., M.R. Finiasz, J.A. Notrica and M.A. Lazzari, 1994, Platelet aggregation inhibition by mononuclear leukocytes, Thromb Res, 73, 205-14.

Schiffrin, E.L., 2001, Role of endothelin-1 in hypertension and vascular disease, Am J Hypertens, 14, 83S-89S.

Schiffrin, E.L., J.B. Park, H.D. Intengan and R.M. Touyz, 2000, Correction of arterial structure and endothelial dysfunction in human essential hypertension by the angiotensin receptor antagonist losartan, Circulation, 101, 1653-9.

Schmidt, H.H., H. Hofmann, U. Schindler, Z.S. Shutenko, D.D. Cunningham and M. Feelisch, 1996, No .NO from NO synthase, Proc Natl Acad Sci U S A, 93, 14492-7.

Schmidt-Ott, K.M., S. Kagiyama and M.I. Phillips, 2000, The multiple actions of angiotensin II in atherosclerosis, Regul Pept, 93, 65-77.

Schonbeck, U. and P. Libby, 2001, CD40 signaling and plaque instability, Circ Res, 89, 1092-103.

Schonbeck, U., F. Mach, G.K. Sukhova, E. Atkinson, E. Levesque, M. Herman, P. Graber, P. Basset and P. Libby, 1999, Expression of stromelysin-3 in atherosclerotic lesions: regulation via CD40-CD40 ligand signaling *in vitro* and *in vivo*, J Exp Med, 189, 843-53.

Schonbeck, U., F. Mach, G.K. Sukhova, C. Murphy, J.Y. Bonnefoy, R.P. Fabunmi and P. Libby, 1997, Regulation of matrix metalloproteinase expression in human vascular smooth muscle cells by T lymphocytes: a role for CD40 signaling in plaque rupture?, Circ Res, 81, 448-54.

Schonbeck, U., G.K. Sukhova, K. Shimizu, F. Mach and P. Libby, 2000, Inhibition of CD40 signaling limits evolution of established atherosclerosis in mice, Proc Natl Acad Sci U S A, 97, 7458-63.

Schoonmaker, G.C., R.W. Fallet and P.K. Carmines, 2000, Superoxide anion curbs nitric oxide modulation of afferent arteriolar ANG II responsiveness in diabetes mellitus, Am J Physiol Renal Physiol, 278, F302-9.

Schroder, H., D.C. Leitman, B.M. Bennett, S.A. Waldman and F. Murad, 1988, Glyceryl trinitrate-induced desensitization of guanylate cyclase in cultured rat lung fibroblasts, J Pharmacol Exp Ther, 245, 413-8.

Schroeder, S., M.D. Enderle, R. Ossen, C. Meisner, A. Baumbach, M. Pfohl, C. Herdeg, M. Oberhoff, H.U. Haering and K.R. Karsch, 1999, Noninvasive determination of endothelium-mediated vasodilation as a screening test for coronary artery disease: pilot study to assess the predictive value in comparison with angina pectoris, exercise electrocardiography, and myocardial perfusion imaging, Am Heart J, 138, 731-9.

Schror, K., B. Ahland, P. Weiss and E. Konig, 1988, Stimulation of coronary vascular PGI_2 by organic nitrates, Eur Heart J, 9 Suppl A, 25-32.

Schulman, S.P., P.J. Goldschmidt-Clermont, E.J. Topol, R.M. Califf, F.I. Navetta, J.T. Willerson, N.C. Chandra, A.D. Guerci, J.J. Ferguson, R.A. Harrington, A.M. Lincoff, S.J. Yakubov, P.F. Bray, R.D. Bahr, C.L. Wolfe, P.G. Yock, H.V. Anderson, T.W. Nygaard, S.J. Mason, M.B. Effron, A. Fatterpacker, S. Raskin, J. Smith, L. Brashears and G. Gerstenblith., 1996, Effects of integrelin, a platelet glycoprotein IIb/IIIa receptor antagonist, in unstable angina. A randomized multicenter trial, Circulation, 94, 2083-9.

Schwartz, G.G., A.G. Olsson, M.D. Ezekowitz, P. Ganz, M.F. Oliver, D. Waters, A. Zeiher, B.R. Chaitman, S. Leslie and T. Stern, 2001, Effects of atorvastatin on early recurrent ischemic events in acute coronary syndromes: the MIRACL study: a randomized controlled trial, JAMA, 285, 1711-8.

Schwarz, U.R., J. Geiger, U. Walter and M. Eigenthaler, 1999, Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of activating and inhibitory signal transduction pathways in human platelets-definition and detection of ticlopidine/clopidogrel effects, Thromb Haemost, 82, 1145-52.

Schwemmer, M., O. Sommer and E. Bassenge, 2001, Angiotensin receptor blocker losartan suppresses platelet activity by interfering with thromboxane signaling, Cardiovasc Drugs Ther, 15, 301-7.

Scorza, G., D. Pietraforte and M. Minetti, 1997, Role of ascorbate and protein thiols in the release of nitric oxide from S-nitroso-albumin and S-nitroso-glutathione in human plasma, Free Radic Biol Med, 22, 633-42.

Segal, B.H., T.L. Leto, J.I. Gallin, H.L. Malech and S.M. Holland, 2000, Genetic, biochemical, and clinical features of chronic granulomatous disease, Medicine, 79, 170-200.

Selak, M.A., M. Chignard and J.B. Smith, 1988, Cathepsin G is a strong platelet agonist released by neutrophils, Biochem J, 251, 293-9.

Seligman, B.G., A. Biolo, C.A. Polanczyk, J.L. Gross and N. Clausell, 2000, Increased plasma levels of endothelin 1 and von Willebrand factor in patients with type 2 diabetes and dyslipidemia, Diabetes Care, 23, 1395-400.

Sellke, F.W., P.R. Myers, J.N. Bates and D.G. Harrison, 1990, Influence of vessel size on the sensitivity of porcine coronary microvessels to nitroglycerin, Am J Physiol, 258, H515-20.

Seno, T., N. Inoue, D. Gao, M. Okuda, Y. Sumi, K. Matsui, S. Yamada, K.I. Hirata, S. Kawashima, R. Tawa, S. Imajoh-Ohmi, H. Sakurai and M. Yokoyama, 2001, Involvement of NADH/NADPH oxidase in human platelet ROS production, Thromb Res, 103, 399-409.

Serebruany, V.L., A.B. Alford, A.F. Meister, S.Y. Fuzaylov, W.A. Gattis, P.A. Gurbel and C.M. O'Connor, 2001, Clinical Utility of the Platelet Function Analyzer (PFA-100) for the Assessment of the Platelet Status in Patients with Congestive Heart Failure (EPCOT trial), Thromb Res, 101, 427-33.

Serne, E.H., C.D. Stehouwer, J.C. ter Maaten, P.M. ter Wee, J.A. Rauwerda, A.J. Donker and R.O. Gans, 1999, Microvascular function relates to insulin sensitivity and blood pressure in normal subjects, Circulation, 99, 896-902.

Shaffer, J.E., B.J. Han, W.H. Chern and F.W. Lee, 1992, Lack of tolerance to a 24-hour infusion of S-nitroso N-acetylpenicillamine (SNAP) in conscious rabbits, J Pharmacol Exp Ther, 260, 286-93.

Shapiro, M.J., E.J. Weiss, T.R. Faruqi and S.R. Coughlin, 2000, Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin, J Biol Chem, 275, 25216-21.

Sharpe, M.A. and C.E. Cooper, 1998, Reactions of nitric oxide with mitochondrial cytochrome c: a novel mechanism for the formation of nitroxyl anion and peroxynitrite, Biochem J, 332, 9-19.

Shattil, S.J., H. Kashiwagi and N. Pampori, 1998, Integrin signaling: the platelet paradigm, Blood, 91, 2645-57.

Sheikh, S. and G.B. Nash, 1996, Continuous activation and deactivation of integrin CD11b/CD18 during de novo expression enables rolling neutrophils to immobilize on platelets, Blood, 87, 5040-50.

Shenker, A., P. Goldsmith, C.G. Unson and A.M. Spiegel, 1991, The G protein coupled to the thromboxane A_2 receptor in human platelets is a member of the novel Gq family, J Biol Chem, 266, 9309-13.

Shepherd, J., S.M. Cobbe, I. Ford, C.G. Isles, A.R. Lorimer, P.W. MacFarlane, J.H. McKillop and C.J. Packard, 1995, Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group, N Engl J Med, 333, 1301-7.

Shikano, K., E.H. Ohlstein and B.A. Berkowitz, 1987, Differential selectivity of endothelium-derived relaxing factor and nitric oxide in smooth muscle, Br J Pharmacol, 92, 483-5.

Shinozaki, K., A. Kashiwagi, Y. Nishio, T. Okamura, Y. Yoshida, M. Masada, N. Toda and R. Kikkawa, 1999, Abnormal biopterin metabolism is a major cause of impaired endothelium-dependent relaxation through nitric oxide/O_2^- imbalance in insulin-resistant rat aorta, Diabetes, 48, 2437-45.

Shinozaki, K., Y. Nishio, T. Okamura, Y. Yoshida, H. Maegawa, H. Kojima, M. Masada, N. Toda, R. Kikkawa and A. Kashiwagi, 2000, Oral administration of tetrahydrobiopterin prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats, Circ Res, 87, 566-73.

Shiomi, M., T. Ito, Y. Hirouchi and M. Enomoto, 2001, Fibromuscular cap composition is important for the stability of established atherosclerotic plaques in mature WHHL rabbits treated with statins, Atherosclerosis, 157, 75-84.

Shiose, A., J. Kuroda, K. Tsuruya, M. Hirai, H. Hirakata, S. Naito, M. Hattori, Y. Sakaki and H. Sumimoto, 2001, A Novel Superoxide-producing NAD(P)H Oxidase in Kidney, J Biol Chem, 276, 1417-1423.

Siess, W., 1989, Molecular mechanisms of platelet activation, Physiol Rev, 69, 58-178.

Siess, W., K.J. Zangl, M. Essler, M. Bauer, R. Brandl, C. Corrinth, R. Bittman, G. Tigyi and M. Aepfelbacher, 1999, Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions, Proc Natl Acad Sci U S A, 96, 6931-6.

Sievert, H., G. Selzer, W. Schneider, G. Kober, M. Kaltenbach and W.D. Bussmann, 1989, Coronary stenosis dilation by low dose intravenous nitroglycerin, Eur Heart J, 10 Suppl F, 134-6.

Simon, D.I., Z. Chen, H. Xu, C.Q. Li, J. Dong, L.V. McIntire, C.M. Ballantyne, L. Zhang, M.I. Furman, M.C. Berndt and J.A. Lopez, 2000, Platelet glycoprotein ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18), J Exp Med, 192, 193-204.

Simoons, M.L., M.J. de Boer, M.J. van den Brand, A.J. van Miltenburg, J.C. Hoorntje, G.R. Heyndrickx, L.R. van der Wieken, D. de Bono, W. Rutsch, T.F. Schaibl, 1994, Randomized trial of a GPIIb/IIIa platelet receptor blocker in refractory unstable angina. European Cooperative Study Group, Circulation, 89, 596-603.

Singal, P.K., N. Khaper, V. Palace and D. Kumar, 1998, The role of oxidative stress in the genesis of heart disease, Cardiovasc Res, 40, 426-32.

Sinzinger, H., I. Virgolini, J. O'Grady, F. Rauscha and P. Fitscha, 1992, Modification of platelet function by isosorbide dinitrate in patients with coronary artery disease, Thromb Res, 65, 323-35.

Skatchkov, M.P., D. Sperling, U. Hink, A. Mulsch, D.G. Harrison, I. Sindermann, T. Meinertz and T. Munzel, 1999, Validation of lucigenin as a chemiluminescent probe to monitor vascular superoxide as well as basal vascular nitric oxide production, Biochem Biophys Res Commun, 254, 319-24.

Skyrme-Jones, R.A., R.C. O'Brien, K.L. Berry and I.T. Meredith, 2000, Vitamin E supplementation improves endothelial function in type I diabetes mellitus: a randomized, placebo-controlled study, J Am Coll Cardiol, 36, 94-102.

Smith, J.A. and M.J. Weidemann, 1993, Further characterization of the neutrophil oxidative burst by flow cytometry, J Immunol Methods, 162, 261-8.

Smith, J.W., S.R. Steinhubl, A.M. Lincoff, J.C. Coleman, T.T. Lee, R.S. Hillman and B.S. Coller, 1999, Rapid platelet-function assay: an automated and quantitative cartridge-based method, Circulation, 99, 620-5.

Smith, W.L. and D.L. DeWitt, 1995, Biochemistry of prostaglandin endoperoxide H synthase-1 and synthase-2 and their differential susceptibility to nonsteroidal anti-inflammatory drugs, Semin Nephrol, 15, 179-94.

Sneddon, J.M. and J.R. Vane, 1988, Endothelium-derived relaxing factor reduces platelet adhesion to bovine endothelial cells, Proc Natl Acad Sci U S A, 85, 2800-4.

Solzbach, U., B. Hornig, M. Jeserich and H. Just, 1997, Vitamin C improves endothelial dysfunction of epicardial coronary arteries in hypertensive patients, Circulation, 96, 1513-9.

Sorescu, D., M.J. Somers, B. Lassegue, S. Grant, D.G. Harrison and K.K. Griendling, 2001, Electron spin resonance characterization of the NAD(P)H oxidase in vascular smooth muscle cells, Free Radic Biol Med, 30, 603-12.

Sorescu, D., D. Weiss, B. Lassegue, R.E. Clempus, K. Szocs, G.P. Sorescu, L. Valppu, M.T. Quinn, J.D. Lambeth, J.D. Vega, W.R. Taylor and K.K. Griendling, 2002, Superoxide production and expression of nox family proteins in human atherosclerosis, Circulation, 105, 1429-35.

Souza, H.P., L.C. Souza, V.M. Anastacio, A.C. Pereira, M.L. Junqueira, J.E. Krieger, P.L. da Luz, O. Augusto and F.R. Laurindo, 2000, Vascular oxidant stress early after balloon injury: evidence for increased NAD(P)H oxidoreductase activity, Free Radic Biol Med, 28, 1232-42.

Spano, G.M., R. La Mancusa, G. Pettirossi, F.M. Pulcinelli, P.P. Gazzaninga and C. Cordova, 1993, Circadian variations in platelet aggregability in non insulin dependent diabetes patients (NIDDM), Clin Ter, 142, 19-22.

Spasojevic, I., S.I. Liochev and I. Fridovich, 2000, Lucigenin: redox potential in aqueous media and redox cycling with O_2^- production, Arch Biochem Biophys, 373, 447-50.

Spisani, S., A.L. Giuliani, T. Cavalletti, M. Zaccarini, L. Milani, R. Gavioli and S. Traniello, 1992, Modulation of neutrophil functions by activated platelet release factors, Inflammation, 16, 147-58.

Stamler, J., M. Cunningham and J. Loscalzo, 1988, Reduced thiols and the effect of intravenous nitroglycerin on platelet aggregation, Am J Cardiol, 62, 377-80.

Stamler, J., O. Vaccaro, J.D. Neaton and D. Wentworth, 1993, Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial, Diabetes Care, 16, 434-44.

Stamler, J.S., O. Jaraki, J. Osborne, D.I. Simon, J. Keaney, J. Vita, D. Singel, C.R. Valeri and J. Loscalzo, 1992a, Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin, Proc Natl Acad Sci U S A, 89, 7674-7.

Stamler, J.S. and J. Loscalzo, 1991, The antiplatelet effects of organic nitrates and related nitroso compounds *in vitro* and *in vivo* and their relevance to cardiovascular disorders, J Am Coll Cardiol, 18, 1529-36.

Stamler, J.S., D.I. Simon, J.A. Osborne, M.E. Mullins, O. Jaraki, T. Michel, D.J. Singel and J. Loscalzo, 1992b, S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds, Proc Natl Acad Sci U S A, 89, 444-8.

Stamler, J.S., D.J. Singel and J. Loscalzo, 1992c, Biochemistry of nitric oxide and its redox-activated forms, Science, 258, 1898-902.

Stanger, O., W. Renner, G. Khoschsorur, B. Rigler and T.C. Wascher, 2001, NADH/NADPH oxidase $p22^{phox}$ C242T polymorphism and lipid peroxidation in coronary artery disease, Clin Physiol, 21, 718-22.

Steering Committee of the Physicians' Health Study Research Group., 1988a, Findings from the aspirin component of the ongoing Physicians' Health Study, N Engl J Med, 318, 262-4.

Steering Committee of the Physicians' Health Study Research Group., 1988b, The physicians' health study: aspirin for the primary prevention of myocardial infarction, N Engl J Med, 318, 924-6.

Steering Committee of the Physicians' Health Study Research Group., 1989, Final report on the aspirin component of the ongoing Physicians' Health Study, N Engl J Med, 321, 129-35.

Steering Committee, Transdermal Nitroglycerin Cooperative Study., 1991, Acute and chronic antianginal efficacy of continuous twenty-four-hour application of transdermal nitroglycerin., Am J Cardiol, 68, 1263-73.

Stefanadis, C., C. Stratos, H. Boudoulas, C. Kourouklis and P. Toutouzas, 1990, Distensibility of the ascending aorta: comparison of invasive and non-invasive techniques in healthy men and in men with coronary artery disease, Eur Heart J, 11, 990-6.

Steinberg, H.O., G. Brechtel, A. Johnson, N. Fineberg and A.D. Baron, 1994, Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release, J Clin Invest, 94, 1172-9.

Stenestrand, U. and L. Wallentin, 2001, Early statin treatment following acute myocardial infarction and 1-year survival, JAMA, 285, 430-6.

Stewart, S., D.W. Voss, D.L. Northey and J.D. Horowitz, 1996, Relationship between plasma perhexiline concentration and symptomatic status during short-term perhexiline therapy, Ther Drug Monit, 18, 635-9.

