Vasomotor and anti-oxidant effects of anti-ischaemic therapies.

Elizabeth Liberts

A thesis submitted to The University of Adelaide as the requirement for the degree of Doctor of Philosophy

The Cardiology Unit,

The Queen Elizabeth Hospital,

Department of Medicine,

The University of Adelaide

Submitted in August 2006

Table of contents

Abstract

Nitric oxide (NO) plays a fundamental role in vascular homeostasis including modulating vasomotor tone, platelet adhesion and aggregation. The majority of coronary risk factors, as well as the presence of overt ischaemic heart disease have been associated with anomalies of NO-mediated biological effect, including impaired endothelium mediated vasodilatation, nett increases in platelet aggregation, and increased activation of neutrophils. Although in many circumstances NO release is impaired, it is increasingly apparent that in most of these pathological states there is associated reduction in tissue responsiveness to NO. This impaired responsiveness may be mediated by either "scavenging" of released NO by free radicals such as superoxide (O_2^-) or by dysfunction of the NO/soluble guanylate cyclase second messenger system. This phenomenon of "tissue NO resistance", which occurs both in vasculature and in platelets, carries independently adverse prognosis and also implies reduction in the therapeutic efficacy of the NO donors such as organic nitrates, which are commonly utilised in treatment of unstable coronary artery disease and myocardial infarction. A variety of agents have been shown to improve responsiveness to NO, including ACE inhibitors, perhexiline and possibly statins.

The current studies examine various aspects of NO responses, including: the methodology for assessment of vascular NO responses, and identification of factors responsible for poor responsiveness and pharmacological modulation of these responses. The first study investigated the utility of pulse wave analysis for measurement of responses to glycerol trinitrate (GTN) administered both orally and intravenously, in patients with coronary artery disease and in controls. The second component of the work

investigated whether oxidative stress, an important factor in the pathogenesis of coronary artery disease and postulated to be involved in impaired responsiveness to NO, was associated with the presence of stable or unstable coronary artery disease. Subsequently, the effects of two drug treatments, perhexiline and the ACE inhibitor ramipril, were investigated for potential effects on NO responsiveness and oxidative stress markers in vasculature, platelets and neutrophils.

These studies demonstrated that in normal subjects that oral administration of greater than 200μg of GTN induces near-maximal responses as measured by changes in augmentation index (AIx, by pulse wave analysis), which may obscure the detection of changes in sensitivity to GTN. Intravenous infusion of GTN at the rate of 5μg/min (lower end of clinically utilised infusion rates) produced a similar magnitude of reduction in AIx, in patients with stable angina pectoris. This technique was also utilised during the transition from low dose intravenous GTN to oral ISDN (10mg) to minimise potential "rebound" phenomenon (rebound vasoconstriction following withdrawal of vasodilator therapy) and it was found that this regimen is associated with no loss of vasodilator effect, as measured by AIx.

Investigation of the possible potentiation of vascular and extra vascular NO responsiveness by perhexiline in patients with stable angina pectoris, demonstrated that perhexiline has no effect on apparent arterial stiffness either alone or in combination with intravenous GTN and that in this patient cohort with normal platelet function perhexiline also has no effect on platelet NO responsiveness. However, the study also demonstrated

that in vivo administration of low infusion rates of GTN inhibits neutrophil O_2^- release, and that this effect is potentiated by pre-treatment with perhexiline.

Oxidative stress, as a potential source of differential NO responsiveness between patient groups was examined utilising two different markers, malondialdehyde (MDA, a lipid peroxidation product) and ex vivo neutrophil superoxide release, for comparison between three patient groups. The study demonstrated that both acute coronary syndromes (ACS) and (marginally) stable angina pectoris (SAP) are associated with incremental oxidative stress. This study also demonstrated that there was no correlation between levels of the two markers in individual patients and therefore it was concluded that no single estimate of either superoxide release or of MDA would prove of value as a correlate of extent of ongoing ischaemia in individual patients.

Ramipril therapy in patients, who are at high risk of cardiovascular events (as delineated in the HOPE trial), was associated with improved function of the NO system in vasculature and in platelets. This study demonstrated that ramipril sensitises platelets to the anti-aggregatory effects of NO but that this effect occurs predominantly in those patients with poor initial responses to NO. However it failed to demonstrate a decrease in oxidative stress, utilising MDA as a marker, or sensitisation of the platelet soluble guanylate cyclase system during ramipril treatment.

The experiments described in this thesis therefore demonstrate that two agents with differential regional effects on oxidative stress exert NO-potentiating effects: perhexiline predominantly in neutrophils and ramipril in platelets (and presumably also in blood vessels). On the other hand, conventional measures of oxidative stress provide only arbitrary value as regards either degree of haemostatic disturbance, and are unlikely to be of value for individual patient categorisation or assignment of therapeutic priorities.

Declaration

With the exception of the first 12 patients in chapter 4, which were included in my Honours thesis, submitted to the Department of Physiology in 2001, this work contains no material which has been accepted for the award of any other degree or diploma in any university or 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 is 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.

Elizabeth Liberts

(August 2006)

Acknowledgements

Firstly I would like to thank my supervisors Dr Jenny Kennedy and Professor John Horowitz for their guidance and support. I also wish to thank all of the people in the Cardiology Laboratories at the Queen Elizabeth Hospital, in particular Geraldine Murphy for unending support and teaching me laboratory techniques, Dr Scott Willoughby, for his support and guidance and for completing the platelet studies in chapter 4 and 6 (along with Dr Yuily Chirkov and Jackie Stepien-Hulleman), Tamila Heresztyn for performing the ADMA assay in chapter 6 and Sue Leslie for assistance with recruitment of patients, clinical assessment and applanation tonometry measurement in chapter 6.

Having been the recipient of a University of Adelaide Postgraduate Research Scholarship, I would like to thank the University of Adelaide for their support.

I would also like to thank all the people who I have met along the way, particularly all of my employers and colleagues at my many casual jobs for their support and understanding. Finally I would like to acknowledge the assistance of my family for their encouragement, support and understanding.

Addenda

Insert on Pg 14, line 11 following "Ruth et al., 1993)": Furthermore, it has been demonstrated that PKG can reduce intracellular calcium concentration and hence inhibit contraction via phosphorylation of several proteins involved in intracellular calcium regulation. Phosphorylation of phospholamban (Raeymaekers et al. 1988) leads to activation of sarcoplasmic reticulum calcium ATPase (SERCA) (Cornwell et al. 1991)which results in rapid uptake of calcium into the SR, thereby decreasing intracellular calcium concentration. Phosphorylation of 1,4,5-inositoltriphosphate (IP_3) receptor associated cGMP kinase substrate (IRAG) inhibits IP_3 induced release of calcium from the sarcoplasmic reticulum (Schlossmann et al. 2000), which also contributes to decreased intracellular calcium concentration. Activation of calcium dependant potassium channels by NO has been demonstrated both directly and via PKG and results in hyperpolarisation of the cell, which both counteracts the membrane depolarising signal (Bolotina et al. 1994; Li et al. 1998) and inhibits voltage gated calcium channels.

Insert on page 20, line 16:

1.2.3.3 Effects of NO on apoptosis

NO also plays an important role in regulation of cellular apoptosis. High concentrations of NO (such as those seen during iNOS activation or during in vitro exposure) and resultant peroxynitrite formation are known be important in immune mediated cytotoxicity (Drapier et al. 1988), and have been shown to stimulate apoptotic pathways (Messmer et al. 1995); however, NO has also been associated with inhibition of apoptosis. Several mechanisms have been suggested to mediate this effect including both direct S-nitrosylation of cysteine residues of caspase enzymes and cGMP dependant inhibition of caspases and suppression of cytochrome c release from the mitochondria (Kim et al. 1997; Li et al. 1997; Rossig et al. 1999).

Insert on page 31, line 13 after "second higher peak." and substitute the following for the rest of the paragraph: The second peak results from reflection of the incident

wave from the conductance vessels, which is a product both of the stiffness of these vessels and the speed with which the pressure wave is reflected. The difference between the incident and reflected waves is known as the augmentation index (AIx). Therefore changes in AIx reflect changes in stiffness of conductance vessels and/or the speed with which the wave is reflected, however these parameters are inherently linked and both contribute to measured arterial stiffness.

Insert page 70, line 20, after "anginine tablet)": placed under the tongue for 10 minutes, as compared to a 500μg sublingual tablet held under the tongue for 3 minutes as in (Wilkinson et al. 2002). **Replace "This dose" with**: This 300μg dose

Insert on page 99, line 5 following "systolic blood pressure of 24±4mmHg": (decrease of 17±14mmHg mean arterial pressure (MAP)). **Insert on page 99, line 7, between "systolic BP, 3.3±1mmHg" and "AIx, 1.5±1.5%, p=NS)".**: MAP, 3±2mmHg,

Insert on page 115, line 14 after "acute coronary events.": (Brennan et al. 2003) demonstrated that among patients presenting with acute chest pain, elevation of plasma myeloperoxidase concentrations predicts risk of infarction and of other adverse events.

Add to the figure legend to figure 5.1 (page 126): median values outlined.