Stork, T., H. Eichstadt, M. Mockel, R. Gareis, T. Bodemann and R. Muller, 1997, Hemodynamic action of captopril in coronary patients with heart failure tolerant to nitroglycerin, Clin Cardiol, 20, 999-1004.

Strandberg, T.E., H. Vanhanen and M.J. Tikkanen, 2000, Associations between change in C-reactive protein and serum lipids during statin treatment, Ann Med, 32, 579-83.

Stuehr, D.J., O.A. Fasehun, N.S. Kwon, S.S. Gross, J.A. Gonzalez, R. Levi and C.F. Nathan, 1991, Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyleneiodonium and its analogs, Faseb J, 5, 98-103.

Subbanagounder, G., A.D. Watson and J.A. Berliner, 2000, Bioactive products of phospholipid oxidation: isolation, identification, measurement and activities, Free Radic Biol Med, 28, 1751-61.

Suh, Y.A., R.S. Arnold, B. Lassegue, J. Shi, X. Xu, D. Sorescu, A.B. Chung, K.K. Griendling and J.D. Lambeth, 1999, Cell transformation by the superoxide-generating oxidase Mox1, Nature, 401, 79-82.

Sumi, D., T. Hayashi, N.K. Thakur, M. Jayachandran, Y. Asai, H. Kano, H. Matsui and A. Iguchi, 2001, A HMG-CoA reductase inhibitor possesses a potent anti-atherosclerotic effect other than serum lipid lowering effects-the relevance of endothelial nitric oxide synthase and superoxide anion scavenging action, Atherosclerosis, 155, 347-57.

Surdacki, A., M. Nowicki, J. Sandmann, D. Tsikas, R.H. Boeger, S.M. Bode-Boeger, O. Kruszelnicka-Kwiatkowska, F. Kokot, J.S. Dubiel and J.C. Froelich, 1999, Reduced urinary excretion of nitric oxide metabolites and increased plasma levels of asymmetric dimethylarginine in men with essential hypertension, J Cardiovasc Pharmacol, 33, 652-8.

Sussex, B.A., N.R. Campbell, M.K. Raju and D.W. McKay, 1994, The antianginal efficacy of isosorbide dinitrate therapy is maintained during diuretic treatment, Clin Pharmacol Ther, 56, 229-34.

Sutsch, G., J.H. Kim, C. Bracht and W. Kiowski, 1997, Lack of cross-tolerance to short-term linsidomine in forearm resistance vessels and dorsal hand veins in subjects with nitroglycerin tolerance, Clin Pharmacol Ther, 62, 538-45.

Suwaidi, J.A., S. Hamasaki, S.T. Higano, R.A. Nishimura, D.R. Holmes, Jr. and A. Lerman, 2000, Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction, Circulation, 101, 948-54.

Suzuki, Y., J. Kajikuri, K. Suzumori and T. Itoh, 2000, Mechanisms underlying the reduced endothelium-dependent relaxation in human omental resistance artery in pre-eclampsia, J Physiol, 527 Pt 1, 163-74.

Swahn, E. and L. Wallentin, 1987, Platelet reactivity in unstable coronary artery disease, Thromb Haemost, 57, 302-5.

Szczeklik, A., R.J. Gryglewski, R. Nizankowski, J. Musial, R. Pieton and J. Mruk, 1978, Circulatory and anti-platelet effects of intravenous prostacyclin in healthy men, Pharmacol Res Commun, 10, 545-56.

Szocs, K., B. Lassegue, D. Sorescu, L.L. Hilenski, L. Valppu, T.L. Couse, J.N. Wilcox, M.T. Quinn, J.D. Lambeth and K.K. Griendling, 2002, Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury, Arterioscler Thromb Vasc Biol, 22, 21-7.

Szuster-Ciesieiska, A., J. Daniluk and M. Kanderfer-Szerszer, 2001, Oxidative stress in blood of patients with alcohol-related pancreatitis, Pancreas, 22, 261-6.

Szuwart, T., T. Brzoska, T.A. Luger, T. Filler, E. Peuker and R. Dierichs, 2000, Vitamin E reduces platelet adhesion to human endothelial cells *in vitro*, Am J Hematol, 65, 1-4.

Tabibiazar, R., A.H. Jamali and S.G. Rockson, 2001, Formulating clinical strategies for angiotensin antagonism: a review of preclinical and clinical studies, Am J Med, 110, 471-80.

Taddei, S., A. Virdis, P. Mattei and A. Salvetti, 1993, Vasodilation to acetylcholine in primary and secondary forms of human hypertension, Hypertension, 21, 929-33.

Takahara, K., R. Murray, G.A. FitzGerald and D.J. Fitzgerald, 1990, The response to thromboxane A_2 analogues in human platelets. Discrimination of two binding sites linked to distinct effector systems, J Biol Chem, 265, 6836-44.

Takano, S., J. Kimura, I. Matsuoka and T. Ono, 1999, No requirement of $P2X_1$ purinoceptors for platelet aggregation, Eur J Pharmacol, 372, 305-9.

Takatsu, H., H. Tasaki, H.N. Kim, S. Ueda, M. Tsutsui, K. Yamashita, T. Toyokawa, Y. Morimoto, Y. Nakashima and T. Adachi, 2001, Overexpression of EC-SOD suppresses endothelial-cell-mediated LDL oxidation, Biochem Biophys Res Commun, 285, 84-91.

Takeshita, S., T Isshiki, M. Ochiai, T. Ishikawa, Y. Nishiyama, T. Fusano, H. Toyoizumi, K. Kondo, Y. Ono and T. Sato, 1997, Systemic inflammatory responses in acute coronary syndrome: Increased activity observed in polymorphonuclear leukocytes but not in T-lymphocytes, Atherosclerosis, 135, 187-92.

Tannous, M., R.A. Rabini, A. Vignini, N. Moretti, P. Fumelli, B. Zielinski, L. Mazzanti and B. Mutus, 1999, Evidence for iNOS-dependent peroxynitrite production in diabetic platelets, Diabetologia, 42, 539-44.

Tarpey, M.M. and I. Fridovich, 2001, Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite, Circ Res, 89, 224-36.

Tarpey, M.M., C.R. White, E. Suarez, G. Richardson, R. Radi and B.A. Freeman, 1999, Chemiluminescent detection of oxidants in vascular tissue. Lucigenin but not coelenterazine enhances superoxide formation, Circ Res, 84, 1203-11.

Tatarko, M. and J.A. Bumpus, 1997, Further studies on the inactivation by sodium azide of lignin peroxidase from Phanerochaete chrysosporium, Arch Biochem Biophys, 339, 200-9.

Tateson, J.E., S. Moncada and J.R. Vane, 1977, Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets, Prostaglandins, 13, 389-97.

Tawakol, A., M. Forgione, M. Stuehlinger, N. Alpert, J. Cooke, J. Loscalzo, A. Fischman, M. Creager and H. Gewirtz, 2002, Homocysteine impairs coronary microvascular dilator function in humans, J Am Coll Cardiol, 40, 1501-5.

Taylor, B.S. and D.A. Geller, 2000, Molecular regulation of the human inducible nitric oxide synthase (iNOS) gene, Shock, 13, 413-24.

Taylor, R.R., M. Sturm, R. Vandongen, J. Strophair and L.J. Beilin, 1987, Whole blood platelet aggregation is not affected by cigarette smoking but is sex-related, Clin Exp Pharmacol Physiol, 14, 665-71.

Tcheng, J.E., S.G. Ellis, B.S. George, D.J. Kereiakes, N.S. Kleiman, J.D. Talley, A.L. Wang, H.F. Weisman, R.M. Califf and E.J. Topol, 1994, Pharmacodynamics of chimeric glycoprotein IIb/IIIa integrin antiplatelet antibody Fab 7E3 in high-risk coronary angioplasty, Circulation, 90, 1757-64.

Tcheng, J.E., R.A. Harrington, K. Kottke-Marchant, N.S. Kleiman, S.G. Ellis, D.J. Kereiakes, M.J. Mick, F.I. Navetta, J.E. Smith and S.J. Worley, 1995, Multicenter, randomized, double-blind, placebo-controlled trial of the platelet integrin glycoprotein IIb/IIIa blocker Integrelin in elective coronary intervention. IMPACT Investigators, Circulation, 91, 2151-7.

Teranishi, K. and O. Shimomura, 1997, Coelenterazine analogs as chemiluminescent probe for superoxide anion, Anal Biochem, 249, 37-43.

Test, S.T. and S.J. Weiss, 1984, Quantitative and temporal characterization of the extracellular H_2O_2 pool generated by human neutrophils, J Biol Chem, 259, 399-405.

Thadani, U., H.L. Fung, A.C. Darke and J.O. Parker, 1982, Oral isosorbide dinitrate in angina pectoris: comparison of duration of action an dose-response relation during acute and sustained therapy, Am J Cardiol, 49, 411-9.

Thadani, U., S.F. Hamilton, E. Olson, J. Anderson, W. Voyles, R. Prasad and S.M. Teague, 1986, Transdermal nitroglycerin patches in angina pectoris. Dose titration, duration of effect, and rapid tolerance, Ann Intern Med, 105, 485-92.

Thadani, U., S.F. Hamilton, E. Olson, J.L. Anderson, R. Prasad, W. Voyles, R. Doyle, E. Kirsten and S.M. Teague, 1987, Duration of effects and tolerance of slow-release isosorbide-5-mononitrate for angina pectoris, Am J Cardiol, 59, 756-62.

Thadani, U., C.R. Maranda, E. Amsterdam, L. Spaccavento, R.G. Friedman, R. Chernoff, S. Zellner, J. Gorwit and P.H. Hinderaker, 1994, Lack of pharmacologic tolerance and rebound angina pectoris during twice-daily therapy with isosorbide-5-mononitrate, Ann Intern Med, 120, 353-9.

Thaulow, E., J. Erikssen, L. Sandvik, H. Stormorken and P.F. Cohn, 1991, Blood platelet count and function are related to total and cardiovascular death in apparently healthy men, Circulation, 84, 613-7.

The CONSENSUS Trial Study Group., 1987, Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS), N Engl J Med, 316, 1429-35.

The EPIC Investigators., 1994, Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty, N Engl J Med, 330, 956-61.

The EPILOG Investigators., 1997, Platelet glycoprotein IIb/IIIa receptor blockade and low-dose heparin during percutaneous coronary revascularization, N Engl J Med, 336, 1689-96.

The PURSUIT Trial Investigators., 1998, Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. Platelet Glycoprotein IIb/IIIa in Unstable Angina: Receptor Suppression Using Integrilin Therapy, N Engl J Med, 339, 436-43.

The RESTORE Investigators., 1997, Effects of platelet glycoprotein IIb/IIIa blockade with tirofiban on adverse cardiac events in patients with unstable angina or acute myocardial infarction undergoing coronary angioplasty. Randomized Efficacy Study of Tirofiban for Outcomes and REstenosis, Circulation, 96, 1445-53.

The RISC Group., 1990, Risk of myocardial infarction and death during treatment with low dose aspirin and intravenous heparin in men with unstable coronary artery disease, Lancet, 336 (8719), 827-30.

The SALT Collaborative Group., 1991, Swedish Aspirin Low-Dose Trial (SALT) of 75 mg aspirin as secondary prophylaxis after cerebrovascular ischaemic events, Lancet, 338 (8779), 1345-9.

The SOLVD Investigators., 1991, Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure, N Engl J Med, 325, 293-302.

The SYMPHONY Investigators., 2000, Comparison of sibrafiban with aspirin for prevention of cardiovascular events after acute coronary syndromes: a randomised trial. Sibrafiban versus Aspirin to Yield Maximum Protection from Ischemic Heart Events Post-acute Coronary Syndromes, Lancet, 355 (9201), 337-45.

Theilmeier, G., J.R. Chan, C. Zalpour, B. Anderson, B.Y. Wang, A. Wolf, P.S. Tsao and J.P. Cooke, 1997, Adhesiveness of mononuclear cells in hypercholesterolemic humans is normalized by dietary L-arginine, Arterioscler Thromb Vasc Biol, 17, 3557-64.

Theroux, P. and V. Fuster, 1998, Acute coronary syndromes: unstable angina and non-Q-wave myocardial infarction, Circulation, 97, 1195-206.

Theroux, P., D. Waters, J. Lam, M. Juneau and J. McCans, 1992, Reactivation of unstable angina after the discontinuation of heparin, N Engl J Med, 327, 141-5.

Thomas, J.S., M.F. McConnell, T.G. Bell and G.A. Padgett, 1992, Platelet aggregation and dense granule secretion in a colony of dogs with spontaneous hypertension, J Hypertens, 10, 1493-8.

Thompson, P.D., N.M. Moyna, C. Michael White, K.M. Weber, S. Giri and D.D. Waters, 2002, The effects of hydroxy-methyl-glutaryl co-enzyme A reductase inhibitors on platelet thrombus formation, Atherosclerosis, 161, 301-6.

Thompson, S.G., J. Kienast, S.D. Pyke, F. Haverkate and J.C. van de Loo, 1995, Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group, N Engl J Med, 332, 635-41.

Thomson, L., M. Trujillo, R. Telleri and R. Radi, 1995, Kinetics of cytochrome C^{2+} oxidation by peroxynitrite: implications for superoxide measurements in nitric oxide-producing biological systems, Arch Biochem Biophys, 319, 491-7.

Thorne, S., M.J. Mullen, P. Clarkson, A.E. Donald and J.E. Deanfield, 1998, Early endothelial dysfunction in adults at risk from atherosclerosis: different responses to L-arginine, J Am Coll Cardiol, 32, 110-6.

Ting, H.H., F.K. Timimi, K.S. Boles, S.J. Creager, P. Ganz and M.A. Creager, 1996, Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus, J Clin Invest, 97, 22-8.

Ting, H.H., F.K. Timimi, E.A. Haley, M.A. Roddy, P. Ganz and M.A. Creager, 1997, Vitamin C improves endothelium-dependent vasodilation in forearm resistance vessels of humans with hypercholesterolemia, Circulation, 95, 2617-22.

Tofler, G.H., D. Brezinski, A.I. Schafer, C.A. Czeisler, J.D. Rutherford, S.N. Willich, R.E. Gleason, G.H. Williams and J.E. Muller, 1987, Concurrent morning increase in platelet aggregability and the risk of myocardial infarction and sudden cardiac death, N Engl J Med, 316, 1514-8.

Togashi, H., H. Shinzawa, T. Matsuo, Y. Takeda, T. Takahashi, M. Aoyama, K. Oikawa and H. Kamada, 2000, Analysis of hepatic oxidative stress status by electron spin resonance spectroscopy and imaging, Free Radic Biol Med, 28, 846-53.

Tokuue, J., J. Hayashi, Y. Hata, K. Nakahara and Y. Ikeda, 1996, Enhanced platelet aggregability under high shear stress after treadmill exercise in patients with effort angina, Thromb Haemost, 75, 833-7.

Tomaselli, L., C. Cerletti, G. de Gaetano, A. Notarbartolo, G. Davi and M. Pupillo, 1990, Normal platelet function, but increased platelet activation *in vivo* in diabetic patients, Thromb Haemost, 64, 604.

Tomoda, F., M. Takata, S. Kagitani, H. Kinuno, K. Yasumoto, S. Tomita and H. Inoue, 1999, Different platelet aggregability during mental stress in two stages of essential hypertension, Am J Hypertens, 12, 1063-70.

Topol, E.J., R.M. Califf, H.F. Weisman, S.G. Ellis, J.E. Tcheng, S. Worley, R. Ivanhoe, B.S. George, D. Fintel, M. Weston, 1994, Randomised trial of coronary intervention with antibody against platelet IIb/IIIa integrin for reduction of clinical restenosis: results at six months. The EPIC Investigators, Lancet, 343 (8902), 881-6.

Topol, E.J., J.J. Ferguson, H.F. Weisman, J.E. Tcheng, S.G. Ellis, N.S. Kleiman, R.J. Ivanhoe, A.L. Wang, D.P. Miller, K.M. Anderson and R.M. Califf, 1997, Long-term protection from myocardial ischemic events in a randomized trial of brief integrin beta3 blockade with percutaneous coronary intervention. EPIC Investigator Group. Evaluation of Platelet IIb/IIIa Inhibition for Prevention of Ischemic Complication, JAMA, 278, 479-84.

Topol, E.J., D.B. Mark, A.M. Lincoff, E. Cohen, J. Burton, N. Kleiman, D. Talley, S. Sapp, J. Booth, C.F. Cabot, K.M. Anderson and R.M. Califf, 1999, Outcomes at 1 year and economic implications of platelet glycoprotein IIb/IIIa blockade in patients undergoing coronary stenting: results from a multicentre randomised trial. EPISTENT Investigators. Evaluation of Platelet IIb/IIIa Inhibitor for Stenting, Lancet, 354 (9195), 2019-24.

Tornvall, P., L. Chirkova, K.D. Toverud, J.D. Horowitz and Y. Chirkov, 1999, Native and oxidized low density lipoproteins enhance platelet aggregation in whole blood, Thromb Res, 95, 177-83.

Tortorella, C., G. Piazzollo, F. Spanccavento, F.Vella, L.Pace and S. Antonaci, 2000, Regulatory role of extracellular matrix proteins in neutrophil respiratory burst during aging, Mech Ageing Dev, 119, 69-82.

Tosi, M.F. and A. Hamedani, 1992, A rapid, specific assay for superoxide release from phagocytes in small volumes of whole blood, Am J Clin Pathol, 97, 566-73.

Touyz, R.M. and E.L. Schiffrin, 1994, Blunted inhibition by insulin of agonist-stimulated calcium, pH and aggregatory responses in platelets from hypertensive patients, J Hypertens, 12, 1255-63.

Treasure, C.B., J.L. Klein, W.S. Weintraub, J.D. Talley, M.E. Stillabower, A.S. Kosinski, J. Zhang, S.J. Boccuzzi, J.C. Cedarholm and R.W. Alexander, 1995, Beneficial effects of cholesterol-lowering therapy on the coronary endothelium in patients with coronary artery disease, N Engl J Med, 332, 481-7.

Treasure, C.B., J.A. Vita, D.A. Cox, R.D. Fish, J.B. Gordon, G.H. Mudge, W.S. Colucci, M.G. Sutton, A.P. Selwyn and R.W. Alexander, 1990, Endothelium-dependent dilation of the coronary microvasculature is impaired in dilated cardiomyopathy, Circulation, 81, 772-9.

Trepakova, E.S., R.A. Cohen and V.M. Bolotina, 1999, Nitric oxide inhibits capacitative cation influx in human platelets by promoting sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase-dependent refilling of Ca^{2+} stores, Circ Res, 84, 201-9.

Trip, M.D., V.M. Cats, F.J. van Capelle and J. Vreeken, 1990a, Platelet hyperreactivity and prognosis in survivors of myocardial infarction, N Engl J Med, 322, 1549-54.

Trip, M.D., V.M. Cats, F.J. van Capelle and J. Vreeken, 1990b, Platelet hyperreactivity and prognosis in survivors of myocardial infarction, N Engl J Med, 322, 1549-54.

Trovati, M., G. Anfossi, F. Cavalot, P. Massucco, E. Mularoni and G. Emanuelli, 1988, Insulin directly reduces platelet sensitivity to aggregating agents. Studies *in vitro* and *in vivo*, Diabetes, 37, 780-6.

Trovati, M., G. Anfossi, F. Cavalot, S. Vitali, P. Massucco, E. Mularoni, P. Schinco, G. Tamponi and G. Emanuelli, 1986, Studies on mechanisms involved in hypoglycemia-induced platelet activation, Diabetes, 35, 818-25.

Trovati, M., G. Anfossi, P. Massucco, L. Mattiello, C. Costamagna, V. Piretto, E. Mularoni, F. Cavalot, A. Bosia and D. Ghigo, 1997, Insulin stimulates nitric oxide synthesis in human platelets and, through nitric oxide, increases platelet concentrations of both guanosine-3', 5'-cyclic monophosphate and adenosine-3', 5'-cyclic monophosphate, Diabetes, 46, 742-9.

Trovati, M., P. Massucco, L. Mattiello, E. Mularoni, F. Cavalot and G. Anfossi, 1994, Insulin increases guanosine-3',5'-cyclic monophosphate in human platelets. A mechanism involved in the insulin anti-aggregating effect, Diabetes, 43, 1015-9.

Trovati, M., P. Massucco, L. Mattiello, V. Piretto, F. Cavalot, E. Mularoni and G. Anfossi, 1996, The insulin-induced increase of guanosine-3',5'-cyclic monophosphate in human platelets is mediated by nitric oxide, Diabetes, 45, 768-70.

Trovati, M., E.M. Mularoni, S. Burzacca, M.C. Ponziani, P. Massucco, L. Mattiello, V. Piretto, F. Cavalot and G. Anfossi, 1995, Impaired insulin-induced platelet antiaggregating effect in obesity and in obese NIDDM patients, Diabetes, 44, 1318-22.

Tsikas, D., M. Ikic, K.S. Tewes, M. Raida and J.C. Frolich, 1999, Inhibition of platelet aggregation by S-nitroso-cysteine via cGMP-independent mechanisms: evidence of inhibition of thromboxane A_2 synthesis in human blood platelets, FEBS Lett, 442, 162-6.

Tsuchida, S., T. Maki and K. Sato, 1990, Purification and characterization of glutathione transferases with an activity toward nitroglycerin from human aorta and heart. Multiplicity of the human class Mu forms, J Biol Chem, 265, 7150-7.

Tsuji, T., K. Nagata, J. Koike, N. Todoroki and T. Irimura, 1994, Induction of superoxide anion production from monocytes and neutrophils by activated platelets through the P-selectin-sialyl Lewis X interaction, J Leukoc Biol, 56, 583-7.

Tsutamoto, T., M. Kinoshita, T. Hisanaga, Y. Maeda, K. Maeda, A. Wada, D. Fukai and S. Yoshida, 1995, Comparison of hemodynamic effects and plasma cyclic guanosine monophosphate of nicorandil and nitroglycerin in patients with congestive heart failure, Am J Cardiol, 75, 1162-5.

Turton, M.B. and T. Deegan, 1974, Circadian variations of plasma catecholamine, cortisol and immunoreactive insulin concentrations in supine subjects, Clin Chim Acta, 55, 389-97.

Ubatuba, F.B., S. Moncada and J.R. Vane, 1979, The effect of prostacyclin (PGT_2) on platelet behaviour. Thrombus formation *in vivo* and bleeding time, Thromb Haemost, 41, 425-35.

Uematsu, M., Y. Ohara, J.P. Navas, K. Nishida, T.J. Murphy, R.W. Alexander, R.M. Nerem and D.G. Harrison, 1995, Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress, Am J Physiol, 269, C1371-8.

Unger, P., G. Berkenboom and J. Fontaine, 1993, Interaction between hydralazine and nitrovasodilators in vascular smooth muscle, J Cardiovasc Pharmacol, 21, 478-83.

Unger, S.A., M.A. Robinson and J.D. Horowitz, 1997, Perhexiline improves symptomatic status in elderly patients with severe aortic stenosis, Aust N Z J Med, 27, 24-8.

Urban, P., C. Macaya, H.J. Rupprecht, F. Kiemeneij, H. Emanuelsson, A. Fontanelli, M. Pieper, T. Wesseling and L. Sagnard, 1998, Randomized evaluation of anticoagulation versus antiplatelet therapy after coronary stent implantation in high-risk patients: the multicenter aspirin and ticlopidine trial after intracoronary stenting (MATTIS), Circulation, 98, 2126-32.

Usui, M., H. Matsuoka, H. Miyazaki, S. Ueda, S. Okuda and T. Imaizumi, 1998, Increased endogenous nitric oxide synthase inhibitor in patients with congestive heart failure, Life Sci, 62, 2425-30.

Valant, P.A. and D.H. Haynes, 1993, The Ca^{2+}-extruding ATPase of the human platelet creates and responds to cytoplasmic pH changes, consistent with a 2 Ca^{2+}/nH^{+} exchange mechanism, J Membr Biol, 136, 215-30.

Vallance, P., J. Collier and S. Moncada, 1989, Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man, Lancet, 2 (8670), 997-1000.

Vallance, P., A. Leone, A. Calver, J. Collier and S. Moncada, 1992a, Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure, Lancet, 339 (8793), 572-5.

Vallance, P., A. Leone, A. Calver, J. Collier and S. Moncada, 1992b, Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis, J Cardiovasc Pharmacol, 20 Suppl 12, S60-2.

Valles, J., M.T. Santos, A.J. Marcus, L.B. Safier, M.J. Broekman, N. Islam, H.L. Ullman and J. Aznar, 1993, Downregulation of human platelet reactivity by neutrophils. Participation of lipoxygenase derivatives and adhesive proteins, J Clin Invest, 92, 1357-65.

Van Buren, T., W. Vleeming, M.M. Krutzen, T. Van de Kuil, W.H. Gispen and D.J. De Wildt, 1998, Vascular responses of isolated mesenteric resistance and basilar arteries from short- and long-term diabetic rats, Naunyn Schmiedebergs Arch Pharmacol, 358, 663-70.

van der Wal, A.C., A.E. Becker, C.M. van der Loos and P.K. Das, 1994, Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology, Circulation, 89, 36-44.

Vane, J.R. and R.M. Botting, 1995, New insights into the mode of action of anti-inflammatory drugs, Inflamm Res, 44, 1-10.

Varon, D., R. Dardik, B. Shenkman, S. Kotev-Emeth, N. Farzame, I. Tamarin and N. Savion, 1997, A new method for quantitative analysis of whole blood platelet interaction with extracellular matrix under flow conditions, Thromb Res, 85, 283-94.

Varon, D., I. Lashevski, B. Brenner, R. Beyar, N. Lanir, I. Tamarin and N. Savion, 1998, Cone and platelet analyzer: monitoring glycoprotein IIb/IIIa antagonists and von Willebrand disease replacement therapy by testing platelet deposition under flow conditions, Am Heart J, 135, S187-93.

Vasquez-Vivar, J., N. Hogg, P. Martasek, H. Karoui, K.A. Pritchard, Jr. and B. Kalyanaraman, 1999, Tetrahydrobiopterin-dependent inhibition of superoxide generation from neuronal nitric oxide synthase, J Biol Chem, 274, 26736-42.

Vasquez-Vivar, J., N. Hogg, K.A. Pritchard, Jr., P. Martasek and B. Kalyanaraman, 1997, Superoxide anion formation from lucigenin: an electron spin resonance spin-trapping study, FEBS Lett, 403, 127-30.

Vasquez-Vivar, J., B. Kalyanaraman, P. Martasek, N. Hogg, B.S. Masters, H. Karoui, P. Tordo and K.A. Pritchard, Jr., 1998, Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors, Proc Natl Acad Sci U S A, 95, 9220-5.

Verma, S., F. Lovren, A.S. Dumont, K.J. Mather, A. Maitland, T.M. Kieser, C.R. Triggle and T.J. Anderson, 2000, Tetrahydrobiopterin improves endothelial function in human saphenous veins, J Thorac Cardiovasc Surg, 120, 668-71.

Violi, F., A. Ghiselli, L. Iuliano, C. Alessandri, C. Cordova and F. Balsano, 1988, Influence of hydroxyl radical scavengers on platelet function, Haemostasis, 18, 91-8.

Vita, J.A., C.B. Treasure, E.G. Nabel, J.M. McLenachan, R.D. Fish, A.C. Yeung, V.I. Vekshtein, A.P. Selwyn and P. Ganz, 1990, Coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease, Circulation, 81, 491-7.

Vita, J.A., A.C. Yeung, M. Winniford, J.M. Hodgson, C.B. Treasure, J.L. Klein, S. Werns, M. Kern, D. Plotkin, W.J. Shih, Y. Mitchel and P. Ganz, 2000, Effect of cholesterol-lowering therapy on coronary endothelial vasomotor function in patients with coronary artery disease, Circulation, 102, 846-51.

Vu, T.K., D.T. Hung, V.I. Wheaton and S.R. Coughlin, 1991, Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation, Cell, 64, 1057-68.

Wada, T., K. Kodaira, K. Fujishiro, K. Maie, E. Tsukiyama, T. Fukumoto, T. Uchida and S. Yamazaki, 1994, Correlation of ultrasound-measured common carotid artery stiffness with pathological findings, Arterioscler Thromb, 14, 479-82.

Wagner, C.L., M.A. Mascelli, D.S. Neblock, H.F. Weisman, B.S. Coller and R.E. Jordan, 1996, Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets, Blood, 88, 907-14.

Waldman, S.A. and F. Murad, 1987, Cyclic GMP synthesis and function, Pharmacol Rev, 39, 163-96.

Waldman, S.A., R.M. Rapoport, R. Ginsburg and F. Murad, 1986, Desensitization to nitroglycerin in vascular smooth muscle from rat and human, Biochem Pharmacol, 35, 3525-31.

Wallace, D.C., 1978, Perhexiline maleate in the treatment of angina pectoris. Five years of personal clinical experience, Med J Aust, 2, 466, 493-5.

Wallen, N.H., A. Andersson and P. Hjemdahl, 1994, Effects of treatment with oral isosorbide dinitrate on platelet function *in vivo*; a double-blind placebo-controlled study in patients with stable angina pectoris, Br J Clin Pharmacol, 38, 63-70.

Wallen, N.H., C. Held, N. Rehnqvist and P. Hjemdahl, 1995, Platelet aggregability *in vivo* is attenuated by verapamil but not by metoprolol in patients with stable angina pectoris, Am J Cardiol, 75, 1-6.

Wallen, N.H., C. Held, N. Rehnqvist and P. Hjemdahl, 1997, Effects of mental and physical stress on platelet function in patients with stable angina pectoris and healthy controls, Eur Heart J, 18, 807-15.

Wallentin, L.C., 1991, Aspirin (75 mg/day) after an episode of unstable coronary artery disease: long-term effects on the risk for myocardial infarction, occurrence of severe angina and the need for revascularization. Research Group on Instability in Coronary Artery Disease in Southeast Sweden, J Am Coll Cardiol, 18, 1587-93.

Wallerath, T., I. Gath, W.E. Aulitzky, J.S. Pollock, H. Kleinert and U. Forstermann, 1997, Identification of the NO synthase isoforms expressed in human neutrophil granulocytes, megakaryocytes and platelets, Thromb Haemost, 77, 163-7.

Wang, E.Q., W.I. Lee and H.L. Fung, 2002, Lack of critical involvement of endothelial nitric oxide synthase in vascular nitrate tolerance in mice, Br J Pharmacol, 135, 299-302.

Wang, G.R., Y. Zhu, P.V. Halushka, T.M. Lincoln and M.E. Mendelsohn, 1998, Mechanism of platelet inhibition by nitric oxide: *in vivo* phosphorylation of thromboxane receptor by cyclic GMP-dependent protein kinase, Proc Natl Acad Sci U S A, 95, 4888-93.

Wang, H.D., S. Xu, D.G. Johns, Y. Du, M.T. Quinn, A.J. Cayatte and R.A. Cohen, 2001, Role of NADPH oxidase in the vascular hypertrophic and oxidative stress response to angiotensin II in mice, Circ Res, 88, 947-53.

Wang, J., N. Seyedi, X.B. Xu, M.S. Wolin and T.H. Hintze, 1994, Defective endothelium-mediated control of coronary circulation in conscious dogs after heart failure, Am J Physiol, 266, H670-80.

Wang, W., S. Wang, L. Yan, P. Madara, A. Del Pilar Cintron, R.A. Wesley and R.L. Danner, 2000, Superoxide production and reactive oxygen species signaling by endothelial nitric-oxide synthase, J Biol Chem, 275, 16899-903.

Wang, Y., D.C. Newton and P.A. Marsden, 1999, Neuronal NOS: gene structure, mRNA diversity, and functional relevance, Crit Rev Neurobiol, 13, 21-43.

Ward, C., T.H. Wong, J. Murray, I. Rahman, C. Haslett, E.R. Chilvers and A.G. Rossi, 2000, Induction of human neutrophil apoptosis by nitric oxide donors: evidence for a caspase-dependent, cyclic-GMP-independent, mechanism, Biochem Pharmacol, 59, 305-14.

Ware, J.A., P.C. Johnson, M. Smith and E.W. Salzman, 1986, Inhibition of human platelet aggregation and cytoplasmic calcium response by calcium antagonists: studies with aequorin and quin2, Circ Res, 59, 39-42.

Warnholtz, A., H. Mollnau, M. Oelze, M. Wendt and T. Munzel, 2001, Antioxidants and endothelial dysfunction in hyperlipidemia, Curr Hypertens Rep, 3, 53-60.

Warnholtz, A., G. Nickenig, E. Schulz, R. Macharzina, J.H. Brasen, M. Skatchkov, T. Heitzer, J.P. Stasch, K.K. Griendling, D.G. Harrison, M. Bohm, T. Meinertz and T. Munzel, 1999, Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system, Circulation, 99, 2027-33.

Wassmann, S., U. Laufs, A.T. Baumer, K. Muller, K. Ahlbory, W. Linz, G. Itter, R. Rosen, M. Bohm and G. Nickenig, 2001, HMG-CoA reductase inhibitors improve endothelial dysfunction in normocholesterolemic hypertension via reduced production of reactive oxygen species, Hypertension, 37, 1450-7.

Wassmann, S., U. Laufs, K. Muller, C. Konkol, K. Ahlbory, A.T. Baumer, W. Linz, M. Bohm and G. Nickenig, 2002, Cellular antioxidant effects of atorvastatin *in vitro* and *in vivo*, Arterioscler Thromb Vasc Biol, 22, 300-5.

Watanabe, H., M. Kakihana, S. Ohtsuka and Y. Sugishita, 1997a, Effects of enalapril during continuous nitrate therapy: analysis of diameter of coronary arteries and platelet cyclic guanosine monophosphate, Am Heart J, 134, 614-21.

Watanabe, H., M. Kakihana, S. Ohtsuka and Y. Sugishita, 1997b, Randomized, double-blind, placebo-controlled study of supplemental vitamin E on attenuation of the development of nitrate tolerance, Circulation, 96, 2545-50.

Watanabe, H., M. Kakihana, S. Ohtsuka and Y. Sugishita, 1998a, Preventive effects of angiotensin-converting enzyme inhibitors on nitrate tolerance during continuous transdermal application of nitroglycerin in patients with chronic heart failure, Jpn Circ J, 62, 353-8.

Watanabe, H., M. Kakihana, S. Ohtsuka and Y. Sugishita, 1998b, Randomized, double-blind, placebo-controlled study of ascorbate on the preventive effect of nitrate tolerance in patients with congestive heart failure, Circulation, 97, 886-91.

Watanabe, H., M. Kakihana, S. Ohtsuka and Y. Sugishita, 1998c, Randomized, double-blind, placebo-controlled study of the preventive effect of supplemental oral vitamin C on attenuation of development of nitrate tolerance, J Am Coll Cardiol, 31, 1323-9.

Waters, D.D., 2001, Early pharmacologic intervention and plaque stability in acute coronary syndromes, Am J Cardiol, 88, 30K-36K.

Waters, D.D. and P.Y. Hsue, 2001, What is the role of intensive cholesterol lowering in the treatment of acute coronary syndromes?, Am J Cardiol, 88, 7J-16J.