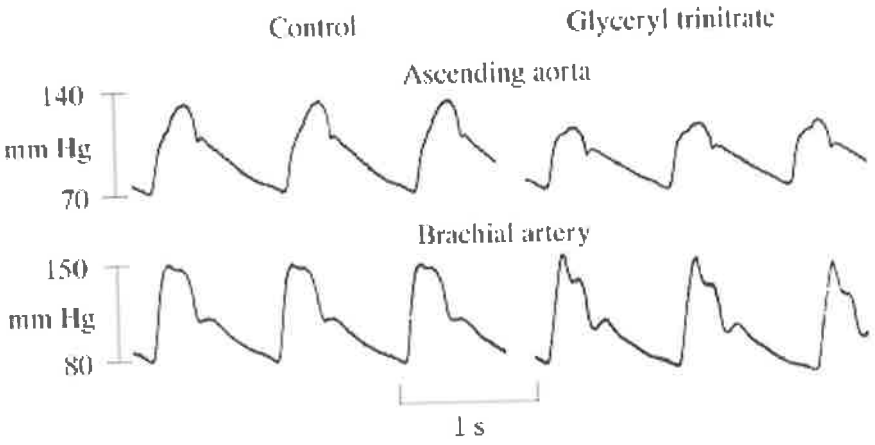
Insert on page 153, line 5 after "activity or expression": In the current study, effects of ramipril were examined only in relatively short-term follow-up: it remains questionable whether the effects seen after 3 months are representative of those associated with long-term treatment, as in the HOPE study.

Insert in table 6.1, Pg 154 between DBP(SD) and HR(SD):

MAP (SD)	103 (13)	103 (14)	NS

Insert after Figure 1.2 on page 32:

Figure 1.3: Effects of administration of GTN on both radial and central pressure waveforms, recorded via applanation tonometry (from (Jiang et al. 2002)).



Insert following page 10:

Publications for Elizabeth Liberts (nee Kelly):

Effects of perhexiline and nitroglycerin vascular, neutrophil and platelet function in patients with stable angina pectoris. EA Liberts, SR Willoughby, JA Kennedy and JD Horowitz. Eur J Pharmacol, 2007 Mar 29;560(1):49-55.

Withdrawal of Intravenous Glyceryl Trinitrate: Absence of Rebound Phenomena with Transition to Oral Isosorbide Dinitrate. EA Kelly, RM Ahmed, JD Horowitz. Clin Exp Pharmacol Physiol. 2005;32:269-72.

Abstracts:

2006 European Society of Cardiology congress, Barcelona, Spain. The effect of ramipril on platelet function in patients with high cardiovascular risk: A double-blind randomized placebo-controlled evaluation of ramipril on platelet nitric oxide responsiveness. S.R. Willoughby, S. Rajendran, E.A. Kelly, T. Heresztyn, S. Leslie, Y.Y. Chirkov, J.D. Horowitz.

2005 Frontiers in Vascular Medicine, Melbourne. Elevated oxidative stress markers are associated with symptomatic status in patients with coronary artery disease. EA Kelly, JA Kennedy, MA Arstall and JD Horowitz

2005 Cardiac Society of Australia and New Zealand, Perth.
1) Elevated oxidative stress markers are associated with symptomatic status in patients with coronary artery disease. EA Kelly, JA Kennedy, MA Arstall and JD Horowitz.

2) Hypertension is associated with impairment of platelet responsiveness to nitric oxide in a HOPE-type patient cohort. SR Willoughby, S Rajendran, EA Kelly, S Leslie, T Heresztyn, M Dronavalli, YY Chirkov and JD Horowitz.

2004 Cardiac Society of Australia and New Zealand, Brisbane. Inhibition of Neutrophil Superoxide Release by Nitroglycerine. EA Kelly, JA Kennedy and John D Horowitz.

2003 Cardiac Society of Australia and New Zealand, Adelaide. Acute glucose loading in normal volunteers does not affect platelet function, nitric oxide responsiveness, superoxide content or arterial stiffness. SR Willoughby, MI Worthley, EA Kelly and JD Horowitz.

2002 Australian Health and Medical Research Congress, Melbourne. Acute effects of intravenous nitroglycerine infusion on augmentation index: Lack of evidence for de novo nitric oxide resistance. EA Kelly, JA Kennedy and John D Horowitz.

2002 Australian Society for Medical Research, South Australian Branch annual scientific meeting, Adelaide. Effects of perhexiline on tissue nitric oxide responsiveness. EA Kelly, JA Kennedy and John D Horowitz.

Additional references from page 173 onwards:

Bolotina, V. M., S. Najibi, et al. (1994). "Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle." Nature **368**(6474): 850-3.

Brennan, M. L., M. S. Penn, et al. (2003). "Prognostic value of myeloperoxidase in patients with chest pain." N Engl J Med **349**(17): 1595-604.

Cornwell, T. L., K. B. Pryzwansky, et al. (1991). "Regulation of sarcoplasmic reticulum protein phosphorylation by localized cyclic GMP-dependent protein kinase in vascular smooth muscle cells." Mol Pharmacol **40**(6): 923-31.

Drapier, J. C., J. Wietzerbin, et al. (1988). "Interferon-gamma and tumor necrosis factor induce the L-arginine-dependent cytotoxic effector mechanism in murine macrophages." Eur J Immunol **18**(10): 1587-92.

Fetics, B., E. Nevo, et al. (1999). "Parametric model derivation of transfer function for noninvasive estimation of aortic pressure by radial tonometry." IEEE Trans Biomed Eng **46**(6): 698-706.

Jiang, X. J., M. F. O'Rourke, et al. (2002). "Quantification of glyceryl trinitrate effect through analysis of the synthesised ascending aortic pressure waveform." Heart **88**(2): 143-8.

Karamanoglu, M., M. F. O'Rourke, et al. (1993). "An analysis of the relationship between central aortic and peripheral upper limb pressure waves in man." Eur Heart J **14**(2): 160-7.

Kim, Y. M., R. V. Talanian, et al. (1997). "Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms." J Biol Chem **272**(49): 31138-48.

Li, J., T. R. Billiar, et al. (1997). "Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation." Biochem Biophys Res Commun **240**(2): 419-24.

Li, P. L., M. W. Jin, et al. (1998). "Effect of selective inhibition of soluble guanylyl cyclase on the K(Ca) channel activity in coronary artery smooth muscle." Hypertension **31**(1 Pt 2): 303-8.

Messmer, U. K., E. G. Lapetina, et al. (1995). "Nitric oxide-induced apoptosis in RAW 264.7 macrophages is antagonized by protein kinase C- and protein kinase A-activating compounds." Mol Pharmacol **47**(4): 757-65.

Raeymaekers, L., F. Hofmann, et al. (1988). "Cyclic GMP-dependent protein kinase phosphorylates phospholamban in isolated sarcoplasmic reticulum from cardiac and smooth muscle." Biochem J **252**(1): 269-73.

Rossig, L., B. Fichtlscherer, et al. (1999). "Nitric oxide inhibits caspase-3 by S-nitrosation in vivo." J Biol Chem **274**(11): 6823-6.

Schlossmann, J., A. Ammendola, et al. (2000). "Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta." Nature **404**(6774): 197-201.

Takazawa, K., M. F. O'Rourke, et al. (1996). "Estimation of ascending aortic pressure from radial arterial pressure using a generalised transfer function." Z Kardiol **85** **Suppl 3**: 137-9.

Wilkinson, I. B., I. R. Hall, et al. (2002). "Pulse-wave analysis: clinical evaluation of a noninvasive, widely applicable method for assessing endothelial function." Arterioscler Thromb Vasc Biol **22**(1): 147-52.

Corrigenda

Correct references for page 31, line 2 are (Karamanoglu et al. 1993; Takazawa et al. 1996; Fetics et al. 1999)

Change page 44 line 18, from "more efficient" to "less efficient".

Change page 70, line 12 from 'heart rate variability' to 'changes in heart rate'

Change legend to Figure 4.2 on page 107, to "solid line = control, dashed line = perhexiline."

Exchange table 3.3, page 85 for this version:

Patient Cohort	UAP/NQAMI (n=16)	SAP (n=10)	P values
Age	69±10	59±13	0.036
Gender (M:F)	9:7	6:4	NS
% NQAMI	44	0	-
Coronary Risk Factors (%)			
Smoking	12.5	20	NS
Hypertension	56	50	NS
Diabetes Mellitus	37.5	10	NS
Hypercholesterolaemia	75	70	NS
Concurrent Pharmacotherapy (%)			
Aspirin	100	100	NS
Heparin	81	0	-
ACE inhibitor	40	40	NS
ß-adrenoceptor antagonist	31	20	NS
Calcium channel antagonist	81	70	NS
Statin	50	70	NS

Table 3.3. Characteristics of patients with unstable angina pectoris/non-Q-wave infarction (UAP/NQAMI) compared with those with stable angina pectoris. P values greater than 0.1 and shown as NS.