Waters, D.D., M. Juneau, D. Gossard, G. Choquette and M. Brien, 1989, Limited usefulness of intermittent nitroglycerin patches in stable angina, J Am Coll Cardiol, 13, 421-5.

Watson, S.P. and J. Gibbins, 1998, Collagen receptor signalling in platelets: extending the role of the ITAM, Immunol Today, 19, 260-4.

Wattanapitayakul, S.K. and J.A. Bauer, 2001, Oxidative pathways in cardiovascular disease: roles, mechanisms, and therapeutic implications, Pharmacol Ther, 89, 187-206.

Weber, A.A., S. Reimann and K. Schror, 1999, Specific inhibition of ADP-induced platelet aggregation by clopidogrel *in vitro*, Br J Pharmacol, 126, 415-20.

Weber, C., W. Erl, K.S. Weber and P.C. Weber, 1997, HMG-CoA reductase inhibitors decrease CD11b expression and CD11b-dependent adhesion of monocytes to endothelium and reduce increased adhesiveness of monocytes isolated from patients with hypercholesterolemia, J Am Coll Cardiol, 30, 1212-7.

Weidtmann, A., R. Scheithe, N. Hrboticky, A. Pietsch, R. Lorenz and W. Siess, 1995, Mildly oxidized LDL induces platelet aggregation through activation of phospholipase A_2, Arterioscler Thromb Vasc Biol, 15, 1131-8.

Weir, M.R. and V.J. Dzau, 1999, The renin-angiotensin-aldosterone system: a specific target for hypertension management, Am J Hypertens, 12, 205S-213S.

Weiss, H.J., 1991, von Willebrand factor and platelet function, Ann N Y Acad Sci, 614, 125-37.

Weissberg, P.L., 2000, Atherogenesis: current understanding of the causes of atheroma, Indian Heart J, 52, 467-72.

Wennmalm, A., J. Nowak and T. Bjuro, 1990, Excretion of thromboxane A_2 and prostacyclin metabolites before and after exercise testing in patients with and without signs of ischemic heart disease, Circulation, 82, 1737-43.

Werns, S.W., W.E. Rote, J.H. Davis, T. Guevara and B.R. Lucchesi, 1994, Nitroglycerin inhibits experimental thrombosis and reocclusion after thrombolysis, Am Heart J, 127, 727-37.

Westerbacka, J., I. Wilkinson, J. Cockcroft, T. Utriainen, S. Vehkavaara and H. Yki-Jarvinen, 1999, Diminished wave reflection in the aorta. A novel physiological action of insulin on large blood vessels, Hypertension, 33, 1118-22.

Weyrich, A.S., M.R. Elstad, R.P. McEver, T.M. McIntyre, K.L. Moore, J.H. Morrissey, S.M. Prescott and G.A. Zimmerman, 1996, Activated platelets signal chemokine synthesis by human monocytes, J Clin Invest, 97, 1525-34.

Weyrich, A.S., T.M. McIntyre, R.P. McEver, S.M. Prescott and G.A. Zimmerman, 1995, Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-alpha secretion. Signal integration and NF-kappa B translocation, J Clin Invest, 95, 2297-303.

White, C.R., V. Darley-Usmar, W.R. Berrington, M. McAdams, J.Z. Gore, J.A. Thompson, D.A. Parks, M.M. Tarpey and B.A. Freeman, 1996, Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits, Proc Natl Acad Sci U S A, 93, 8745-9.

Whittle, B.J., S. Hamid, P. Lidbury and A.C. Rosam, 1985, Specificity between the anti-aggregatory actions of prostacyclin, prostaglandin E_1 and D_2 on platelets, Adv Exp Med Biol, 192, 109-25.

Wilkinson, I.B., J.R. Cockcroft and D.J. Webb, 1998a, Pulse wave analysis and arterial stiffness, J Cardiovasc Pharmacol, 32 Suppl 3, S33-7.

Wilkinson, I.B., S.A. Fuchs, I.M. Jansen, J.C. Spratt, G.D. Murray, J.R. Cockcroft and D.J. Webb, 1998b, Reproducibility of pulse wave velocity and augmentation index measured by pulse wave analysis, J Hypertens, 16, 2079-84.

Wilkinson, I.B., I.R. Hall, H. MacCallum, I.S. Mackenzie, C.M. McEniery, B.J. van der Arend, Y.E. Shu, L.S. MacKay, D.J. Webb and J.R. Cockcroft, 2002, Pulse-wave analysis: clinical evaluation of a noninvasive, widely applicable method for assessing endothelial function, Arterioscler Thromb Vasc Biol, 22, 147-52.

Wilkinson, I.B., H. MacCallum, D.F. Rooijmans, G.D. Murray, J.R. Cockcroft, J.A. McKnight and D.J. Webb, 2000, Increased augmentation index and systolic stress in type 1 diabetes mellitus, QJM, 93, 441-8.

Williams, C.S. and R.N. DuBois, 1996, Prostaglandin endoperoxide synthase: why two isoforms?, Am J Physiol, 270, G393-400.

Williams, D.L., 1996, S-nitrosothiols and role of metal ions in decomposition to nitric oxide, Methods Enzymol, 268, 299-308.

Willich, S.N., 1999, European survey on circadian variation of angina pectoris (ESCVA): design and preliminary results, J Cardiovasc Pharmacol, 34 Suppl 2, S9-13.

Willis, A.L., D.L. Smith, M. Loveday, J. Fulks, C.H. Lee, L. Hedley and D. VanAntwerp, 1989, Selective anti-platelet aggregation synergism between a prostacyclin-mimetic, RS93427 and the nitrodilators sodium nitroprusside and glyceryl trinitrate, Br J Pharmacol, 98, 1296-302.

Willoughby, S.R., Y.Y. Chirkov, J.A. Kennedy, G.A. Murphy, L.P. Chirkova and J.D. Horowitz, 1998, Inhibition of long-chain fatty acid metabolism does not affect platelet aggregation responses, Eur J Pharmacol, 356, 207-13.

Willoughby, S.R., L.P. Chirkova, J.D. Horowitz and Y.Y. Chirkov, 1996, Multiple agonist induction of aggregation: an approach to examine anti-aggregating effects *in vitro*, Platelets, 7, 329-333.

Willoughby, S.R., S. Stewart, Y.Y. Chirkov, J.A. Kennedy, A.S. Holmes and J.D. Horowitz, 2002, Benefical clinical effects of perhexiline in patients with stable angina pectoris and acute coronary syndormes are associated with potentiation of platelet responsiveness to nitric oxide., Eur Heart J, 23, 1946-54.

Wingler, K., S. Wunsch, R. Kreutz, L. Rothermund, M. Paul and H.H. Schmidt, 2001, Upregulation of the vascular NAD(P)H-oxidase isoforms Nox1 and Nox4 by the renin-angiotensin system *in vitro* and *in vivo*, Free Radic Biol Med, 31, 1456-64.

Wink, D.A., M.B. Grisham, J.B. Mitchell and P.C. Ford, 1996, Direct and indirect effects of nitric oxide in chemical reactions relevant to biology, Methods Enzymol, 268, 12-31.

Winlaw, D.S., G.A. Smythe, A.M. Keogh, C.G. Schyvens, P.M. Spratt and P.S. Macdonald, 1994, Increased nitric oxide production in heart failure, Lancet, 344 (8919), 373-4.

Winlaw, D.S., G.A. Smythe, A.M. Keogh, C.G. Schyvens, P.M. Spratt and P.S. Macdonald, 1995, Nitric oxide production and heart failure, Lancet, 345 (8946), 390-1.

Winniford, M.D., P.L. Kennedy, P.J. Wells and L.D. Hillis, 1986, Potentiation of nitroglycerin-induced coronary dilatation by N-acetylcysteine, Circulation, 73, 138-42.

Winther, K., G. Gleerup and T. Hedner, 1991, Platelet function and fibrinolytic activity in hypertension: differential effects of calcium antagonists and beta-adrenergic receptor blockers, J Cardiovasc Pharmacol, 18 Suppl 9, S41-4.

Winther, K., C.M. Jespersen, B. Rydberg, G. Thamsborg and T. Hedner, 1990, Dose-dependent effects of verapamil and nifedipine on *in vivo* platelet function in normal volunteers, Eur J Clin Pharmacol, 39, 291-3.

Winther, K. and E. Rein, 1990, Exercise-induced platelet aggregation in angina and its possible prevention by beta 1-selective blockade, Eur Heart J, 11, 819-23.

Wisenberg, G., C. Roks, P. Nichol and M.D. Goddard, 1989, Sustained effect of and lack of development of tolerance to controlled-release isosorbide-5-mononitrate in chronic stable angina pectoris, Am J Cardiol, 64, 569-76.

Wolfram, G., U. Meyer, U. Scheske, M. Horn, C. Drummer, M. Spannagl and R. Gerzer, 1996, Effect of organic nitrates on *ex vivo* platelet aggregation and fibrinolysis in man, Eur J Med Res, 1, 291-8.

Woods, J.D., J.S. Edwards and J.M. Ritter, 1993, Inhibition by nitroprusside of platelet calcium mobilization: evidence for reduced sensitivity to nitric oxide in essential hypertension, J Hypertens, 11, 1369-73.

Wort, S.J., J.A. Mitchell and T.W. Evans, 2001, Inducible nitric oxide synthase: a tissue-specific affair?, Am J Physiol Lung Cell Mol Physiol, 280, L387-9.

WOSCOPS, 1998, Influence of pravastatin and plasma lipids on clinical events in the West of Scotland Coronary Prevention Study (WOSCOPS), Circulation, 97, 1440-5.

Wymann, M.P., V. von Tscharner, D.A. Deranleau and M. Baggiolini, 1987, The onset of the respiratory burst in human neutrophils. Real-time studies of H_2O_2 formation reveal a rapid agonist-induced transduction process, J Biol Chem, 262, 12048-53.

Xia, Y., V.L. Dawson, T.M. Dawson, S.H. Snyder and J.L. Zweier, 1996, Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury, Proc Natl Acad Sci U S A, 93, 6770-4.

Xia, Y., L.J. Roman, B.S. Masters and J.L. Zweier, 1998a, Inducible nitric-oxide synthase generates superoxide from the reductase domain, J Biol Chem, 273, 22635-9.

Xia, Y., A.L. Tsai, V. Berka and J.L. Zweier, 1998b, Superoxide generation from endothelial nitric-oxide synthase. A Ca^{2+}/calmodulin-dependent and tetrahydrobiopterin regulatory process, J Biol Chem, 273, 25804-8.

Xia, Y. and J.L. Zweier, 1997, Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages, Proc Natl Acad Sci U S A, 94, 6954-8.

Xu, K.Y., 2000, Does nitric oxide synthase catalyze the synthesis of superoxide?, FEBS Lett, 474, 252-3.

Xu, W.F., H. Andersen, T.E. Whitmore, S.R. Presnell, D.P. Yee, A. Ching, T. Gilbert, E.W. Davie and D.C. Foster, 1998, Cloning and characterization of human protease-activated receptor 4, Proc Natl Acad Sci U S A, 95, 6642-6.

Yao, S.K., J.C. Ober, A. Gonenne, F.J. Clubb, Jr., A. Krishnaswami, J.J. Ferguson, H.V. Anderson, M. Gorecki, L.M. Buja and J.T. Willerson, 1993, Active oxygen species play a role in mediating platelet aggregation and cyclic flow variations in severely stenosed and endothelium-injured coronary arteries, Circ Res, 73, 952-67.

Yao, S.K., J.C. Ober, A. Krishnaswami, J.J. Ferguson, H.V. Anderson, P. Golino, L.M. Buja and J.T. Willerson, 1992, Endogenous nitric oxide protects against platelet aggregation and cyclic flow variations in stenosed and endothelium-injured arteries, Circulation, 86, 1302-9.

Yin, K., P.S. Lai, A. Rodriguez, B.W. Spur and P.Y. Wong, 1995, Antithrombotic effects of peroxynitrite: inhibition and reversal of aggregation in human platelets, Prostaglandins, 50, 169-78.

Yoshimoto, H., A. Suehiro and E. Kakishita, 1999, Exogenous nitric oxide inhibits platelet activation in whole blood, J Cardiovasc Pharmacol, 33, 109-15.

Yoshizumi, M., M.A. Perrella, J.C. Burnett, Jr. and M.E. Lee, 1993, Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life, Circ Res, 73, 205-9.

Youssefian, T., J.M. Masse, F. Rendu, J. Guichard and E.M. Cramer, 1997, Platelet and megakaryocyte dense granules contain glycoproteins Ib and IIb-IIIa, Blood, 89, 4047-57.

Yusuf, S., R. Collins, S. MacMahon and R. Peto, 1988, Effect of intravenous nitrates on mortality in acute myocardial infarction: an overview of the randomised trials, Lancet, 1 (8594), 1088-92.

Yusuf, S., P. Sleight, J. Pogue, J. Bosch, R. Davies and G. Dagenais, 2000, Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators, N Engl J Med, 342, 145-53.

Zahavi, M., J. Zahavi, R. Schafer, E. Firsteter and S. Laniado, 1989, Abnormal typical pattern of platelet function and thromboxane generation in unstable angina, Thromb Haemost, 62, 840-5.

Zalba, G., F.J. Beaumont, G. San Jose, A. Fortuno, M.A. Fortuno, J.C. Etayo and J. Diez, 2000, Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats, Hypertension, 35, 1055-61.

Zavoico, G.B. and M.B. Feinstein, 1984, Cytoplasmic Ca^{2+} in platelets is controlled by cyclic AMP: antagonism between stimulators and inhibitors of adenylate cyclase, Biochem Biophys Res Commun, 120, 579-85.

Zeiher, A.M., H. Drexler, H. Wollschlager and H. Just, 1991, Modulation of coronary vasomotor tone in humans. Progressive endothelial dysfunction with different early stages of coronary atherosclerosis, Circulation, 83, 391-401.

Zeiher, A.M., V. Schachinger and J. Minners, 1995, Long-term cigarette smoking impairs endothelium-dependent coronary arterial vasodilator function, Circulation, 92, 1094-100.

Zhang, H., A. Schmeisser, C.D. Garlichs, K. Plotze, U. Damme, A. Mugge and W.G. Daniel, 1999, Angiotensin II-induced superoxide anion generation in human vascular endothelial cells: role of membrane-bound NADH-/NADPH-oxidases, Cardiovasc Res, 44, 215-22.

Zhang, J., S.J. Shattil, M.C. Cunningham and S.E. Rittenhouse, 1996, Phosphoinositide 3-kinase gamma and p85/phosphoinositide 3-kinase in platelets. Relative activation by thrombin receptor or beta-phorbol myristate acetate and roles in promoting the ligand-binding function of alphaIIbbeta3 integrin, J Biol Chem, 271, 6265-72.

Zhao, B., C.H. Rickert, T.J. Filler, B. Liu, P.F. Verhallen and R. Dierichs, 1995, Adhesion of washed blood platelets *in vitro* is advanced, accelerated, and enlarged by oxidized low-density lipoprotein, Am J Hematol, 49, 177-82.

Zhu, Y., Y. Hojo, U. Ikeda, M. Takahashi and K. Shimada, 2000, Interaction between monocytes and vascular smooth muscle cells enhances matrix metalloproteinase-1 production, J Cardiovasc Pharmacol, 36, 152-61.

Zimrin, D., N. Reichek, K.T. Bogin, G. Aurigemma, P. Douglas, B. Berko and H.L. Fung, 1988, Antianginal effects of intravenous nitroglycerin over 24 hours, Circulation, 77, 1376-84.

Zoccali, C., S. Bode-Boger, F. Mallamaci, F. Benedetto, G. Tripepi, L. Malatino, A. Cataliotti, I. Bellanuova, I. Fermo, J. Frolich and R. Boger, 2001, Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study, Lancet, 358 (9299), 2113-7.

Zucker, M.B. and J. Peterson, 1968, Inhibition of adenosine diphosphate-induced secondary aggregation and other platelet functions by acetylsalicylic acid ingestion, Proc Soc Exp Biol Med, 127, 547-51.

Zurbano, M.J., I. Anguera, M. Heras, E. Roig, M. Lozano, G. Sanz and G. Escolar, 1999, Captopril administration reduces thrombus formation and surface expression of platelet glycoprotein IIb/IIa in early postmyocardial infarction stage, Arterioscler Thromb Vasc Biol, 19, 1791-5.

Chapter 7

Appendix

Appendix Table 1:
Patient number differences for coronary risk factors and anti-anginal pharmacotherapy (Comparison of SAP vs ACS patients)

Determinants	Odds Ratio	95% CI	p
Male subjects	0.99	0.42 to 2.30	1.0
Age ≥ 70 years	0.56	0.24 to 1.30	0.21
Diabetes	1.35	0.56 to 3.24	0.66
Hypertension	1.49	0.65 to 3.42	0.40
Hypercholesterolaemia	1.77	0.76 to 4.10	0.21
Smoking	0.37	0.11 to 1.30	0.15
Medications			
Aspirin	1.29	0.41 to 4.10	0.77
Nitrates	0.02	0.0009 to 0.28	< 0.01
ACE Inhibition	1.73	0.67 to 4.40	0.34
SH-donors	0.63	0.25 to 1.59	0.36
Perhexiline	10.2	2.01 to 20.31	< 0.01
Statins	1.43	0.51 to 4.11	0.60
Ca^{2+} antagonists	0.75	0.32 to 1.72	0.53
β-adrenoceptor antagonists	1.55	0.59 to 4.02	0.47

From section 2.3.4.1. Significantly greater numbers of SAP patients were undergoing perhexiline pharmacotherapy than ACS patients. More ACS patients were receiving nitrate compared to SAP patients (Fishers exact test). SAP = stable angina pectoris; ACS = acute coronary syndrome; CI = confidence interval.

Appendix Table 2:
Platelet responsiveness to SNP and its relationship to disease state, gender and aspirin pharmacotherapy (Gaussian distribution)

Disease State	Gender	Aspirin pharmacotherapy	KS	p
NV	Males	Yes	N/A	NS
		No	0.14	NS
	Females	Yes	N/A	N/A
		No	0.19	NS
SAP	Males	Yes	0.11	NS
		No	N/A	N/S
	Females	Yes	0.09	NS
		No	N/A	N/A
ACS	Males	Yes	0.16	NS
		No	0.22	NS
	Females	Yes	0.19	NS
		No	N/A	N/A

N/A = insufficient numbers to perform test. Kolmogorov-Smirnov test requires >4 subjects, NS = not significant. Platelet responsiveness to SNP (10μM) in blood samples obtained from NVs, SAP patients and ACS patients was examined to see if it conformed to a Gaussian distribution utilizing the Kolmogorov-Smirnov test. Each subject was classified according to disease state, gender and aspirin use.

Appendix Table 3:
Platelet responsiveness to SNP
3-way ANOVA contingency table

Determinants	F	p
Subjects	5.93	0.0036
Gender	1.32	0.25
Aspirin	0.45	0.50
Interactions		
Subjects x Gender	0.11	0.89
Subjects x Aspirin	0.28	0.75
Gender x Aspirin	0.03	0.86
Subjects x Gender x Aspirin	1.19	0.31

From section 2.3.4.3. Platelet responsiveness to SNP (10μM) was assessed by 3-way ANOVA. Subjects = NVs, SAP patients and ACS patients. (Bartlett's statistic = 4.19, p = 0.18).

Appendix Table 4:
Patient number differences for coronary risk factors and anti-anginal pharmacotherapy (SAP and ACS patients)

Determinants	Odds Ratio	95% CI	p
Male subjects	0.38	0.12 to 1.28	0.155
Diabetes	0.16	0.04 to 0.56	< 0.01
Hypertension	1.06	0.37 to 3.10	1.0
Hypercholesterolaemia	14.5	3.7 to 55.7	< 0.01
Smoking	0.71	0.19 to 2.52	0.75
Medications			
Aspirin	0.76	0.16 to 3.50	1.0
Nitrates	0.005	0.0003 to 0.12	< 0.01
ACE Inhibition	1.77	0.59 to 5.23	0.42
NAC	0.09	0.0004 to 1.95	0.07
Perhexiline	4.19	1.03 to 16.9	0.067
Statins	1.88	0.69 to 5.09	0.32
Ca^{2+} Antagonists	0.94	0.31 to 2.85	1.0
β-adrenoceptor antagonists	1.07	0.29 to 3.90	1.0

From Section 2.5.4.1. Significantly greater numbers of ACS patients had diabetes. Significantly more SAP subjects had hypercholesterolaemia compared to ACS patients. Not surprisingly more ACS patients were receiving nitrates than SAP patients with a trend towards more SAP patients being treated with perhexiline. SAP = stable angina pectoris; ACS = acute coronary syndrome.

Appendix Table 5.
Patient number differences for coronary risk factors and pharmacotherapy (Comparison of SAP vs ACS patients)

Determinant	*Odds ratio*	*95% CI*	*p*
Age ≥ 70 yrs	0.054	0.328, 0.987	0.054
Smoking	0.486	0.227, 1.043	0.079
Aspirin	2.008	1.006, 4.008	0.054
Nitrates	0.492	0.279, 0.865	0.019

From section 3.5.1. No significant difference in subject numbers between SAP and ACS patients were observed for the numbers of male subjects, diabetes mellitus, hypertension and hypercholesterolaemia. Numbers of subjects ≥ 70 years of age and the numbers of current smokers tended towards increased numbers in the ACS cohort compared to SAP subjects. No significant differences in subject numbers between SAP and ACS patients was observed for treatment with ACE inhibitors, SH-donors, perhexiline, statins, Ca^{2+} antagonists or β-adrenoceptor antagonists.

Appendix Table 6.
Patient number differences for coronary risk factors and anti-anginal pharmacotherapy (Comparison of SAP and ACS patients with NIPs)

Coronary risk factors	Diagnosis	Odds ratio	95% CI	p
Male gender	SAP	0.41	0.18, 0.93	< 0.01
	ACS	0.57	0.27, 1.20	0.1813
Age ≥ 70 yrs	SAP	0.22	0.07, 0.67	< 0.01
	ACS	0.12	0.04, 0.37	< 0.01
Diabetes	SAP	0.08	0.01, 0.62	< 0.01
	ACS	0.06	0.01, 0.51	< 0.01
Hypertension	SAP	0.31	0.13, 0.75	< 0.01
	ACS	0.29	0.12, 0.66	< 0.01
Hypercholesterolaemia	SAP	0.15	0.05, 0.44	< 0.01
	ACS	0.18	0.06, 0.49	< 0.01
Smoking	SAP	1.20	0.37, 3.81	0.76
	ACS	0.58	0.21, 1.61	0.36
Medications				
Aspirin	SAP	0.08	0.01, 0.06	< 0.01
	ACS	6.60	2.93, 14.9	< 0.01
Nitrates	SAP	0.15	0.05, 0.41	< 0.01
	ACS	0.07	0.02, 0.19	< 0.01
ACE Inhibitors	SAP	0.37	0.12, 1.18	0.13
	ACS	0.43	0.14, 1.32	0.16
SH-donors	SAP	0.43	0.09, 2.11	0.51
	ACS	0.25	0.05, 1.09	0.05
Perhexiline	SAP	0.17	0.02, 1.37	0.11
	ACS	0.21	0.03, 1.60	0.13
Statins	SAP	0.30	0.11, 0.87	0.026
	ACS	0.45	0.16, 1.25	0.133
Ca^{2+} Antagonists	SAP	0.47	0.21, 1.06	0.072
	ACS	0.46	0.22, 0.98	0.058
β-adrenoceptor antagonists	SAP	0.18	0.04, 0.85	0.019
	ACS	0.31	0.06, 1.37	0.178

From section 3.5.1. For almost all possible determinants there were significantly greater proportions of SAP and ACS patients compared to NIPs (differences determined by Fisher's exact test).

Appendix Table 7:
Platelet response to ADP (Gaussian distribution)

Disease State	Gender	Aspirin pharmacotherapy	KS	p
NV	Males	Yes	N/A	N/A
		No	0.14	NS
	Females	Yes	N/A	N/A
		No	0.1	NS
NIP	Males	Yes	0.16	NS
		No	0.17	NS
	Females	Yes	0.16	NS
		No	0.21	NS
SAP	Males	Yes	0.11	NS
		No	0.17	NS
	Females	Yes	0.11	NS
		No	N/A	N/A
ACS	Males	Yes	0.07	NS
		No	0.17	NS
	Females	Yes	0.11	NS
		No	0.17	NS

From section 3.5.2. The extent of platelet aggregation in response to ADP (1μM) was assessed for normality according to disease state, gender and aspirin use. Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population the level of significance for the above data population was >0.1 and were therefore determined to conform to a Gaussian distribution N.A = insufficient numbers to perform analysis

Appendix Table 8:
Significant results from Bonferroni's post hoc multiple comparison test

Subject Description	Aggregability (Mean ± SEM)	vs	Subject Description	Aggregability (Mean ± SEM)	p
Female SAP +ASA	12.9 ± 0.9	vs	Male SAP +ASA	8.1 ± 0.5	< 0.01
Male ACS +ASA	9.2 ± 0.5	vs	Female SAP +ASA	12.9 ± 0.9	< 0.05
Female ACS +ASA	12.7 ± 0.7	vs	Males SAP +ASA	8.1 ± 0.5	< 0.01
Males ACS -ASA	12.3 ± 1.0	vs	Males SAP +ASA	8.1 ± 0.5	< 0.01
Females ACS -ASA	12.6 ± 1.1	vs	Males SAP +ASA	8.1 ± 0.5	< 0.01
Males ACS +ASA	9.2 ± 0.5	vs	Females ACS +ASA	12.7 ± 0.7	< 0.01

From Table 3.5. Following a 3-way ANOVA a number of significant differences in the extent of platelet aggregation were observed utilizing Bonferroni's post hoc multiple comparison test. +ASA = aspirin use, -ASA = no aspirin use, SEM = standard error of the mean.

Appendix Table 9:
Significant results from Bonferroni's post hoc multiple comparison test

Subject Description	SNP Responsiveness (Mean ± SEM)	vs	Subject Description	SNP Responsiveness (Mean ± SEM)	p
NV Male	60.1 ± 3.9	vs	SAP Male	39.9 ± 3.5	< 0.05
SAP Male	39.9 ± 3.5	vs	NV Female	63.2 ± 5.0	< 0.01
SAP Male	39.9 ± 3.5	vs	NIP Female	67.3 ± 7.6	< 0.05
SAP Female	38.7 ± 3.5	vs	NV Male	60.1 ± 3.9	< 0.05
SAP Female	38.7 ± 3.5	vs	NV Female	63.2 ± 5.0	< 0.05
SAP Female	38.7 ± 3.5	vs	NIP Female	67.3 ± 7.6	< 0.05
ACS Male	35.6 ± 2.6	vs	NV Male	60.1 ± 3.9	< 0.01
ACS Male	35.6 ± 2.6	vs	NV Female	63.2 ± 5.0	< 0.01
ACS Male	35.6 ± 2.6	vs	NIP Male	60.8 ± 7.6	< 0.01
ACS Male	35.6 ± 2.6	vs	NIP Female	67.3 ± 7.6	< 0.01
ACS Female	31.4 ± 3.1	vs	NV Male	60.1 ± 3.9	< 0.01
ACS Female	31.4 ± 3.1	vs	NV Female	63.2 ± 5.0	< 0.01
ACS Female	31.4 ± 3.1	vs	NIP Male	60.8 ± 7.6	< 0.01
ACS Female	31.4 ± 3.1	vs	NIP Female	67.3 ± 7.6	< 0.01

From section 3.5.3 (Table 3.8). Following a 2-way ANOVA a number of significant differences in the extent of platelet responsiveness to SNP (10μM) were observed using Bonferroni's post hoc multiple comparison test. NV = normal volunteers, NIP = non-ischaemic patient, SAP = stable angina pectoris, ACS = acute coronary syndrome, SEM = standard error of the mean.

Appendix Table 10.
Platelet responsiveness to SNP relationship to disease state, gender and aspirin pharmacotherapy (Gaussian distribution)

Disease State	Gender	Aspirin pharmacotherapy	KS	p
NIP	Males	Yes	N/A	N/A
		No	0.23	NS
	Females	Yes	0.16	NS
		No	N/A	N/A
SAP	Males	Yes	0.06	NS
		No	0.18	NS
	Females	Yes	0.15	NS
		No	N/A	N/A
ACS	Males	Yes	0.09	NS
		No	0.13	NS
	Females	Yes	0.14	NS
		No	0.13	NS

From section 3.5.3. The extent of platelet responsiveness to SNP (10μM) was assessed for normality according to disease state, gender and aspirin use. Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population the level of significance for the above data population was >0.1 and were therefore determined to conform to a Gaussian distribution N.A = insufficient numbers to perform analysis

Appendix Table 11.
Platelet responsiveness to SNP, relationship to disease state, gender and aspirin pharmacotherapy (Gaussian distribution)

Disease State	Gender	Aspirin pharmacotherapy	KS	p
NV	Males	Yes	N/A	N/A
		No	N/A	N/A
	Females	Yes	N/A	N/A
		No	0.22	NS
SAP	Males	Yes	0.17	NS
		No	N/A	N/A
	Females	Yes	0.19	NS
		No	N/A	N/A
ACS	Males	Yes	0.11	NS
		No	0.12	NS
	Females	Yes	0.16	NS
		No	0.10	NS

From section 3.5.3. The extent of platelet responsiveness to NTG (100μM) was assessed for normality according to disease state, gender and aspirin use. Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population the level of significance for the above data population was >0.1 and were therefore determined to conform to a Gaussian distribution N.A = insufficient numbers to perform analysis.

Appendix Table 12.
Platelet responsiveness to SNP and its relationship to disease state, gender and aspirin pharmacotherapy (Gaussian distribution) ACS patients

Disease State	Gender	Aspirin pharmacotherapy	KS	p
UAP	Males	Yes	0.15	NS
		No	0.17	NS
	Females	Yes	0.21	NS
		No	0.17	NS
NQAMI	Males	Yes	0.15	NS
		No	0.19	NS
	Females	Yes	0.21	NS
		No	0.19	NS

From section 3.5.3. Data representing the degree of platelet responsiveness to SNP (10μM) within the ACS subject cohort was assessed for normality according to the final diagnosis of either UAP or NQAMI, gender and aspirin use. Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population the level of significance for the above data population was >0.1 and were therefore determined to conform to a Gaussian distribution N.A = insufficient numbers to perform analysis.

Appendix Table 13.
Platelet responsiveness to NTG, relationship to disease state, gender and aspirin pharmacotherapy (Gaussian distribution) ACS patients

Disease State	Gender	Aspirin pharmacotherapy	KS	p
UAP	Males	Yes	0.13	NS
		No	0.22	NS
	Females	Yes	0.15	NS
		No	0.24	NS
NQAMI	Males	Yes	0.15	NS
		No	0.14	NS
	Females	Yes	N/A	N/A
		No	0.17	NS

From section 3.5.3. Data representing the degree of platelet responsiveness to NTG (100μM) was assessed for normality according to the final diagnosis of either UAP/NQAMI, gender and aspirin use. Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population the level of significance for the above data population was >0.1 and were therefore determined to conform to a Gaussian distribution N.A = insufficient numbers to perform analysis.

Appendix Table 14.
Platelet response to ADP versus platelet responsiveness to SNP

Subject cohort	Gender	Regression Analysis			ANCOVA Summary		Outcome
		r	F	p	F	p	
N.V	Males	-0.35	2.00	0.18	0.58	0.45	Pooled
	Females	-0.16	0.26	0.62			
NIP	Males	-0.22	0.44	0.53	0.66	0.43	Pooled
	Females	-0.08	0.06	0.81			
SAP	Males	-0.54	20.30	<0.01	3.39	0.068	Pooled
	Females	-0.39	4.07	0.06			
ACS (MI)	Males	-0.14	0.49	0.49	0.009	0.93	Pooled
	Females	-0.57	7.68	0.01			
ACS (UAP)	Males	-0.48	14.2	0.0004	0.018	0.89	Pooled
	Females	-0.14	0.56	0.46			
ACS	MI	-0.38	7.30	0.009	0.004	0.95	Pooled
	UAP	-0.38	13.52	0.004			

The extent of ADP (1μM) induced platelet aggregation versus the degree of platelet responsiveness to SNP (10μM) within all the patient cohorts was analyzed according to gender. A significant negative correlation between platelet aggregability and SNP responsiveness was observed for male SAP patients, female NQAMI patients, male UAP patients and both the pooled populations of MI and UAP subjects. By ANCOVA genders were determined not to be a significant determinant and hence were analyzed by clinical condition only. See Table 3.14 for further analysis.

Appendix Table 15:
Platelet response to ADP versus platelet responsiveness to NTG

Subject cohort	Gender	Regression Analysis			ANCOVA Summary		Outcome
		r	F	p	F	p	
NV	Males	-0.09	0.03	0.87	0.87	0.37	Pooled
	Females	-0.17	0.23	0.64			
NIP	Males	-0.89	7.74	0.11	2.49	0.15	Pooled
	Females	-0.18	0.22	0.65			
SAP	Males	-0.73	16.03	< 0.01	0.26	0.62	Pooled
	Females	-0.14	0.25	0.63			
ACS(MI)	Males	-0.15	0.50	0.49	0.08	0.78	Pooled
	Females	-0.54	3.27	0.11			
ACS (UAP)	Males	-0.08	0.16	0.69	0.41	0.53	Pooled
	Females	-0.16	0.4	0.53			
ACS	MI	-0.32	3.56	0.068	7.42	0.008	Separate
	UAP	-0.11	0.47	0.50			

The extent of ADP (1μM) induced platelet aggregation versus the degree of platelet responsiveness towards NTG (100μM) within all the patient cohorts was analyzed according to gender. Male SAP patients were the only subject class to demonstrate a significant negative correlation between the extent of aggregation and the degree of NTG responsiveness. All subject populations demonstrated no significant differences between genders and were accordingly pooled. However, patients with a MI as distinct from UAP were not significantly different. For further analysis see Table 3.15.

Appendix Table 16:
Age versus platelet responsiveness to SNP

Subject cohort	Gender	Regression Analysis			ANCOVA Summary		Outcome
		r	F	p	F	p	
NV	Males	-0.09	0.11	0.73	1.12	0.30	Pooled
	Females	-0.28	1.13	0.31			
NIP	Males	0.41	1.8	0.21	0.09	0.76	Pooled
	Females	-0.02	0.004	0.94			
SAP	Males	0.05	0.11	0.74	0.03	0.86	Pooled
	Females	0.03	0.02	0.88			
ACS	Males	-0.03	0.07	0.79	0.91	0.34	Pooled
	Females	-0.06	0.14	0.70			

An age relationship for the degree of SNP responsiveness was firstly assessed according to gender within each subject cohort. No significant correlation or differences between regression analysis was found for each gender and clinical condition. Accordingly these results were pooled and further analyzed in Table 3.17.

Appendix Table 17:
Age versus platelet responsiveness to NTG

Subject cohorts	Gender	Regression Analysis			ANCOVA Summary		Outcome
		r	F	p	F	p	
NV	Males	-0.75	5.19	0.09	2.60	0.13	Pooled
	Females	-0.1	0.06	0.81			
NIP	Males	-0.78	4.6	0.12	0.62	0.45	Pooled
	Females	-0.46	2.2	0.17			
SAP	Males	-0.35	1.9	0.19	2.59	0.12	Pooled
	Females	-0.22	0.53	0.47			
ACS	Males	-0.13	0.76	0.39	0.0001	0.99	Pooled
	Females	0.18	0.91	0.35			

An age relationship for the degree of NTG responsiveness was firstly assessed according to gender within each subject cohort. No significant correlation or differences between regression analysis was found for each gender and clinical condition. Accordingly these results were pooled and further analyzed in Table 3.18.