Exchange table 4.1, page 111, for this version:

	Control Group n=22	Perhexiline Group n=17	P values
Risk Factors			
Age(±SEM)	57±0.6	62±0.6	NS
Male gender	13 (59%)	10 (59%)	NS
Current smoking	5 (23%)	5 (29%)	NS
Hypertension	12 (55%)	10 (59%)	NS
Hypercholesterolemia	18 (82%)	13 (76%)	NS
Diabetes Mellitus	4 (18%)	3 (18%)	NS
Drug Treatment			
Statin	16 (73%)	11 (65%)	NS
β-Adrenoceptor antagonist	8 (36%)	3 (18%)	NS
Aspirin	21 (95%)	17 (100%)	NS
ACE inhibitor/AT1 receptor antagonist	10 (46%)	9 (53%)	NS
Ca antagonist	14 (64%)	13 (76%)	NS
Number of Stenosed/occluded vessels†			
0	3 (14%)	0	NS
1	9 (41%)	6 (35%)	NS
2	10 (45%)	5 (29%)	NS
3	0	6 (35%)	0.004

Table 4.1: Characteristics of patients in control and perhexiline groups. All p values greater than 0.1 are shown as NS. †: significant stenosis = <50% stenosis in a major epicardial vessel

Exchange table 5.1, page 125, for this version

	Normal n=14	SAP n=30	ACS n=32	P value
Age	53±3	60±2	60±1	NS
Gender (% male)	57	63	60	NS
Coronary Risk Factors (%)				
Smoking	0	30	47	NS
Hypertension	0	47	50	NS
Diabetes Mellitus	0	17	31	NS
Hypercholesterolemia	0	77	75	NS
Concurrent Pharmacotherapy (%)*				
Aspirin	0	100	100	NS
ACE inhibitor/AII receptor antagonist	0	43	38	NS
ß-adrenoceptor antagonist	0	30	25	NS
Calcium channel antagonist	0	60	69	NS
Statin	0	63	41	0.08

Table 5.1. Patient characteristics. All three groups were compared with respect to age and gender, but only SAP and ACS were compared with respect to other factors. * All patients in the ACS group also received intravenously infused GTN (2.5-10µg/min). Patients with SAP were studied before and after infusion of GTN. 38% of ACS patients also received intravenous N-acetylcysteine. P values greater than 0.1 are shown as NS.

Chapter 1: Introduction

1.1. The endothelium and vascular homeostasis

At its most basic, the role of the circulatory system is primarily to transport blood around the body. This function is carried out utilising a pump, the heart, and a system of pipes, blood vessels. However on closer examination the system is not so simple and the blood vessels have a much more complex role in maintenance of vascular homeostasis.

High pressures at which blood is pumped from the heart, requires arteries to be strong but able to stretch to absorb and transfer pressure. Different demands in different tissues also require blood vessels to dilate and constrict to enable distribution of blood throughout the arterial tree, yet the necessity for transport from blood to tissues requires them to also be permeable.

The elastic characteristics of arteries are determined largely by their structure and the level of dilatation and constriction is determined by release of endogenous mediators of smooth muscle tone. Both components are important for normal function of arteries. Disruption to one or both elements creates the potential for a loss of normal function, for example, hypertension may develop due to loss of elasticity of blood vessels with age or hypotension may occur due to release of vasoactive mediators during sepsis.

The vascular endothelium is also an important component of the innate immune system. Under normal conditions the endothelium produces anti-inflammatory cytokines (which may include nitric oxide), inhibits adhesion of inflammatory cells and platelets and produces anti-oxidants to limit oxidative stress. Upon activation by the presence of foreign antigens or by mechanical damage, the endothelium expresses receptors which stimulate adhesion of inflammatory cells and platelets and also secretes pro-aggregatory

and stimulatory cytokines. This process is designed to protect the integrity of the vascular endothelium; however there is a significant body of evidence suggesting that activation of this system may contribute to vascular pathology such as that seen in atherosclerosis, resulting in loss of vascular homeostasis.

1.2 Physiology of nitric oxide (NO)

1.2.1 Synthesis, mechanisms of action and degradation of NO

In the endothelium, NO is produced from L-arginine in a reaction catalysed by endothelial NO synthase (eNOS) (Palmer et al., 1988a; Palmer et al., 1988b). NO is then rapidly degraded via a reaction with superoxide (O_2^-), which leads to the formation of peroxynitrite and to loss of biological effect of NO. Vascular smooth muscle cell relaxation and subsequent vasodilatation occurs when NO binds to the haem-iron domain in the N-terminal domain of the enzyme soluble guanylate cyclase (sGC), which catalyses the conversion of guanosine tri-phosphate (GTP) to cyclic guanosine monophosphate (cGMP) and activates signal transduction pathways that lead to smooth muscle relaxation (Rapoport et al., 1983). The precise cellular pathways leading to smooth muscle relaxation down-stream of cGMP are still not completely understood. However it is known that NO stimulates an increase in the intracellular concentration of cGMP which activates a family of enzymes known as the cGMP dependent protein kinases or protein kinase G (PKG) (Pfeifer et al., 1998). These enzymes relax smooth muscle by modulating intracellular calcium concentration either by preventing entry or

by stimulating removal of calcium from within cells. Contraction of smooth muscle cells occurs via receptor activated generation of inositol 1,4,5-triphosphate, which induces release of calcium from intracellular stores. This in turn activates voltage gated calcium channels to stimulate calcium influx. Increased intracellular calcium concentration activates Ca^{2+}/calmodulin dependant myosin light chain kinase which phosphorylates myosin light chain kinase and activates myosin light chain ATPase, leading to contraction (Alioua et al., 1995; Archer et al., 1994; Fukao et al., 1999; Zhou et al., 1996). The intracellular mechanism by which cGMP and PKG are able to inhibit this process remains unclear, however it has been demonstrated that PKG can inhibit hydrolysis of phosphatidyl inositol 4,5 bisphosphate to its active form, inositol 1,4,5 triphosphate (Rapoport et al., 1989; Ruth et al., 1993).

It has also been suggested that PKG may be involved in regulation of thin filaments by phosphorylation of thin filament binding proteins such as VASP (vasodilatory-stimulated phosphoprotein) (Reinhard et al., 1995) and heat shock protein 20 (Beall et al., 1997), leading to disruption of cross bridge formation. It is likely that a combination of these mechanisms is involved in smooth muscle relaxation in response to NO. VASP phosphorylation has also been utilised as an index of levels of activation of the NO/cGMP pathway, although this pathway is not exclusively activated by cGMP (Reinhard et al., 1995).

1.2.2 Endothelium dependant vasodilatation

Early investigations demonstrated the presence of a layer of cells lining the inside of blood vessels; however the function of this layer was unknown. The importance of NO in modulating vascular tone was discovered during investigations which demonstrated the dependence of vasodilatation in response to acetylcholine (ACh) on the presence of intact endothelium. This suggested that responses were reliant on a vasoactive intermediate released from the endothelium (Furchgott and Zawadzki, 1980) and acting on the outer muscle layer to induce relaxation. This intermediate was initially termed endothelium derived relaxing factor (EDRF). EDRF release has subsequently been demonstrated from a variety of different vessels including both arteries and veins of varying sizes and in response to stimuli including: altered flow (shear stress) and chemical mediators such as acetylcholine, bradykinin, and substance P (Ignarro et al., 1988a).

EDRF was identified as NO (Ignarro et al., 1988b; Palmer et al., 1987) in experiments which demonstrated that responses to ACh were initiated by activation of soluble guanylate cyclase (sGC) and subsequent elevation of intracellular cyclic guanosine monophosphate levels (cGMP) (Rapoport et al., 1983), a mechanism of action which had already been demonstrated for organic nitrates (see figure 1.1).

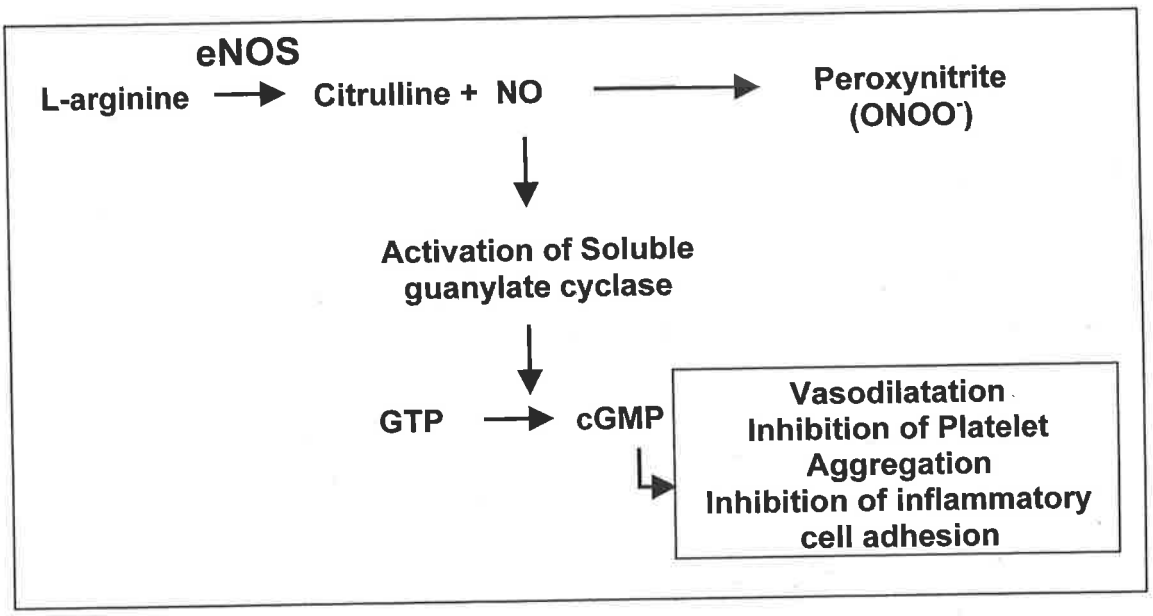


Figure 1.1. Schema of production and action of NO.