Appendix Table 18:
Platelet responsiveness to SNP as a function of the number of coronary risk factors

Disease State	Gender	Risk factors	KS	p
SAP	Males	1	0.28	NS
		2	0.16	NS
		3	0.13	NS
		4	0.2	NS
	Females	1	0.16	NS
		2	0.21	NS
		3	0.16	NS
		4	N/A	N/A
ACS	Males	1	0.24	NS
		2	0.18	NS
		3	0.11	NS
		4	0.09	NS
	Females	1	0.33	NS
		2	0.22	NS
		3	0.13	NS
		4	0.26	NS

Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population the level of significance for the above data population was >0.1 and were therefore determined to conform to a Gaussian distribution N.A = insufficient numbers to perform analysis.

Appendix Table 19:
Platelet responsiveness to NTG as a function of the number of coronary risk factors

Disease State	*Gender*	*Risk factors*	*KS*	*p*
SAP	*Males*	1	N/A	N/A
		2	0.14	NS
		3	0.21	NS
		4	N/A	N/A
	Females	1	N/A	N/A
		2	N/A	N/A
		3	0.25	NS
		4	N/A	N/A
ACS	*Males*	1	0.27	NS
		2	0.16	NS
		3	0.11	NS
		4	0.22	NS
	Females	1	0.29	NS
		2	0.19	NS
		3	0.18	NS
		4	N/A	N/A

From section 3.5.7 of chapter 3. Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population the level of significance for the above data population was >0.1 and were therefore determined to conform to a Gaussian distribution N.A = insufficient numbers to perform analysis.

Appendix Table 20
Diurnal variability in platelet response to ADP

Time	*Gaussian distribution*	*Males*	*Females*
0-hrs	KS	0.11	0.13
	p	NS	NS
4-hrs	KS	0.12	0.12
	p	NS	NS
8-hrs	KS	0.11	0.21
	p	NS	NS
24-hrs	KS	0.12	0.14
	p	NS	NS

From section 4.5.3.1 of chapter 4. Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population, the level of significance for the above data population was > 0.1 and were therefore determined to conform to a Gaussian distribution. NS = not significant.

Appendix Table 21:
Acute nitrate effects on platelet response to ADP

Time	Treatment	Gender	Gaussian distribution	p
0-hrs	ISMN	Males	KS	0.22
			p	ns
		Females	KS	0.16
			p	ns
	TD-NTG	Males	KS	0.17
			p	ns
		Females	KS	0.19
			p	ns
4-hrs	ISMN	Males	KS	0.11
			p	ns
		Females	KS	0.15
			p	ns
	TD-NTG	Males	KS	0.16
			p	ns
		Females	KS	0.20
			p	ns
8-hrs	ISMN	Males	KS	0.10
			p	ns
		Females	KS	0.15
			p	ns
	TD-NTG	Males	KS	0.09
			p	ns
		Females	KS	0.27
			p	ns
24-hrs	ISMN	Males	KS	0.09
			p	ns
		Females	KS	0.16
			p	ns
	TD-NTG	Males	KS	0.16
			p	ns
		Females	KS	0.19
			p	ns

From section 4.5.3.2 of chapter 4. Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population, the level of significance for the above data population was > 0.1 and were therefore determined to conform to a Gaussian distribution. NS = not significant.

Appendix Table 22:
Chronic nitrate effects on platelet response to ADP

Time	Treatment	Gender	Gaussian distribution	p
0-hrs	ISMN	Males	KS	0.20
			p	ns
		Females	KS	0.21
			p	ns
	TD-NTG	Males	KS	0.10
			p	ns
		Females	KS	0.13
			p	ns
4-hrs	ISMN	Males	KS	0.2
			p	ns
		Females	KS	0.11
			p	ns
	TD-NTG	Males	KS	0.18
			p	ns
		Females	KS	0.13
			p	ns
8-hrs	ISMN	Males	KS	0.15
			p	ns
		Females	KS	0.17
			p	ns
	TD-NTG	Males	KS	0.11
			p	ns
		Females	KS	0.12
			p	ns

From section 4.5.3.3 of chapter 4. Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population, the level of significance for the above data population was > 0.1 and were therefore determined to conform to a Gaussian distribution. NS = not significant.

Appendix Table 23:
Differences between treatment phases and nitrate preparations (platelet responsiveness to ADP)

Treatment phase	Therapy	Gender	Gaussian distribution	p
Acute	ISMN	Males	KS	0.12
			p	ns
		Females	KS	0.15
			p	ns
	TD-NTG	Males	KS	0.14
			p	ns
		Females	KS	0.27
			p	ns
Chronic	ISMN	Males	KS	0.17
			p	ns
		Females	KS	0.15
			p	ns
	TD-NTG	Males	KS	0.10
			p	ns
		Females	KS	0.14
			p	ns

From section 4.5.3.5. Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population, the level of significance for the above data population was > 0.1 and were therefore determined to conform to a Gaussian distribution. NS = not significant.

Appendix Table 24:
Differences between treatment phases and nitrate preparations (platelet responsiveness to NTG)

Treatment phase	Therapy	Gaussian distribution	p
Acute	ISMN	KS	0.07
		p	ns
	TD-NTG	KS	0.15
		p	ns
Chronic	ISMN	KS	0.16
		p	ns
	TD-NTG	KS	0.11
		p	ns

From section 4.5.3.5. Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population, the level of significance for the above data population was > 0.1 and were therefore determined to conform to a Gaussian distribution. NS = not significant.

Appendix Table 25:
Differences between treatment phases and nitrate preparations (platelet responsiveness to SNP)

Treatment phase	*Therapy*	*Gaussian distribution*	*p*
Acute	*ISMN*	KS	0.10
		p	ns
	TD-NTG	KS	0.12
		p	ns
Chronic	*ISMN*	KS	0.13
		p	ns
	TD-NTG	KS	0.14
		p	ns

From section 4.5.3.5. Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population, the level of significance for the above data population was > 0.1 and were therefore determined to conform to a Gaussian distribution. NS = not significant.

Appendix Table 26:
Differences between treatment phases and nitrate preparations (Pre-aggregation LDCL)

Time	*Therapy*	*Treatment*	*Gaussian distribution*	*p*
0-hrs	*ISMN*	*Acute*	KS	0.17
			p	ns
		Chronic	KS	0.23
			p	ns
	TD-NTG	*Acute*	KS	0.18
			p	ns
		Chronic	KS	0.21
			p	ns
4-hrs	*ISMN*	*Acute*	KS	0.22
			p	ns
		Chronic	KS	0.14
			p	ns
	TD-NTG	*Acute*	KS	0.17
			p	ns
		Chronic	KS	0.19
			p	ns
8-hrs	*ISMN*	*Acute*	KS	0.26
			p	ns
		Chronic	KS	0.17
			p	ns
	TD-NTG	*Acute*	KS	0.17
			p	ns
		Chronic	KS	0.17
			p	ns

From section 4.5.3.5.Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population (pre-aggregation LDCL), the level of significance for the above data population was > 0.1 and were therefore determined to conform to a Gaussian distribution. NS = not significant.

Appendix Table 27:

Pre-aggregation LDLC:- three-way ANOVA contingency table

Determinant	*F*	*p*
Phase	0.62	0.43
Treatment	2.02	0.15
Time	0.48	0.61
Interactions		
Phase x Treatment	3.87	0.051
Phase x Time	0.31	0.73
Treatment x Time	0.46	0.63
Phase x Treatment x Time	1.09	0.34

From section 4.5.3.5. Differences between nitrate treatment phases (acute/chronic) and treatment regimens (ISMN/TD-NTG) for the extent of pre-aggregation LDCL (12.5μM lucigenin) was examined by 3-way ANOVA (Bartlett's statistic for homogeneity of variances = 6.2, p = 0.86).

Appendix Table 28:
Differences between treatment phases and nitrate preparations (Aggregation-associated LDCL)

Time	*Therapy*	*Treatment*	*Gaussian distribution*	*p*
0-hrs	*ISMN*	*Acute*	KS	0.18
			p	ns
		Chronic	KS	0.15
			p	ns
	TD-NTG	*Acute*	KS	0.16
			p	ns
		Chronic	KS	0.19
			p	ns
4-hrs	*ISMN*	*Acute*	KS	0.12
			p	ns
		Chronic	KS	0.18
			p	ns
	TD-NTG	*Acute*	KS	0.21
			p	ns
		Chronic	KS	0.16
			p	ns
8-hrs	*ISMN*	*Acute*	KS	0.23
			p	ns
		Chronic	KS	0.22
			p	ns
	TD-NTG	*Acute*	KS	0.17
			p	ns
		Chronic	KS	0.18
			p	ns

From section 4.5.3.5.Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population, the level of significance for the above data population was > 0.1 and were therefore determined to conform to a Gaussian distribution. NS = not significant.

Appendix Table 29:
Aggregation-associated LDCL, three-way ANOVA contingency table

Determinant	F	p
Phase	1.91	0.17
Treatment	3.80	0.05
Time	1.34	0.27
Interactions		
Phase x Treatment	0.67	0.41
Phase x Time	0.31	0.73
Treatment x Time	1.16	0.31
Phase x Treatment x Time	0.053	0.95

From section 4.5.3.5. Differences between nitrate treatment phases (acute/chronic) and regimens (ISMN/TD-NTG) on the extent of aggregation-associated LDCL (lucigenin 12.5μM) was examined by 3-way ANOVA (Bartlett's statistic for homogeneity of variances = 13.3, p = 0.27).

Appendix Table 30:
Differences between treatment phases and nitrate preparations (systolic blood pressure)

Time	Therapy	Treatment	Gaussian distribution	p
0-hrs	ISMN	Acute	KS	0.18
			p	ns
		Chronic	KS	0.11
			p	ns
	TD-NTG	Acute	KS	0.19
			p	ns
		Chronic	KS	0.09
			p	ns
4-hrs	ISMN	Acute	KS	0.18
			p	ns
		Chronic	KS	0.15
			p	ns
	TD-NTG	Acute	KS	0.25
			p	ns
		Chronic	KS	0.14
			p	ns
8-hrs	ISMN	Acute	KS	0.09
			p	ns
		Chronic	KS	0.19
			p	ns
	TD-NTG	Acute	KS	0.14
			p	ns
		Chronic	KS	0.11
			p	ns

From section 4.5.4.Utilizing the Kolmogorov-Smirnov method for assessment of normality within a data population, the level of significance for the above data population was > 0.1 and were therefore determined to conform to a Gaussian distribution. NS = not significant.

Discussion/analysis of the platelet/luminescent variables obtained from those subjects that had vascular parameters examined

From section 4.5.5. The degree of change in platelet response to ADP (1μM), NTG (100μM), SNP (10μM) and the extent of whole blood superoxide detected using LDCL was examined in the cohort of subjects (n = 12) that also has vascular parameters (systolic blood pressure/heart rate/AI(x)) evaluated at the time of blood sampling.

Platelet aggregability

Females

The extent of platelet aggregation for female subjects was analyzed for both the acute and chronic phases. Data representing the degree of aggregability for the female subjects during the "no nitrate" and both acute nitrate treatment phases, was insufficient (n=4) to assess normality by the Kolmogorov-Smirnov method. However, as the data from both the acute and chronic phases of the trial regarding the degree of platelet aggregability in the presence of either ISMN and TD-NTG was representative of a larger (n=34) population that was shown to be normally distributed at all time points throughout the trial, it was assumed that these above populations should undergo analysis by ANOVA.

Males

Data representing the degree of platelet aggregability for male subjects were firstly analyzed for normality across all time points. All data populations conformed to a Gaussian distributions ("no nitrates" Ohrs KS = 0.19, p = n.s; 4hrs KS = 0.2, p = ns; 8 hrs KS = 0.18, p = ns; 24hrs KS = 0.26, p = ns. Acute ISMN: Ohrs KS = 0.27, p = n.s; 4hrs KS = 0.18, p = ns; 8 hrs KS = 0.2, p = ns; 24hrs KS = 0.2, p = ns. Acute TD-NTG: Ohrs KS = 0.22, p = n.s; 4hrs KS = 0.18, p = ns; 8 hrs KS = 0.16, p = ns; 24hrs KS = 0.24, p = ns). These results were mirrored by those of the chronic phase of the trial ("no nitrates" as above; ISMN: Ohrs KS = 0.3, p = n.s; 4hrs KS = 0.16, p = ns; 8 hrs KS = 0.2, p = ns. TD-NTG: Ohrs KS = 0.17, p = n.s; 4hrs KS = 0.21, p = ns; 8 hrs KS = 0.16, p = ns).

Analysis-Acute nitrate effects

The extent of platelet aggregation was analyzed firstly according to either the acute and chronic phases of the trial. By 3-way ANOVA there was no significant difference in

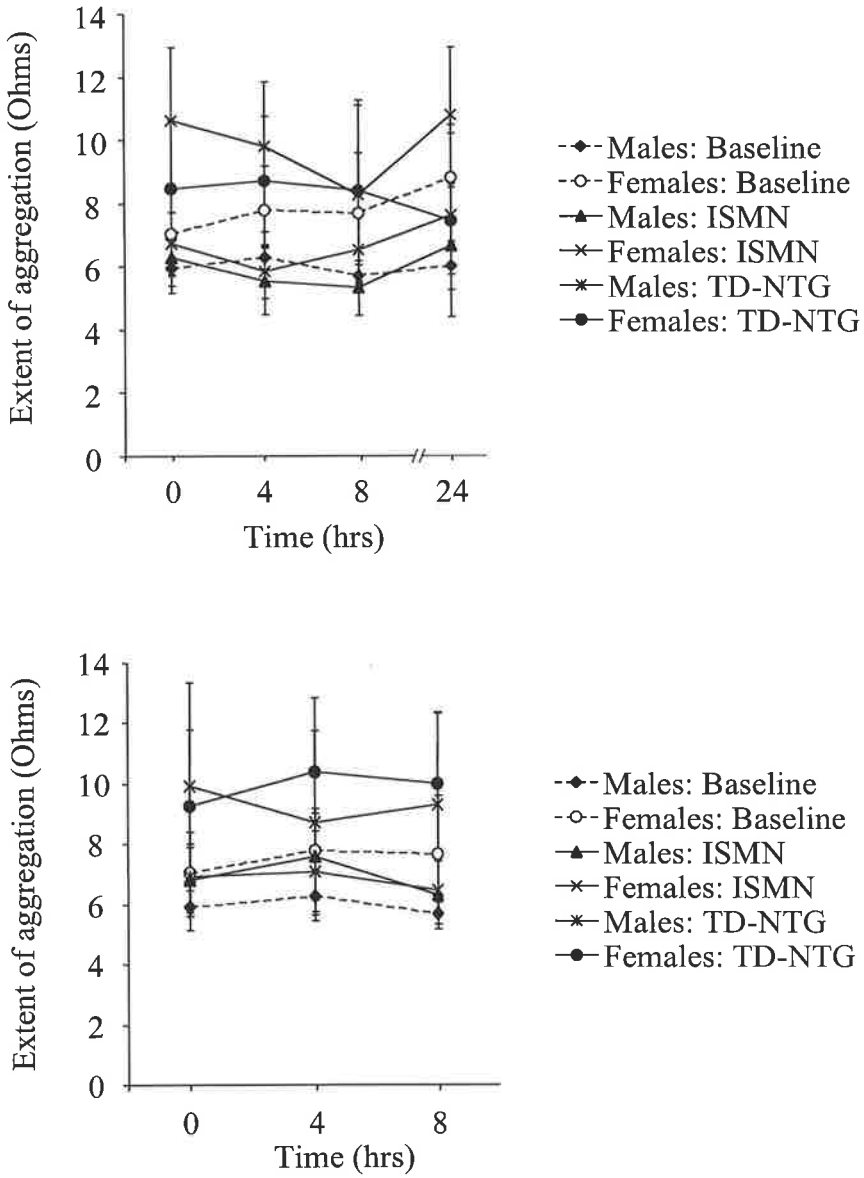
aggregability over the time period, no significant difference between the treatment phases and no significant interactions between any of the determinants. However, as demonstrated previously, a significant difference in aggregability was observed between the genders (Platelet aggregability during acute nitrate administration: Treatment group $F = 1.1$, $p = 0.34$; Time $F = 0.4$, $p = 0.72$; Gender $F = 18.8$ $p < 0.01$; Treatment group x time $F = 0.26$, $p = 0.95$; Treatment group x gender $F = 1.7$, $p = 0.18$; Time x gender $F = 0.065$, $p = 0.97$; Treatment group x time x gender $F = 0.29$, $p = 0.94$; Bartlett's statistic = 29.7, $p = 0.15$). Utilizing Bonferroni's post hoc multiple comparison test, no individual data point as depicted in Appendix Figure 1 upper panel, was significantly different from any other (upper panel of Appendix Figure 1).

Chronic nitrate effects

The effects of chronic nitrate administration of either preparation were then assessed. By 3-way ANOVA there was no significant difference between the treatment groups and across the time period examined. However, as was observed during the acute phase of the trial, a significant gender difference was observed regarding the extent of platelet aggregability. No significant interactions between the determinants was also found (3-way ANOVA: treatment group $F = 1.7$, $p = 0.18$; time $F = 0.1$, $p = 0.9$; Gender $F = 9.2$, $p < 0.01$; treatment group x time $F = 0.05$, $p = 0.99$; treatment group x gender $F = 0.32$, $p = 0.7$; time x gender $F = 0.12$, $p = 0.88$; treatment group x time x gender $F = 0.12$, $p = 0.97$; Bartlett's statistic = 26.5, $p = 0.065$). Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between any of the data points.