1.2.3 Effects outside smooth muscle cells

As well as regulation of smooth muscle tone, NO has been found to be important in many other haemostatic functions. These include modulation of interactions between platelets and inflammatory cells and between these cells and the endothelium.

1.2.3.1 Platelets

The role of platelets in the circulation is to initiate thrombus formation. Platelets become activated in response to agonists such as collagen, collagen-related peptides, prostaglandins and ADP, this signal leads to activation of P-selectin on the platelet surface, which binds to integrins expressed on other platelets, leukocytes or on

endothelial cells (Mickelson et al., 1996; Schafer et al., 2004a). Binding of integrins is followed by fibrinogen binding between platelets at the site of vascular injury. This then stimulates cross-linking and further platelet aggregation and clot formation. Platelet-leukocyte aggregates have also been shown to enhance the inflammatory response at the site of vascular injury.

In normal arteries activation and subsequent aggregation of platelets is inhibited at least in part by NO which is released both from normal endothelium and platelets (Radomski et al., 1987). However, in the presence of atherosclerosis, not only is the endothelium dysfunctional in the sense that NO release is impaired, but it is also damaged and therefore expresses pro-inflammatory and aggregatory stimuli. To add to this pro-aggregatory state, there is considerable evidence that platelets from patients with coronary artery disease and also from asymptomatic individuals with some coronary risk factors are hypo-responsive to the anti-aggregatory effects of NO. This is superficially paradoxical, as originally vasomotor responsiveness to NO was assumed to be "intact" in patients with coronary artery disease (Ludmer et al., 1986); only recently has vasomotor hypo-responsiveness to NO been shown to be associated with endothelial dysfunction (Adams et al., 1998; Schachinger et al., 1999). Therefore, the combination of the pro-aggregatory environment in atherosclerotic vessels, impaired release of anti-aggregatory NO from dysfunctional endothelium and impaired platelet responsiveness contributes to increased risk of thrombotic events. Indeed, a recent study has demonstrated that impaired responsiveness of platelets to NO is associated with poor outcomes in patients following acute coronary syndromes (Willoughby et al., 2005).

1.2.3.2 Inflammatory cells

The endothelium is an important part of the immune system. Endothelial cells express selectins (particularly P and E-selectins) in response to inflammatory stimuli. These selectins interact with those expressed on leukocytes and platelets thereby stimulating expression of other adhesion molecules, the β_2-integrins CD11/CD18 and cell adhesion molecules (CAMs) such as intracellular adhesion molecule-1 (ICAM-1), which is expressed on endothelial cells and binds to receptors on leukocytes. This binding of receptors stimulates both cell types and stimulates the release of pro-inflammatory cytokines such as interleukins, leukotrienes and tumor necrosis factor (TNF) which further amplifies the inflammatory response and stimulates the recruitment of phagocytes such as macrophages (Granger et al., 2004; Krieglstein and Granger, 2001). Once firm adhesion has occurred, leukocytes are then able to migrate through the endothelium to the sight of the inflammatory stimulus where exocytosis of granules, containing cytotoxins such as the O_2^- radical, hypochloric acid (produced by myeloperoxidase) and hydrogen peroxide, which facilitate microbial killing. While this system is an important part of the immune system, dysfunction of this system has been implicated in the development of atherosclerosis and also in plaque instability (Naruko et al., 2002; van der Wal et al., 1993; van der Wal et al., 1994).

In normal endothelium, this process is tightly regulated. However in patients with coronary risk factors a number of factors contribute to dysregulation of this system. Presence of cardiovascular risk factors is associated with increased oxidative stress, due in part to increased expression of NAD(P)H oxidase (Guzik et al., 2000). Increased

oxidative stress stimulates production of NFκB and AP1 leading to increased expression of selectins, CAMs and CD40, which allows binding of inflammatory cells and platelets. This begins a self perpetuating cycle of inflammation and oxidative stress (Clapp et al., 2004; Granger et al., 2004). Oxidised LDL produced in the presence of oxidative stress accumulates in macrophages and causes conversion to foam cells which accumulate in atherosclerotic plaques, weakening them and increasing risk of plaque rupture. In addition, histological studies have demonstrated an increased number of neutrophils in culprit lesions following myocardial infarction, when compared to non-culprit lesions (Naruko et al., 2002). Increased expression of CD11b/CD18 on neutrophils has been associated with risk factors for atherosclerosis, including: diabetes(Shurtz-Swirski et al., 2001), high insulin (Okouchi et al., 2004) and high glucose (Mohanty et al., 2000), increased homocysteine (Pruefer et al., 1999), hypercholesterolaemia (Stokes et al., 2006), unstable angina (Lindmark et al., 2001), correlated with presence of 2 or more traditional coronary risk factors (Berliner et al., 2000). Moreover, neutrophil activation has also been shown to correlate with presence of endothelial dysfunction (Pruefer et al., 1999).

Infusion of NOS inhibitors in small mesenteric vessels has been shown to produce similar pathophysiological effects of those seen with ischaemia/reperfusion injury, characterised by increases in the number of adherent leukocytes. This can be reversed by administration of L-arginine, but not by the inactive analogue D-arginine (Arndt et al., 1993; Kubes et al., 1991; Kurose et al., 1995). These studies therefore suggest that endothelium derived NO inhibits adhesion of leukocytes and is supported by studies

comparing adhesion of neutrophils to arterial versus venous grafts, which show that fewer neutrophils adhere to segments of internal mammary artery than to saphenous vein, an effect that was reversible by administration of N(ω)-nitro-L-arginine methyl ester (L-NAME) (Chello et al., 1998; Secco et al., 2003).

Several studies have suggested that NO (either from the endothelium or from NO-donors such as nicorandil) inhibits activities of neutrophils (Czapski et al., 2002; Muller et al., 2004; Seth et al., 1994). The mechanism by which these effects of NO occurs is uncertain; however there is some evidence to support the hypothesis that it is at least partly mediated by cGMP. Shear stress responses of neutrophils (pseudopod retraction, and suppression of CD18 expression) have been demonstrated to be dependant on cGMP rise induced either by endothelium derived NO of a NO donor (Fukuda et al., 2000), but other studies (Wanikiat et al., 1997) have demonstrated that cGMP analogues have no effect on adhesion or migration. Studies utilising the cGMP elevating agent YC-1 have demonstrated inhibition of O_2^- release from neutrophils, and conversely, stimulation of neutrophils by cGMP lowering agents (Wang et al., 2002).

1.3 Pathophysiology of endothelium and nitric oxide

Soon after the role of NO as a modulator of vascular tone was described, it became evident that this regulatory system was impaired in some groups of patients. Endothelial dysfunction was first described in atherosclerotic sections of the human coronary blood vessels which exhibited paradoxical vasoconstriction in response to acetylcholine, but intact dilator response to NO donors (Ludmer et al., 1986). This

suggested that endothelial release of NO was impaired in the presence of atherosclerotic lesions. Further studies in both animal models of atherosclerosis (Jayakody et al., 1987) and in human vessels (Forstermann et al., 1988; McLenachan et al., 1991; Nabel et al., 1990; Zeiher et al., 1991) supported this observation. However, it was not clear whether endothelial dysfunction was a product of the pathology of these vessels or whether endothelial function was impaired prior to development of significant lesions and possibly contributed to development of atherosclerosis.

Traditionally, endothelial dysfunction is defined as abnormal responses to endothelium dependant agents in the presence of intact response to exogenous sources of NO. However a number of more recent studies have reported impaired responses to both endothelium derived NO and to NO donors (Adams et al., 1998; Schachinger et al., 1999). These studies of endothelial dysfunction used a single high dose (200-300μg, (Forstermann et al., 1988; Ludmer et al., 1986; McLenachan et al., 1991; Nabel et al., 1990; Zeiher et al., 1991) of endothelium independent vasodilators such as GTN to assess endothelium independent vasodilator responses, and were therefore only able to detect changes in maximal response, rather than a shift in the dose response curve.

More recent studies using lower doses of GTN have demonstrated that in the presence of endothelial dysfunction, responsiveness to NO donors may also be impaired suggesting more generalised vasodilator dysfunction. De novo hyporesponsiveness to NO donors, loosely termed "Nitrate resistance" was first described in patients with severe congestive hear failure, where 25-50% of these patients failed to achieve target reduction in left ventricular filling pressure in response to nitrate therapy irrespective of the dose

(Armstrong et al., 1980; Elkayam et al., 1985; Roth et al., 1987). This phenomenon was thought to be due to high tissue pressure which led to an inability of vessels to dilate (Abrams, 1991). This idea was supported by the observation that diuretic therapy could improve nitrate responsiveness. Subsequent studies have demonstrated NO resistance in the absence of severe CHF thereby suggesting the involvement of other mechanisms. Impaired responsiveness to NO donors has been demonstrated in patients with atherosclerosis (Liao et al., 1991), coronary artery disease (Celermajer et al., 1992; Forstermann et al., 1988; Zhang et al., 2000) and risk factors for coronary artery disease, including hypercholesterolemia (Creager et al., 1990; Duffy et al., 1999; Sorensen et al., 1994), cigarette smoking (Celermajer et al., 1992; Zeiher et al., 1995), and diabetes (Clarkson et al., 1996; McVeigh et al., 1994; Watts et al., 1996; Williams et al., 1996). NO resistance has also be demonstrated in platelets where from patients with coronary artery disease (Chirkov et al., 1999) and type 2 diabetes/obesity (Giugliano et al., 1995). NO resistance in coronary arteries (Schachinger et al., 2000) and in platelets (Willoughby et al., 2005) is associated with poor patient outcomes.

Studies investigating the progression of atherosclerosis in human coronary arteries are impractical due to the invasive nature of vascular function testing; therefore this led to investigation of vasodilator function in peripheral blood vessels (most commonly the brachial artery) and demonstration that patients with impaired coronary artery endothelial function also had widespread vasodilator dysfunction (Teragawa et al., 2005). Studies of brachial artery reactivity meant that endothelial function could be

tested more easily by measuring responses to either infusion of acetylcholine or by altering shear stress.

Peripheral vessel studies demonstrated that responses to both acetylcholine and shear stress are impaired in the presence of coronary artery disease (Celermajer et al., 1992) and that poor vessel responsiveness to acetylcholine in this group is associated with increased incidence of cardiovascular events (Heitzer et al., 2001). Endothelial dysfunction is also associated with coronary risk factors including: hypertension (Panza et al., 1990), hypercholesterolaemia (Celermajer et al., 1992; Creager et al., 1990; Sorensen et al., 1994; Verbeuren et al., 1986; Zeiher et al., 1993), diabetes mellitus (Clarkson et al., 1996; McVeigh et al., 1992; Watts et al., 1996) and cigarette smoking (Celermajer et al., 1992; Zeiher et al., 1995). These studies support the hypothesis that endothelial dysfunction can occur in the absence of atherosclerotic plaques, and it has been suggested that endothelial dysfunction may contribute to the development of atherosclerosis.

1.3.1 Biochemical pathophysiology of impaired NO responsiveness

The mechanism(s) of development of impaired NO responsiveness remain poorly understood. There are three likely mechanisms which lead to reduced bioavailability of NO; impaired formation, increased clearance and down-stream impairment, including the production of endogenous inhibitors and inactivation of signal transduction pathways.

Initially it was thought that endothelial dysfunction was caused by depletion of intracellular stores of L-arginine, the substrate for the eNOS enzyme, leading to "uncoupling" of eNOS. While studies investigating the effect of supplementation of L-arginine have failed to consistently improve responsiveness to endothelium dependant vasodilators (Angdin et al., 2001; Cross et al., 2001; Kawano et al., 2002; Quyyumi, 1998; Sato et al., 2000), another mechanism of uncoupling has been investigated. Tetrahydrobiopterin (BH_4), a metabolite of folate, is an essential cofactor for NO production of NO via eNOS. Supplementation of folate has been beneficial in reversing endothelial dysfunction (Assanelli et al., 2004; Doshi et al., 2003; Doshi et al., 2002; Tiefenbacher et al., 2000; Woo et al., 2002; Woo et al., 1999). However, effects of folate or endothelial dysfunction are further complicated by its' homocystine lowering effects. Homocystine is known to impair endothelial function and many studies suggest that the benefit of folate supplementation reflects homocystine lowering effects rather than BH4 mediated effects (Bennett-Richards et al., 2002; Doshi et al., 2002).

Uncoupling of eNOS has two important consequences. Not only does uncoupled eNOS fail to produce NO, but it is also becomes a source of O_2^- (Heitzer et al., 2000), an oxygen radical which reacts with NO to reduce available NO and produce the toxic peroxynitrite radical (Gryglewski et al., 1986; Kasten et al., 1995; Langenstroer and Pieper, 1992; Wolin et al., 1990). While uncoupling of NOS is one source of O_2^-, there are several other potential sources of O_2^-.

NO resistance in both platelets (Chirkov et al., 1999) and vasculature (Laursen et al., 1997; Rajagopalan et al., 1996) is associated with increased O_2^- release. O_2^- release

from isolated blood vessels (internal mammary artery and saphenous vein) also correlates with the number of cardiovascular risk factors (Guzik et al., 2000). There are a number of potential sources of O_2^- within blood vessels including: NAD(P)H oxidase, xanthine oxidase, electron transport chain, nitric oxide synthase and cyclooxygenases. However increased O_2^- in the presence of coronary risk factors appears to occurs predominantly via NAD(P)H oxidase (Rajagopalan et al., 1996; Wang et al., 1998).

NAD(P)H oxidase is a multi-subunit O_2^- producing enzyme, which was originally described in phagocytes where the enzyme is not assembled until the cell is stimulated, and then upon activation the enzyme is assembled and produces large amounts of O_2^- to facilitate microbial killing. Since then it has been found to be widely expressed in a variety of cell types within the cardiovascular system including those which comprise the vessel wall including endothelial and smooth muscle cells and also in phagocytes, and also in many other tissues outside the cardiovascular system. Phagocytic NAD(P)H oxidase consists of two membrane bound subunits, gp91phox (also known as NOX 2) and p22phox and several cytosolic subunits, including Rac2, p47phox and p67phox, which are translocated to the cell membrane upon stimulation of the enzyme. In other tissues the basic structure of the enzyme appears to be similar to the phagocyte enzyme with the exception of the gp91phox/NOX 2 subunit (Sorescu et al., 2002). Several homologues of this subunit have been identified; however the relative expression of these homologues in different tissues remains highly controversial. It appears that NOX 4 is the predominant homologue expressed in the vasculature (Ago et al., 2004; Paravicini et al., 2002; Sorescu et al., 2002). The vascular enzyme differs from and the phagocytic

enzyme in that it does not require stimulation in order to be assembled, and produces much lower levels of O_2^-. The purpose of this enzyme in the vasculature is not well understood, however it is known that low levels of O_2^- stimulate intracellular signalling pathways such as the TGF-β pathway (Cucoranu et al., 2005; Rocic and Lucchesi, 2005; Sturrock et al., 2006).

The precise mechanism(s) by which NAD(P)H oxidase derived O_2^- release is increased is unknown, however one potential contributor is angiotensin II. Incubation of isolated blood vessels with angiotensin II increases O_2^- release (Berry et al., 2000) and increases expression of NOX-1, NOX-4 (Wingler et al., 2001) and p22Phox (Fukui et al., 1997).

O_2^- may also contribute to impaired responses to NO via direct effects on the enzyme sGC, responsible for initiating down stream signal transduction. Increased levels of O_2^- (Kojda et al., 1998; Mulsch et al., 1997) and increased oxidised low-density lipoprotein (Galle et al., 1992) have been shown to cause desensitisation of sGC. However, some studies have found that sGC expression (Bauersachs et al., 1998) or activity (Watanabe et al., 1998) is reduced independent of O_2^- effects.

Studies using isolated arteries from animal models of hypercholesterolaemia (Cooke et al., 1991; Miller et al., 1998; Verbeuren et al., 1986) and spontaneous hypertension (Bauersachs et al., 1998; Laursen et al., 1997) have demonstrated reduced responsiveness to NO donors and also to an NO-independent stimulator of sGC (Mulsch et al., 1997) suggesting that dysfunction occurs down stream of sGC; however, this has not been confirmed in other studies.

Understanding endothelial dysfunction in the presence of atherosclerosis is not only important for our understanding of pathogenesis of the disease, but also has clinical implications. NO is an important endogenous regulator of vascular tone, therefore when this system is impaired in arteries already narrowed by atherosclerosis the ability of these arteries to dilate and increase blood flow in response to increased demand is impaired. Therefore development of an understanding of the mechanisms of development of endothelial dysfunction and consequently potential strategies for reversal is of utmost importance.

1.4 Methodological considerations in assessment of endothelial and platelet function and inflammation

1.4.1 Techniques for measurement of oxidative stress

The term "oxidative stress" is used broadly to describe states in which levels of endogenously produced oxidants, particularly reactive oxygen species such as O_2^-, are increased, implying an imbalance in oxidant-antioxidant homeostasis. Of particular interest is the fact that oxidative stress can be variably mediated by a considerable number of reactive oxygen species as well as non-oxygen derived free radicals. It is generally accepted that oxygen radicals, particularly O_2^- is a major contributor to oxidative stress. Elevated O_2^- levels have been widely demonstrated in association with hyporesponsiveness to NO (Bauersachs et al., 1998; Ellis et al., 2000; Shinozaki et al., 2000; Sydow and Munzel, 2003). There are many potential sources of O_2^- within the vasculature, including: NAD(P)H oxidase, xanthine oxidase, uncoupled eNOS,

cytochrome p450 and the mitochondrial electron transport chain. All of these enzymes have been found in blood vessels, and are capable of producing O_2^-, however studies utilising inhibitors of these enzyme systems suggest that the most likely source of increased production in the presence of coronary risk factors is NAD(P)H oxidase (Berry et al., 2000).