Differences in platelet aggregability between nitrate treatment phases

Data representing the treatment group ("no nitrates", ISMN and TD-NTG) and time was subsequently pooled in order to assess if there was any significant difference between the acute and chronic treatment phases of the trial. Gender as a determinant remained within the analysis as it was found to be significant (above). By 2-way ANOVA there was no significant difference between the acute and chronic phases of the trial. There was also no significant interaction between the two determinants and a non-significant trend towards a significant difference between genders (2-way ANOVA: Gender $F = 3.3$, $p = 0.086$; Acute and chronic phase $F = 0.25$, $p = 0.61$; Gender x acute and chronic phase $F = 0.036$, $p = 0.85$; Bartlett's statistic = 8.92, $p = 0.31$).



Appendix Figure 1: Acute/chronic anti-aggregatory effects of ISMN and TD-NTG

Upper panel: *The acute anti-aggregatory effects of both ISMN and TD-NTG in 12 subjects for both male and female subjects across 24-hrs. 3-way ANOVA treatment group $F = 1.1$, $p = 0.34$; time $F = 0.4$, $p = 0.72$, Gender $F = 18.8$, $p < 0.01$; treatment group x time $F = 0.26$, $p = 0.95$; treatment group x gender $F = 1.7$, $p = 0.18$; time x gender $F = 0.065$, $p = 0.97$; treatment group x time x gender $F = 0.29$, $p = 0.94$.* ***Lower panel:*** *The chronic anti-aggregatory effects of both ISMN and TD-NTG. 3-way ANOVA treatment group $F = 1.7$, $p = 0.18$; time $F = 0.1$, $p = 0.9$, Gender $F = 9.2$, $p < 0.01$; treatment group x time $F = 0.05$, $p = 0.99$; treatment group x gender $F = 0.32$, $p = 0.7$; time x gender $F = 0.12$, $p = 0.88$; treatment group x time x gender $F = 0.19$, $p = 0.97$.*

Platelet responsiveness to NTG (100μM)

As indicated for platelet aggregability, the degree of platelet responsiveness to NTG (100μM) was analyzed for the 12 subjects whom also had their AI(x) assessed at the time of blood sampling.

Acute phase

Firstly, data for the degree of platelet responsiveness to NTG (100μM) for the acute nitrate phase were found to conform to a Gaussian distribution. ("no nitrates" acute 0hrs KS = 0.14, p = ns; 4hrs KS = 0.09, p = ns; 8hrs KS = 0.13, p = ns; 24hrs KS = 0.12, p = ns. ISMN acute 0hrs KS = 0.11, p = ns; 4hrs KS = 0.12, p = ns; 8hrs KS = 0.14, p = ns; 24hrs KS = 0.14, p = ns. TD-NTG acute 0hrs KS = 0.16, p = ns; 4hrs KS = 0.14, p = ns; 8hrs KS = 0.15, p = ns; 24hrs KS = 0.14, p = ns).

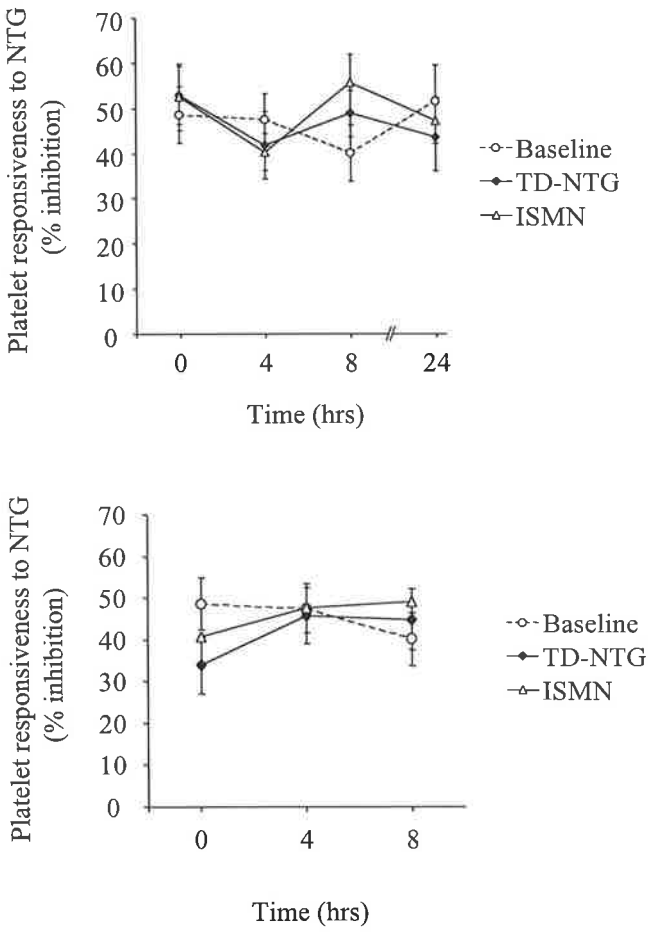
As illustrated in Appendix Figure 2 upper panel and by 2-way ANOVA there was no significant difference between the treatment groups or across the 24-hr time period for the acute phase of the trial (2-way ANOVA: treatment group F = 0.14, p = 0.86; time F = 0.83, p = 0.47; treatment group x time F = 0.75, p = 0.6; Bartlett's statistic = 7.25, p 0.78). No significant interaction between the two determinants was also observed. Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between any of the data points within Appendix Figure 2.

Chronic phase

Much the same as demonstrated above, the data representing the degree of platelet responsiveness to NTG (100μM) during the chronic nitrate phase of the trial were shown to be of a Gaussian distribution. ("no nitrates" 0hrs KS = 0.19, p = ns; 4hrs KS = 0.14, p = ns; 8hrs KS = 0.08, p = ns. ISMN 0hrs KS = 0.16, p = ns; 4hrs KS = 0.13, p = ns; 8hrs KS = 0.18, p = ns. TD-NTG 0hrs KS = 0.13 p = ns; 4hrs KS = 0.15, p = ns; 8hrs KS = 0.2, p = ns). By 2-way ANOVA and as displayed in the lower panel of Appendix Figure 2, there was no significant difference between the treatment groups, across the time period, or any significant interaction between the two determinants (2-way ANOVA Treatment group F = 0.37, p = 0.69; Time F = 0.57, p = 0.56; Treatment group x time F = 0.62, p = 0.64; Bartlett's statistic = 2.0, p = 0.98). Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between any of the data points within the lower panel of Appendix Figure 2.

Differences in platelet responsiveness to NTG (100μM) between the nitrate treatment phases

In order to determine a difference between the acute and chronic phases of the trial for the degree of platelet responsiveness to NTG (100μM), data was pooled across the treatment groups and the time periods. Utilizing a paired *t*-test there was no significant difference between the acute and chronic phases of the trial for the degree of platelet responsiveness to NTG (100μM) (paired *t*-test: $t = 1.56$, $p = 0.15$).



Appendix Figure 2: Influence of acute/chronic nitrate administration on the degree of platelet responsiveness to NTG

***Upper panel**: Acute administration of either TD-NTG or ISMN in 12 subjects had no significant effect on the degree of platelet responsiveness to NTG (100μM). 2-way ANOVA treatment group $F = 0.14$, $p = 0.86$; time $F = 0.83$, $p = 0.47$; treatment group x time $F = 0.75$, $p = 0.6$. **Lower panel**: Chronic administration of either TD-NTG or ISMN also had no significant effect on the degree of platelet responsiveness to NTG (100μM). 2-way ANOVA Treatment group $F = 0.37$, $p = 0.69$; Time $F = 0.57$, $p = 0.56$; Treatment group x time $F = 0.62$, $p = 0.64$.*

Platelet responsiveness to SNP (10μM)

The degree of platelet responsiveness to SNP (10μM) was analyzed for the 12 subjects whom also had their AI(x) assessed at the time of blood sampling.

Acute phase

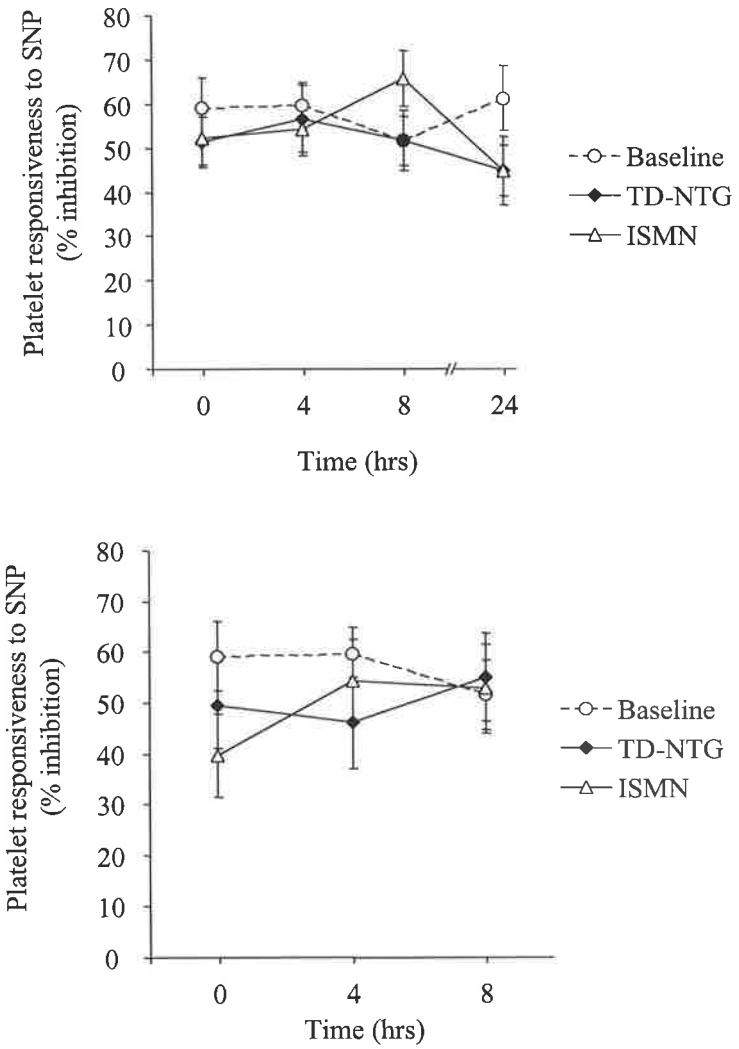
The data representing the degree of platelet responsiveness to SNP (10μM) during the acute nitrate phase of the trial were shown to be of a Gaussian distribution and not to contain any significant differences between the standard deviations across the 24-hr sampling period. ("no nitrates"; 0hrs KS = 0.13, p = ns; 4hrs KS = 0.18, p = ns; 8hrs KS = 0.21, p = ns; 24hrs KS = 0.2, p = ns. ISMN; 0hrs KS = 0.12, p = ns; 4hrs KS = 0.14, p = ns; 8hrs KS = 0.16, p = ns; 24hrs KS = 0.13 p = ns. TD-NTG 0hrs KS = 0.21, p = ns; 4hrs KS = 0.12, p = ns; 8hrs KS = 0.2, p = ns; 24hrs KS = 0.17, p= ns). By 2-way ANOVA and as displayed in Appendix Figure 3 upper panel, there was no significant difference between the treatment groups, across the time period, or any significant interaction between the two determinants (2-way ANOVA Treatment group F = 1.12, p = 0.31; Time F = 0.6 p = 0.59; Treatment group x time F = 1.6, p = 0.39; Bartlett's statistic = 3.2, p = 0.98). Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between any of the data points (upper panel of Appendix Figure 3).

Chronic phase

The data representing the degree of platelet responsiveness to SNP (10μM) during the chronic nitrate phase of the trial were shown to be of a Gaussian distribution and not to contain any significant differences between the standard deviations across the eight hour sampling time period. ("no nitrates" 0hrs KS = 0.13, p = ns; 4hrs KS = 0.17, p = ns; 8hrs KS = 0.2, p = ns. ISMN 0hrs KS = 0.2 p = ns; 4hrs KS = 0.13, p = ns; 8hrs KS = 0.11, p = ns. TD-NTG 0hrs KS = 0.22, p = ns; 4hrs KS = 0.16, p = ns; 8hrs KS = 0.13, p = ns). By 2-way ANOVA and as displayed in Appendix Figure 3 lower panel, there was no significant difference between the treatment groups, across the time period, or any significant interaction between the two determinants (2-way ANOVA Treatment group F = 0.85, p = 0.43; Time F = 0.22, p = 0.8; Treatment group x time F = 0.72, p = 0.57; Bartlett's statistic = 4.3, p = 0.83). Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between any of the data points (lower panel of Appendix Figure 3).

Differences in platelet responsiveness to SNP (10μM) between nitrate treatment phases

Pooling the data for the individual treatment groups ("no nitrates", ISMN and TD-NTG) and time, significant differences between the acute and chronic phases of the trial regarding the degree of platelet responsiveness to SNP (10μM) were examined. By paired *t*-test there was no significant difference between the acute and chronic phase of the trial (paired *t*-test t = 0.73, p = 0.5).



Appendix Figure 3: Influence of acute/chronic nitrate administration on the degree of platelet responsiveness to SNP

***Upper panel**: Acute administration of either TD-NTG or ISMN in 12 subjects had no significant effect on the degree of platelet responsiveness to SNP (10μM). 2-way ANOVA treatment group F = 0.14, p = 0.86; time F = 0.83, p = 0.47; treatment group x time F = 0.75, p = 0.6. **Lower panel**: Chronic administration of either TD-NTG or ISMN also had no significant effect on the degree of platelet responsiveness to SNP (10μM). 2-way ANOVA Treatment group F = 0.37, p = 0.69; Time F = 0.57, p = 0.56; Treatment group x time F = 0.62, p = 0.64.*

Acute and chronic effects of nitrate pharmacotherapy on lucigenin derived chemiluminescence

Data representing the degree of LDCL was analyzed for the 12 subjects whom also had their AI(x) assessed at the time of blood sampling.

Pre-aggregation LDCL-Acute phase

Data for the pre-aggregation LDCL during the acute phase of each nitrate treatment regimen was shown to conform to a Gaussian distribution ("no nitrates"; 0hrs KS = 0.13, p = ns; 4hrs KS = 0.25, p = ns; 8hrs KS = 0.28, p = ns; 24hrs KS = 0.17, p = ns. ISMN; 0hrs KS = 0.24, p = ns; 4hrs KS = 0.2, p = ns; 8hrs KS = 0.29, p = ns; 24hrs KS = 0.22, p = ns. TD-NTG 0hrs KS = 0.16, p = ns; 4hrs KS = 0.2, p = ns; 8hrs KS = 0.22, p = ns; 24hrs KS = 0.22, p= ns). However, heterogeneous variances between the data populations were observed and therefore a log transformation of the data was performed prior to the ANOVA (Bartlett's statistic = 40.2, $p < 0.01$). On the log-transformed data and by 2-way ANOVA a significant difference between the treatment groups ("no nitrates", ISMN and TD-NTG) was observed. There was no significant difference across the 24-hr time period and no significant interaction between the two determinants (2-way ANOVA; Treatment group F = 5.47, $p < 0.01$; time F = 0.74, p = 0.53; treatment group x time F = 0.57, p = 0.75; Bartlett's statistic = 10.5, p = 0.48). Despite demonstrating a significant difference between the treatment groups with the ANOVA Bonferroni's post hoc multiple comparison test was unable to identify any significant differences between any data point on the log transformed data (upper left panel of Appendix Figure 4).

Pre-aggregation LDCL-Chronic phase

Like the luminescence data representing acute pre-aggregation data, all data populations for the pre-aggregation LDCL during the chronic phase of the trial conformed to a Gaussian distribution ("no nitrates"; 0hrs KS = 0.13, p = ns; 4hrs KS = 0.25, p = ns; 8hrs KS = 0.28, p = ns. ISMN; 0hrs KS = 0.25, p = ns; 4hrs KS = 0.18, p = ns; 8hrs KS = 0.16, p = ns. TD-NTG 0hrs KS = 0.24, p = ns; 4hrs KS = 0.26, p = ns; 8hrs KS = 0.23, p = ns). By 2-way ANOVA there was no significant difference between the treatment groups, across the time period and between the two determinants (2-way ANOVA; treatment group F = 0.1, p = 0.9; time F = 0.51, p = 0.6; treatment group x time F = 1.4, p = 0.23; Bartlett's statistic = 6.7, p = 0.56).

Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between any of the data points (lower panel of Appendix Figure 4).

Differences in pre-aggregation LDCL between treatment phases

In order to assess the differences between the acute and chronic phases of the trial for the degree of pre-aggregation LDCL data across the time periods (acute = 0 to 24hrs, chronic = 0 to 8hrs) was pooled. Given that a significant difference within the treatment groups was demonstrated within the acute phase of the pre-aggregation LDCL analysis, the treatment groups ("no nitrates", ISMN, TD-NTG) remained in the analysis to determine a difference between the acute and chronic phases. The data populations for each treatment group during both the acute and chronic phases of the trial, were then shown to conform to a Gaussian distribution (Acute phase: "no nitrates" KS = 0.21, p = ns; ISMN KS = 0.17, p = ns; TD-NTG KS = 0.14, p = ns. Chronic phase: "no nitrates" KS = 0.21, p = ns; ISMN KS = 0.14, p = ns; TD-NTG KS = 0.19, p = ns). By 2-way ANOVA there was no significant difference between the treatment groups and none between the acute and chronic phase of the trial. No significant interaction between the two determinants was also observed (Treatment group F = 1.52, p = 0.23; Acute/chronic F = 0.002, F = 0.96; Treatment group x acute/chronic F = 1.8, p = 0.2; Bartlett's statistic = 5.44, p = 0.36).