Measurement of "oxidative stress" is complicated by the lack of easily accessible direct measures. Three different types of measures of oxidative stress can be utilised. Firstly, the ability of potential sources of oxidants to be activated by a stimulus such as measuring the capacity of NAD(P)H oxidase to produce O_2^- upon stimulation, secondly, the depletion of endogenous anti-oxidants such as reduced glutathione (Ceconi et al., 1988; Curello et al., 1987) can be measured and thirdly levels of proteins and lipids modified by oxidants can be measured. For example, assay of malondialdehyde can provide an indication of lipid peroxidation (Arstall et al., 1995; Lapenna and Cuccurullo, 1993).

1.4.2 Techniques for testing of vascular reactivity/endothelial function

Early studies utilised ex vivo exposure of explanted segments of artery or vein from animal models or taken from patients at the time of coronary artery bypass grafting. Responses were measured to agents such as ACh in organ bath or myograph setups (Jayakody et al., 1987; Ludmer et al., 1986). This approach has the advantage of being able to investigate the effects of many single agents in a simplified environment in a

single experiment. However it does mean that many animals are needed to allow measurement of effects of treatments over time and in humans, does not allow for serial sampling or comparison of responses to normal subjects. Most studies of this type have utilised segments of vessels that appear "normal" as controls, however, as mentioned in the previous section, it has been demonstrated that these vessels do not necessarily behave as normal vessels.

Some studies have utilised quantitative angiography in order to measure responses to ACh at the time of angiography. This technique involves injecting endothelium-dependant vasodilators directly into the coronary arteries at the time of angiography and imaging the artery before and after injection. Response is then measured, usually by a blinded observer (McLenachan et al., 1990; Schachinger et al., 2000; Tiefenbacher et al., 2000). This technique is also limited by inability to recruit truly normal subjects for comparison with patient groups. It is also difficult to obtain follow-up data for trials involving drug treatment.

Demonstration that 'endothelial dysfunction' could be observed in large vascular beds other than the coronaries allowed for the development of techniques which utilised peripheral vessels such as the brachial artery (Anderson et al., 1995; Takase et al., 2006; Teragawa et al., 2005), which is more easily accessible.

The most common methods for measurement of endothelial function utilise either a flow meter inserted into the brachial artery or an ultrasound probe placed on either the brachial or radial artery to measure changes in vessel diameter. Response to intra-arterial infusion of endothelium dependant and/or independent vasodilators such as acetylcholine

and GTN respectively are then measured as changes in flow or vessel diameter (Celermajer et al., 1992; Creager et al., 1990; Panza et al., 1990; Watts et al., 1996). A variation of this technique utilises measurement of responses to reactive hyperaemia, known as flow mediated dilatation (FMD). Reactive hyperaemia is induced by inflation of a cuff and subsequent release, stimulating endothelial release of NO due to shear stress under increased flow (Cross et al., 2001; Varin et al., 2000; Zhang et al., 2000). FMD is often preferred as it does not involve infusion of endothelium dependant vasodilators and does not require arterial cannulation. However there is considerable evidence that other mediators are also released in response to hyperaemia and therefore it is necessary to measure responses in the presence and absence of a NOS inhibitor to establish what proportion of the response is due to NO release. Another important limitation of this technique is that ultrasound results are subjective and the size of the signal is very small and therefore subject to inaccuracy.

1.4.3 Arterial Compliance

More recently a technique known as pulse wave analysis has been utilised, for measurement of 'apparent arterial stiffness'. Apparent arterial stiffness measures the overall stiffness of large arteries in vivo, by examining the way in which the pulse wave is modified as it travels through the vascular tree. This is achieved by mathematical transformation of the pressure waveform recorded in the radial artery, to a representation of the ascending aortic waveform. The most commonly used system (Sphygmocor)

utilises a transfer function derived by O'Rourke and colleagues (Cross et al., 2001; Varin et al., 2000; Zhang et al., 2000). There has been some controversy regarding the accuracy and necessity for this transfer function, with some suggesting that measures derived using this system offer no more information than brachial sphygmomanometer readings (Cameron et al., 1998). Both initial studies by the designers of the system and subsequent studies by others, have demonstrated that derived central measurements are more representative of centrally recorded measures than traditionally utilised sphygmomanometer pressures.

As seen in figure 1.2, in a normal healthy volunteer this waveform is characterised by a single round pressure peak, the incident wave, which is caused by the left ventricular contraction, however in the trace from a patient with hypertension there is an initial pressure peak, but this is followed by a second higher peak, created by the reflection of the incident wave from the conductance vessels. The difference between the incident and reflected waves is known as the augmentation index (AIx), the size of this reflected wave is proportional to the stiffness of the conductance vessels.

This reflected wave arrives at the ascending aorta after the closure of the aortic valve and therefore is not represented in peripheral sphygmomanometer blood pressure, but does contribute to the pressure in the ascending aorta and therefore afterload on the left ventricle and to the potential for development of hypertrophy (O'Rourke and Kelly, 1993). Several studies have now demonstrated that measures of arterial stiffness are better predictors of cardiovascular risk than brachial blood pressure alone (Benetos et al., 1997; Franklin et al., 1999; Hirai et al., 1989).

Arterial stiffness measured using this technique is a product of both elasticity of arteries and the balance of vasodilator and constrictor substances in the system at the time. Therefore, drugs that alter vascular tone should affect AIx. At the commencement of the current studies, few studies had examined the effects of vasoactive drugs on AIx. However there have been some studies published subsequently including the use of the β2 agonist, salbutamol, which has been used to stimulate endothelial release of NO for measurement of endothelial function, and GTN which has been used as control in these studies (Wilkinson et al., 2002a; Wilkinson et al., 2002b).

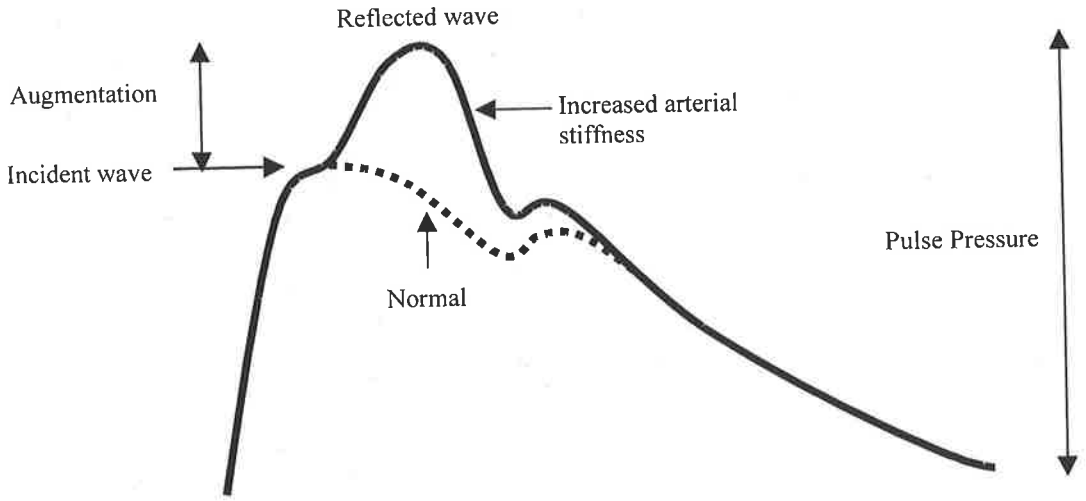


Figure 1.2: Central pulse pressure waveform derived via applanation tonometry

1.4.4 Assessment of platelet function

The central function of platelets is to aggregate in response to appropriate physiological stimuli. The aggregability of platelets in vivo is determined both by levels of endogenous activators and inhibitors of aggregation and also by the ability of the

platelets to respond to these stimuli. Therefore when considering techniques for measurement of platelet function, it is important to measure aggregatory responses to the chosen agonist as well as the ability of anti-aggregatory agents to inhibit this activation.

Aggregation of platelets occurs in stages. Activation results in a change from the typical discoid shape to a more rounded shape with pseudopod projection. Further stimulation results in progressive release of different types of granules beginning with dense granule secretion, followed by secretion of α and finally lysosomal granules; which are only released at very high agonist concentrations. This process is essentially concentration dependant and is also able to amplify itself through release of pro-aggregatory chemokines from granules, however different agonists are able to stimulate with different potencies (Boyd and Davis, 1988; Gewirtz et al., 1985). Thrombin and collagen are considered "strong agonists" as they can induce release of α granules without the need for positive feedback from dense granule release (Koike and Holmsen, 1987). Agonists such as adenosine diphisphate (ADP) and adrenaline cannot give maximal stimulation (even at maximum receptor occupancy) without induction of the positive feedback mechanism. This gives rise to the so-called biphasic response, the first induced by the agonist and the second additive response induced by dense granule release. Once maximal stimulation (the "release reaction") occurs, the reaction is irreversible. This is of particular importance when investigating the effects of anti-aggregatory agents, which are able to modulate aggregability early in the reaction.