Aggregation-associated LDCL-acute phase

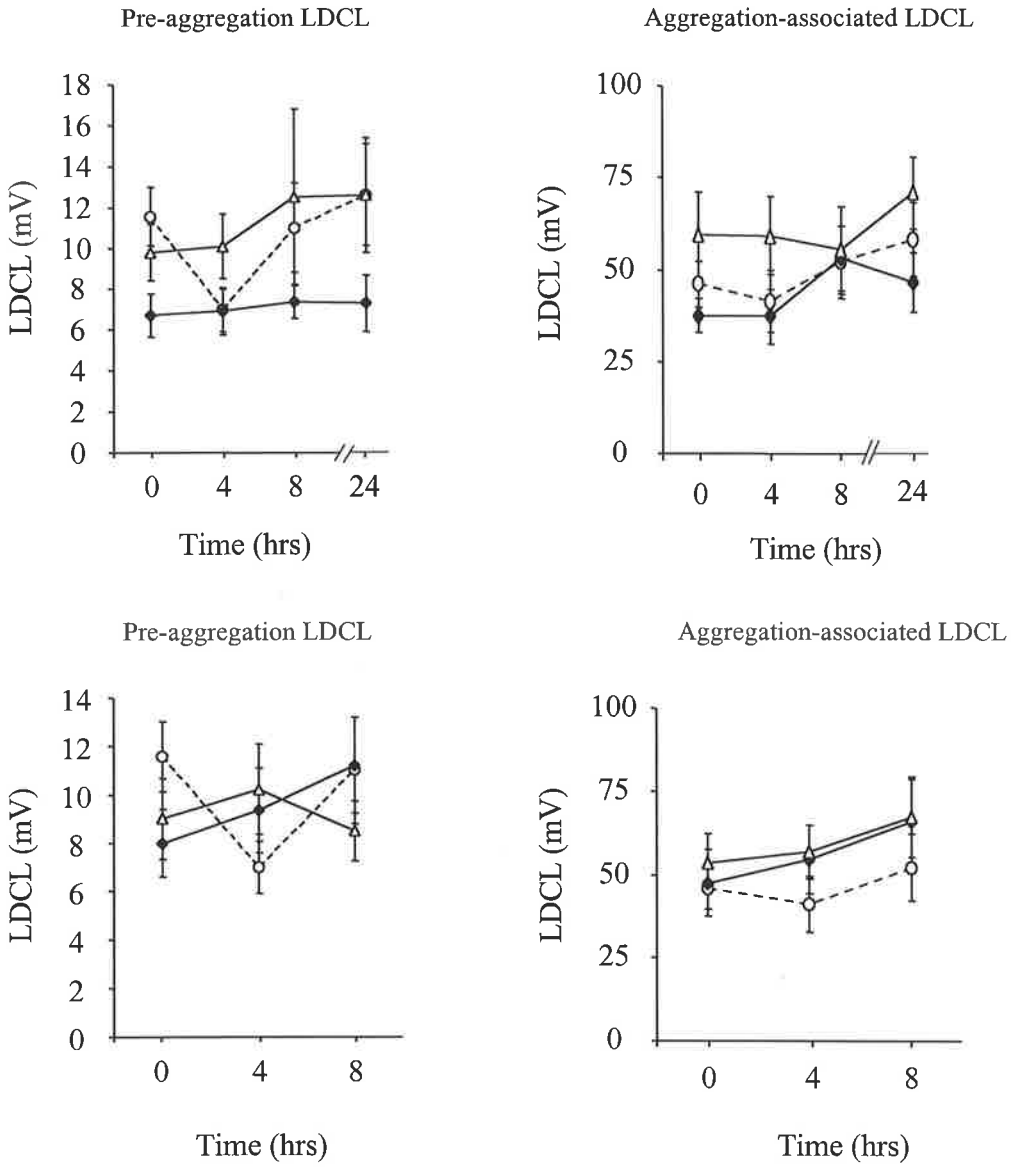
All data populations for the degree of aggregation-associated LDCL (lucigenin 12.5μM) during the acute phase of the trail were found to conform to a Gaussian ("no nitrates"; 0hrs KS = 0.24, p = ns; 4hrs KS = 0.17, p = ns; 8hrs KS = 0.26, p = ns 24hrs KS = 0.21, p = ns. ISMN; 0hrs KS = 0.17, p = ns; 4hrs KS = 0.15, p = ns; 8hrs KS = 0.23, p = ns; 24hrs KS = 0.15, p = ns. TD-NTG 0hrs KS = 0.19, p = ns; 4hrs KS = 0.25, p = ns; 8hrs KS = 0.26, p = ns; 24hrs KS = 0.16, p = ns). By 2-way ANOVA there was a significant difference between the treatment groups ("no nitrates", ISMN, TD-NTG) with no significant difference across the 24-hr time period. There was also no significant interaction between the two determinants (2-way ANOVA; treatment group F = 3.8, p = 0.025; time F = 1.2, p = 0.32; treatment group x time F = 0.33, p = 0.91; Bartlett's statistic = 11.1, p = 0.43). Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between any of the data points (upper right panel of Appendix Figure 4).

Aggregation-associated LDCL-chronic phase

Much the same way as demonstrated above in the acute phase, the data populations for the extent of aggregation-associated LDCL (lucigenin 12.5μM) was shown to conform to a Gaussian distribution ("no nitrates"; 0hrs KS = 0.23, p = ns; 4hrs KS = 0.17, p = ns; 8hrs KS = 0.26, p = ns. ISMN; 0hrs KS = 0.19, p = ns; 4hrs KS = 0.22, p = ns; 8hrs KS = 0.27, p = ns. TD-NTG 0hrs KS = 0.22, p = ns; 4hrs KS = 0.18, p = ns; 8hrs KS = 0.14, p = ns). By 2-way ANOVA there was no significant difference between the treatment groups, across the 8-hr time period. There was also no significant interaction between the two determinants (2-way ANOVA; treatment group F = 1.28, p = 0.28; time F = 1.42, p = 0.25; treatment group x time F = 0.12, p = 0.97; Bartlett's statistic = 6.71, p = 0.57). Utilizing Bonferroni's post hoc multiple comparison test there were no significant differences between any of the data points (lower right panel of Appendix Figure 4).

Differences in aggregation-associated LDCL between the nitrate treatment phases

By pooling the aggregation-associated LDCL data across the time periods and across each treatment a difference between the acute and chronic phases of the trial was examined. Utilizing a paired *t*-test no significant difference between the acute and chronic phases for the degree of aggregation-associated LDCL was observed (paired *t*-test t = 1.05, p = 0.32).



Appendix Figure 4: Influence of acute/chronic nitrate administration on the degree of LDCL

Figure legend: *"no nitrates"; open circle dotted line. TD-NTG; closed diamond solid line. ISMN; open triangle solid line.* ***Upper panels****: Acute administration of either TD-NTG or ISMN in 12 subjects on the degree pre-aggregation LDCL (left) and aggregation-associated LDCL (right). Pre-aggregation LDCL 2-way ANOVA log transformed data treatment group F = 5.47, p < 0.01; time F = 0.74, p = 0.53; treatment group x time F = 0.57 p = 0.75. Aggregation-associated LDCL 2-way ANOVA treatment group F =3.8, p = 0.025; time F = 1.2, p = 0.32; treatment group x time F = 0.33, p = 0.91.* ***Lower panels****: Chronic administration of either TD-NTG or ISMN on the degree of pre-aggregation LDCL (left) and aggregation-associated LDCL (right). 2-way ANOVA pre-aggregation LDCL Treatment group F = 0.1, p = 0.9; Time F = 0.51, p = 0.6; Treatment group x time F = 1.4, p = 0.23. Aggregation-associated LDCL chronic treatment group F = 1.28, p = 0.28; time F = 0.51, p =0.6; treatment group x time F = 0.12, p = 0.97.*

Publications

Chirkov, Y.Y., Holmes, A.S., Chirkova, L.P. & Horowitz, J.D. (1999) Nitrate resistance in platelets from patients with stable angina pectoris.
Circulation, v. 100, pp. 129-134

Chirkov, Y.Y., Holmes, A.S., Willoughby, S.R., Stewart, S., Wuttke, R.D., Sage, P.R. & Horowitz, J.D. (2001) Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets.
Journal of the American College of Cardiology, v. 37(7), pp. 1851-1857

Amendments

Acknowledgements

- The following should be included on page 26 after paragraph 4.

" I would like to comment on the specific contributions Dr Chirkov made regarding the performing of experiments within this thesis. All experiments performed within Chapter 2 were performed by myself with instructions given by Dr Chirkov. Together with a series of additional experiments addressing other questions (performed by Dr Chirkov), the results from section 1 of Chapter 2 formed the basis for the "Circulation" (Chirkov *et al.*, 1999) article. Data from Chapter 3 was derived from experiments performed by a number of people and this should be acknowledged. Experiments were performed by myself, Drs Chirkov, Willoughby and Stewart. As the idea of examining the potential determinants of platelet NO resistance was derived by Dr Chirkov, first authorship was awarded accordingly".

Chapter 1

The following should be changed accordingly:

- Page 34, paragraph 2, line 2:

" an single electron" should be replaced with "a single electron"

Section A

- The following should be included following section A.12 "Effects of *S*-nitrosothiols on platelet function", page 63.

Effect of insulin on platelets

Whilst investigating the effects of insulin on human platelet function Trovati *et al.*, (1988) demonstrated that insulin significantly reduces platelet sensitivity to a number of aggregating agents (ADP, PAF, collagen, adrenaline). Following on from these initial observations the same research group demonstrated that the anti-aggregatory effects of insulin result from an increase in platelet derived nitric oxide generation that promotes an

increase in platelet cGMP / cAMP generation (Trovati *et al.*, 1994, 1996, 1997). The role of insulin in the phenomenon of platelet nitric oxide resistance is discussed in section E.2.1 of the current chapter. However, for a comprehensive review of the literature regarding the effects of insulin on platelet function see Trovati and Anfossi (2002) or Vink *et al.*, 2001.

Complete references:

Trovati M, and Anfossi A. (2002). Influence of insulin and of insulin resistance on platelet and vascular smooth muscle cell function. J Diabet Compl; 16:35-40.

Vink AI., Tomris E, Park TS, Nolan R and Pittenger GL. (2001). Platelet dysfunction in Type 2 diabetes. Diabetes Care: 24:1476-1485.

Section C

- The following should be included at the end of Section C.6.2.1 "Antioxidant pharmacotherapy", page 83, paragraph 3:

"Despite the experimental evidence demonstrating that anti-oxidant pharmacotherapy may ameliorate endothelial dysfunction, results from large clinical trails examining the effects of anti-oxidant therapy on long term clinical outcomes have failed to show any significant benefit with their use. Using vitamin E as an example, the Heart Outcomes Prevention Evaluation (HOPE) study found no significant benefit was derived from taking vitamin E (400U/day) at preventing the incidence of the combined endpoint of MI + cardiovascular death + stroke (RR 1.05, 95% CI 0.95-1.16, p = 0.33) (Yusuf *et al.*, 2000). Similar results were also observed within the Heart Protection Study where a number of vitamin supplements (650mg vitamin C, 25mg vitamin E and 20mg beta-carotene) were examined for their effects on secondary prevention outcomes (Heart Protection Study Collaborative Group, 2002).

Complete references:

Yusuf S, G Dagenais, J Pogue, J Bosch, P Sleight. (2000). Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Study Investigators. New Engl J Med; 342: 154-60.

Heart Protection Study Collaborative Group (2002). MRC/BHF Heart Protection Study of antioxidant vitamin supplementation 20536 high-risk individuals: a randomized placebo-controlled trial. The Lancet: 360: 23-33.

The following should be changed:

- Page 94, paragraph 3, starting with "Perhaps the most ... and ending with Marso *et al.*, 1999) should be replaced with the following:

Subgroup analysis of the major GPIIb/IIIa receptor antagonist trials have provided some indirect evidence suggesting platelets may play a role in the pathogenesis of diabetes mellitus. Significant improvements in clinical outcome have been observed in diabetic patients randomized to receive GPIIb/IIIa receptor antagonists compared to diabetic patients receiving placebo preparations (Bhatt *et al.*, 2000; Lincoff, 2000; Marso *et al.*, 1999).

- The following should be included at the end of section C13 "Aspirin", page 106

Aspirin resistance

Despite receiving therapeutic doses of aspirin a certain proportion of subjects still experience thrombotic events. This absence of the protective effect from aspirin use has been termed "aspirin resistance" with the prevalence of this phenomenon ranging from 10 – 45% of subjects with CAD (Gum *et al.*, 2001).

A number of potential mechanisms behind the phenomenon of aspirin resistance have been suggested. Such mechanisms include activation of platelets via pathways that do not

involve TxA_2, ineffective doses for particular patients or further TxA_2 generation from alternative sources even in the presence of therapeutic concentrations of aspirin. For a review of the incidences and mechanisms of aspirin resistance see the following reviews (FitzGerald 2003; Smout and Stansby 2000)

Recently Eikelboom *et al.*, 2002 utilizing samples obtained from a subset of patients enrolled in the HOPE study, demonstrated that high urinary concentrations of 11-dehydro thromboxane B_2 predict MI or cardiovascular death. That is despite receiving aspirin, those subjects with a high 11-dehydro thromboxane B_2, serving as a marker for the presence of TxA_2 and thus referred to a "aspirin resistant", were shown to have an increased risk of MI or cardiovascular death compared to those subjects with a low 11-dehydro thromboxane B_2 level.

Complete references:

Eikelboom JW, Hirsh J, Witz JI, Johnston M, Yi Q, Yusuf S. (2002). Aspirin-resistant thromboxane biosynthesis and the risk of myocardial infarction, stroke, or cardiovascular death in patients at high risk for cardiovascular events. Circulation; 105:1650-1655.

FitzGerald GA (2003). Parsing an enigma: the pharmacodynamics of aspirin resistance. The Lancet; 361: 542-543.

Gum PA., Kottke-Marchant K, Poggio ED, Gurm H, Welsch PA, Brooks L, Sapp SK and Topol EJ. (2001). Profile and prevalence of aspirin resistance in patients with cardiovascular disease. Am J Cardiol;88:230-235.

Smout J and Stansby G (2002). Aspirin resistance. British J Surgery; 89:4-5.

The following should be changed:

- Page 137, section F "Major residual issues" sentence 1, line 2, the word "also" should be deleted.

Chapter 2

The following should be changed:

- Page 141, paragraph 2, line 2, "(n = 43)" should read "(n = 44)".

The following should be changed:

- Page 180, paragraph 2, line 8, starting with "A relationship ….", should be replaced with the following:

"This relationship was not observed in the NV cohort."

The following should be changed:

- Page 180, paragraph 3, line 5, starting with "A result that is agrees….", should be replaced with the following

"A result that is in agreement with the observations of others (Swahn and Wallentin, 1987)."

The following should be changed:

- Page 181, paragraph 4, line 4, starting with "Combing the subject cohorts….", should be replaced with the following:

"When the data was analyzed according to those with/without a history of angina pectoris (Figure 2.3.6), neither population showed a significant relationship between the baseline extent of platelet aggregation and the extent of change in platelet aggregation post administration of SOD/Catalase".

The following should be changed:

- Page 182, section 2.3.5 "Discussion", paragraph 1, line 8, sentence starting with "Investigating this hypothesis…." Should be replaced with the following:

An examination of intra-platelet superoxide generation is a potentially important research direction, one that largely remains unexplored. Despite this, it was decided not to examine intra-platelet superoxide generation in the current series of experiments.

The following should be changed:

- Page 194, paragraph 2, line 4, "potentate" should read "potentiate".

Chapter 4

- The following should be included at the end of section 4.6.3 "Sensitivity to nitric oxide donors", page 382, after paragraph 2.

"Despite not demonstrating nitrate tolerance induction using systolic blood pressure as a measure of vascular function, acute administration of either nitrate preparation reduced systolic blood pressure compared to the no nitrate period, though not significantly (Figure 4.18). Such trends were not observed following chronic nitrate administration (Figure 4.19). As there was no difference between the phases of treatment, no obvious tolerance to either preparation was concluded using systolic blood pressure as a measure of vascular function. Throughout the literature there are a number of examples demonstrating that nitrate administration to subjects with a history of CAD effectively reduces systolic blood pressure. Taking into consideration evidence throughout the literature and that a trend towards a significant reduction in systolic blood pressure was observed in the 12 patients examined within the current study, it is not unreasonable to conclude that an absence of a significant reduction in systolic blood pressure resulted from type II error. However, it should also be noted that the results observed herein are in agreement with the observations of O'Rourke and Nicholas (2002) in which there was no significant change in systolic blood pressures post administration of TD-NTG (2.5 to 15mg/24hrs), whilst there were significant reductions in AI(x).

Complete reference:

O'Rourke M and Nichols WW. (2002). Potential for use of pulse wave analysis in determining the interaction between sildenafil and glyceryl trinitrate. Clin Cardiol: 25:295-9.

Chapter 5

- The following should be included at the end of section 5.5 "Major study limitations", page 396, paragraph 1:

Routine collection of blood for the determination of C-reactive protein (CRP) levels were not performed on samples from subjects studied in Chapter 3. An estimation of the inflammatory status of these subjects may have provided some insight as to whether a reduced responsiveness to NO is related to the inflammatory status of particular subjects. Furthermore, it may have provided some evidence of an anti-inflammatory effect form statins whose use was associated with a higher NO responsiveness.

At no stage throughout the experiments that constitute this thesis was the menopausal / menstrual status of females recorded. This oversight therefore constitutes a major study limitation. However, it should be acknowledged that males made up the majority of subjects enrolled within each study. Moreover, given the mean age of the female patients examined throughout this thesis was greater than sixty, an effect of menopausal / menstrual status on the results observed herein would be minimal.