There are a multitude of techniques available for assessment of different aspects of platelet function. Early studies were conducted using turbidometric techniques in

platelet rich plasma. This procedure involves separation of platelet rich plasma from whole blood and then uses the change in light transmittance as platelets aggregate to measure aggregation in response to various physiological agonists (Ingerman-Wojenski et al., 1983). This technique can only be performed in platelet rich plasma and therefore the platelets are not in contact with other blood components. Consequently, this technique can only give information about the platelets themselves and not the interaction of platelets with other cell types. Alternatively, impedance aggregometry can be performed in whole blood and therefore is more closely representative in vivo conditions. Impedance aggregometry involves placement of an electrode, consisting of two wires between which an electrical current is passed, into the blood. Upon addition of an agonist, platelets aggregate onto the electrode and increase electrical resistance, this increase can then be measured. Whole blood impedance aggregometry measures response of platelets in the presence of other blood components, such as factors released from white blood cells that may also impact on platelet function in vivo (Steen and Holmsen, 1985). Therefore in the clinical setting it is able to give a more complete picture of effects of disease state and drug treatment on platelets and their environment; however it cannot be used to elucidate the specific cell type that is affected.

More recently the technique of flow cytometry has been utilised to assess platelet and inflammatory cell function. Flow cytometry utilises labelled monoclonal antibodies directed against cell activation markers which are expressed on the surface of activated cells. The labelled antibodies bound to activated cells can be counted and expressed as a percentage of the total cell population. Originally this technique could only be used in

isolated cells and could only measure one marker per sample; however recent refinements of this technique mean that it has become possible to use whole blood and to measure several different markers in each run expanding its utility. Commonly used markers of platelet activation are surface expression of p-selectin and VASP (vasodilator-stimulated phosphoprotein) phosphorylation. P-selectin is a cell adhesion molecule expressed by platelets and inflammatory cells and is involved in binding of platelets to other cell types and to other platelets. P-selectin expression is used as a marker of activation of platelets, and is decreased in response to NO. VASP is a protein that is phosphorylated by both cGMP and cAMP dependant protein kinases (at different sites) therefore phosphorylated VASP is used as a marker of activity of the cGMP pathway and NO bioavailability (Schafer et al., 2003; Schafer et al., 2004b).

1.4.5 Relationship between oxidative stress, endothelial function and platelet function

Oxidative stress, endothelial dysfunction and both platelet hyperaggregability and hyporesponsiveness to NO have all been associated with coronary risk factors and with the presence of coronary artery disease. While the reaction between NO and O_2^- to produce peroxynitrite, is an important physiological mechanism for the degradation of NO and therefore regulation of vascular tone, increased O_2^- associated with coronary risk factors and atherosclerosis can lead to depletion of NO. This depletion of NO may contribute to decreased response to both endogenous and exogenous sources, leading to

endothelial dysfunction and impaired responses to NO donors in vasculature and in platelets and activation of inflammatory cells as seen below in figure 1.3.

Oxidative stress in the presence of endothelial dysfunction has been well established and some studies have demonstrated improvement of endothelial function with antioxidant therapy (Clapp et al., 2004; Ellis et al., 2000; Gruhn et al., 2001; Heitzer et al., 2001; Levine et al., 1996; Wingler et al., 2001). Inflammatory cells contribute to oxidative stress by release of oxygen radicals and it has recently been demonstrated that inflammatory cells may directly impair endothelial function (Sugano et al., 2005). Regulation of inflammatory cell function by NO (particularly from NO donors) remains poorly understood, however some studies have demonstrated NO inhibits parameters of inflammatory cells activation (Chello et al., 1998; Clancy et al., 1992; Dikshit and Sharma, 2002; Lee et al., 2000; Secco et al., 2003). Studies using NOS inhibitors (Fukuda et al., 2000) and comparing adhesion to arterial versus venous grafts (Chello et al., 1998) have demonstrated that endothelium derived NO inhibits adhesion to the endothelium, and therefore we can hypothesise that more adhesion would occur it the presence of endothelial dysfunction. The role of the link between endothelial dysfunction and platelet function is less well established (Dinerman and Mehta, 1990; Forstermann et al., 1989; Serrano et al., 2005); however shared mechanisms and risk factors have been demonstrated.

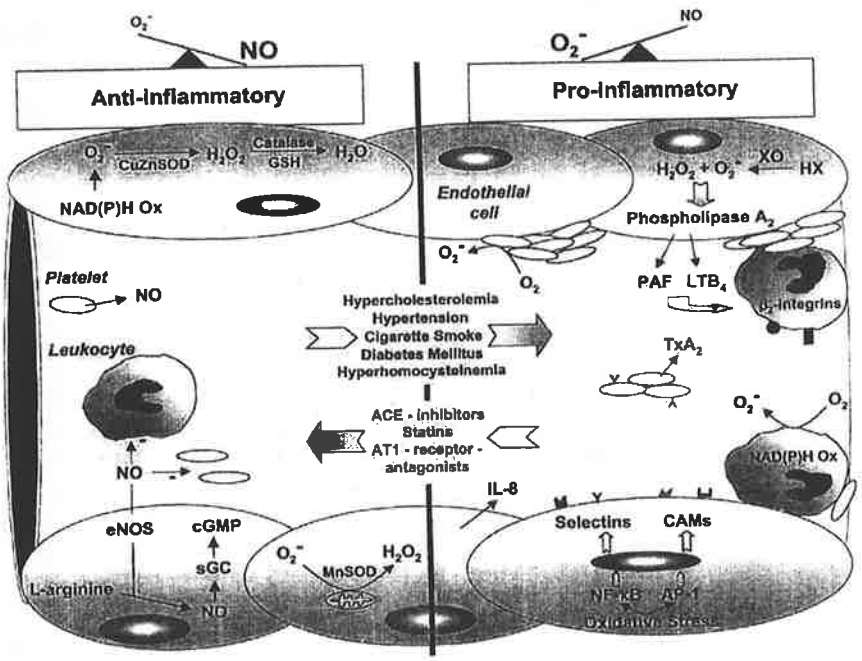


Figure 1.3. Schema of the role of NO/ O_2^- balance in regulating inflammation in endothelial and inflammatory cells from(Granger et al., 2004).

1.5 Therapeutic approaches to endothelial function and nitric oxide resistance: The relevance of oxidative stress

1.5.1 "replacement of NO" – The positives and negatives of NO donors

Nitroglycerine (glyceryl trinitrate; GTN) is an organic nitrate commonly used in the treatment of stable and unstable angina as well as acute coronary syndromes and heart failure. Nitrates are extremely effective in controlling acute angina and improve early (but not late) mortality following infarction.

Organic nitrates were utilised long before their mechanism of action was understood. However, in the late 1970's it was demonstrated nitrates activate sGC (Katsuki et al., 1977). Once EDRF was identified as nitric oxide and thus capable of activating sGC (Murad et al., 1985), it was proposed that nitrates may act by release of NO. This was later supported by studies suggesting NO release from GTN in vascular smooth muscle and endothelial cells (Feelisch and Kelm, 1991). In general, studies have not clearly demonstrated that the radical released from GTN is authentic NO (Kleschyov et al., 2003), however it appears that it is either NO or a closely related molecule.

NO is released in a thiol dependant manner from GTN via enzymatic degradation to 1,2 or 1,3 GDN. The reaction in which 1,2 GDN is produced appears to be associated with the biological effect of GTN and presumably NO release, with levels of this reaction correlating with cGMP production and vasodilatation (Brien et al., 1988).

The enzyme system(s) involved in bioconversion of GTN to produce pharmacologically active NO is still not known. However, there are a number of candidate enzyme systems. One candidate enzyme system is glutathione-S-transferase. Several isoforms of this enzyme are capable of bioconversion of GTN particularly the μ isoform of the enzyme and have been found in vascular smooth muscle (Kenkare et al., 1994; Nigam et al., 1996). However, it is not clear whether NO produced via this enzyme is capable of exerting a pharmacological effect (Kurz et al., 1993; Lau et al., 1992). Studies using inhibitors of glutathione-S-transferase have failed to consistently demonstrate inhibition of GTN induced relaxation (Nigam et al., 1993; Yeates et al., 1989) and GTN response in humans who lack the active isoform of this enzyme have

were normal (Haefeli et al., 1993) providing little support for involvement in production of vasoactive NO.

In 1990 a 160kd enzyme capable of producing NO from GTN was located on the cell membrane of bovine coronary artery smooth muscle cells (Chung and Fung, 1990) The activity of this enzyme was increased by the presence of reduced sulphydryl groups but the enzyme was not glutathione-S-transferase (Chung et al., 1992; Chung and Fung, 1990). These data therefore correspond to those of Ignarro and colleagues (Ignarro et al., 1981) who demonstrated that sGC activation by GTN is sulphydryl dependent. However, the identity of this enzyme remains uncertain and it has not been characterised further.

Bioconversion via the cytochrome P450-NADPH-cytochrome P450 reductase system has been demonstrated in a number of different tissues including rat hepatic microsomes (McDonald and Bennett, 1990; Servent et al., 1989), rat aortic microsomes (Bennett et al., 1992a; McGuire et al., 1998) and rat lung fibroblasts (Schroder, 1992). NO released via this enzyme system has also been shown to stimulate the formation of cGMP (Bennett et al., 1992a). Studies using inhibitors of cytochrome P450 enzymes have produced mixed results, possibly due to the lack of inhibitors for specific enzyme isoforms of the enzyme. The flavoprotein inhibitor diphenyleneiodonium (DPI) inhibits c-DNA expression of rat liver cytochrome P450 reductase and activity of aortic and hepatic microsomal NADPH-cytochrome P450 reductase (McGuire et al., 1998). Some studies using DPI demonstrated reduced vascular relaxation to GTN and c GMP accumulation (McGuire et al., 1994; McGuire et al., 1998; Ratz et al., 2000), but others did not (De la Lande et al., 1996). Therefore, overall this evidence suggests that "NO-

producing: GTN bioconversion" occurs either via the sulphydryl dependent, membrane bound enzyme and/or via cytochrome P450 dependent bioconversion.

A recent paper has suggested that the enzyme aldehyde dehydrogenase (especially mitochondrial aldehyde dehydrogenase-2) may be involved in the bioconversion of GTN to form biologically active NO (Chen et al., 2002b). However, subsequent studies investigating the role of this enzyme system in bioconversion and also in the development of nitrate tolerance (reduced responsiveness following continuous therapy with nitrates) have brought into question the relative contribution of this enzyme system (DiFabio et al., 2003; Mackenzie et al., 2005). Interestingly, GTN is a potent inhibitor of several enzymes which have been proposed as bio-activators, including aldehyde dehydrogenase (Towell et al., 1986) and cytochrome p450 (Bennett et al., 1992b; Schroder, 1992). This finding is of considerable clinical interest.

Utility of nitrates is limited by their loss of effectiveness over time, known as nitrate tolerance. Nitrate tolerance was originally defined as loss of anti-anginal efficacy with long term nitrate therapy (Abrams, 1980; Lee et al., 1978). This led to the introduction of intermittent nitrate therapy, which involved the use of a nitrate free period in order to minimise the extent of nitrate tolerance. However this regimen was often associated with onset of angina during the nitrate free period known as rebound or the "zero hour phenomenon". This phenomenon was attributed to an increase in endogenously produced vasoconstrictors in response to chronic nitrate induced vasodilatation, resulting in "pseudotolerance" (nett vasoconstriction) upon withdrawal of nitrates. In practice it is impossible to distinguish between pseudotolerance and "true"

tolerance in vivo. The only way to measure true tolerance is by examining GTN responses ex vivo; however the relevance of ex vivo vessel studies to in vivo conditions, particularly given the complex nature of the dilator/constrictor relationships is questionable.

There are two potential mechanisms for development of nitrate tolerance. Both increased O_2^- and therefore scavenging of NO and impaired bioconversion have been demonstrated in both humans and in animal models of nitrate tolerance (Forster et al., 1991; Laursen et al., 1996; Sage et al., 2000). The relative contribution of these two mechanisms to development is controversial and difficult to investigate as the specific enzyme(s) responsible have not been fully described. Sage et al (Sage et al., 2000) demonstrated that GTN tolerance induced by 24 hours of GTN infusion in humans essentially reflects impaired GTN biotransformation and is therefore nitrate-specific. Chirkov et al (Chirkov et al., 1997) observed similar changes in platelets.

The clinical utility of nitrates as a replacement for endogenous NO is limited in two important ways: the patients are already hypo-responsive to NO donors (Celermajer et al., 1992; Chirkov et al., 1999; Forstermann et al., 1988; Liao et al., 1991; Zhang et al., 2000) and overcoming this by using higher doses of nitrates increases the risk of both true nitrate tolerance and pseudotolerance. Therefore, given these limitations, are nitrates still clinically relevant? There is little doubt that nitrates are effective in acute management of angina, and the recent AAHeFT study of heart failure in African-Americans (Taylor et al., 2004) demonstrated that in this population, the combination of nitrates and hydralazine is effective in reducing mortality. There is also the potential that in the future

nitrate tolerance will be able to be limited, increasing the therapeutic utility of these agents.

1.5.2 Angiotensin converting enzyme inhibitors

Angiotensin converting enzyme inhibitors (ACEi) are anti-hypertensive agents, which notably but not exclusively inhibit the conversion of angiotensin I to angiotensin II. Angiotensin II in turn is a potent vasoconstrictor, inducer of O_2^- release from blood vessels via induction of NAD(P)H oxidase (Berry et al., 2000; Wingler et al., 2001; Zhang et al., 1999) and pro-aggregatory agent. ACE is also involved in the breakdown of bradykinin, an endothelium dependant vasodilator; therefore ACE inhibitors have the added benefit of increasing vasodilatation by increasing levels of bradykinin. ACE inhibitors (Wiemer et al., 1997) have been consistently shown to inhibit O_2^- release from vessels and are currently the only agents shown to improve both cardiovascular outcomes (Latini et al., 2000; Yusuf et al., 2000b) and endothelial dysfunction (Cashin-Hemphill et al., 1999; Schlaifer et al., 1999). It has been postulated that this improvement in endothelial function may be caused by inhibition of angiotensin II induced O_2^- release via NAD(P)H oxidase (Berry et al., 2000; Wingler et al., 2001).

Few studies have examined the effects of ACE inhibition on responsiveness to NO donors despite their theoretical benefit. Conversely, a study examining potential determinants of platelet NO responsiveness did not show ACE inhibitor therapy to be associated with improved responsiveness to nitrates, although this may be due to the

small number of patients receiving ACE inhibitor therapy (Chirkov et al., 1999). A subsequent study in cardiac failure has shown improved NO responsiveness in patients treated with ACE inhibitors (Chirkov et al., 2002)

1.5.3 Other treatments for endothelial dysfunction

Traditionally endothelial dysfunction is defined as impaired release of NO from the endothelium. Attempts to reverse endothelial dysfunction by supplementation of L-arginine (substrate for eNOS) to prevent uncoupling of eNOS or with antioxidants to decrease levels of O_2^- have had variable results. Endothelial function in a variety of conditions can be transiently improved by either of these treatments (Cooke et al., 1991; Drexler et al., 1991; Heitzer et al., 1996; Keaney et al., 1995; Levine et al., 1996; Ting et al., 1997) however, they do not appear to reduce myocardial ischaemia or cardiovascular risk (Quyyumi, 1998; Stephens et al., 1996; Yusuf et al., 2000a) associated with endothelial dysfunction. Supplementation of tetrahydrobiopterin (with sepiapterin), a co-factor required for NO production via eNOS and a scavenger of O_2^- (Schmidt et al., 1992) also improves endothelial function in animal models (Gruhn et al., 2001; Hong et al., 2001; Shinozaki et al., 2000; Tiefenbacher, 2001; Tiefenbacher et al., 2000), and in some human studies (Heitzer et al., 2000; Tiefenbacher et al., 2000).

HMG-CoA reductase inhibitors or statins are used clinically as cholesterol lowering agents. However large clinical trials investigating the role of statins and cholesterol lowering in modifying cardiovascular risk demonstrated that statins had

beneficial effects that could not be solely attributed to their cholesterol lowering effects. These so-called "pleiotropic effects" include reduction in cardiovascular risk (Byington et al., 1995; Goldberg et al., 1998), reduction in some markers of oxidative stress and reduction in O_2^- release from blood vessels (Wassmann et al., 2002) and possibly improvement in endothelial function(John et al., 2001; John et al., 1998; Tiefenbacher et al., 2004). Multivariate analysis of factors affecting platelet responsiveness to NO in patients with unstable angina (Chirkov et al., 2001) found that statin therapy was a predictor of normalised platelet responsiveness to NO.

1.5.4 Perhexiline: a "Metabolic" anti-ischaemic agent with anti-inflammatory effects

Perhexiline is a prophylactic anti anginal agent that has been shown to cause a decrease in utilisation of long chain fatty acids and an increase in lactate utilisation (Jeffrey et al., 1995) and to inhibit the mitochondrial enzyme CPT-1 in isolated cardiac mitochondria (Kennedy et al., 1996). This enzyme is involved in the transport of long chain fatty acids into the mitochondria. Long chain fatty acid metabolism has been estimated to require 10-15% more oxygen than that of glucose to produce an equivalent amount of ATP (Leidke, 1981) and so may be more efficient during reduced blood flow. A recent study in isolated perfused rat hearts supported this, finding that perhexiline improved diastolic function during ischaemia (Kennedy et al., 2000) however, this has not been investigated in humans. Recently, *in vitro* exposure to perhexiline has been

shown to exhibit O_2^- release (via NAD(P)H oxidase) from isolated neutrophils (Willoughby et al., 2002).

Currently perhexiline is the only agent definitely shown to affect NO resistance. Platelet studies have shown that treatment of patients with both stable and unstable angina with perhexiline improves nitrate responsiveness in platelets. However the effect of perhexiline on vascular responses to NO has not been tested.

Perhexiline was first utilised as an anti-anginal agent in the early 1970's when doses of 200-400mg/day were shown to increase exercise tolerance and decrease anginal frequency in stable angina (Dernier and Colen, 1977; Hitchcock et al., 1977; Masoni et al., 1975; Morledge, 1973; Pepne et al., 1974; Souza, 1973; Teo et al., 1983) and when added to optimal other therapies in refractory angina (Cole et al., 1990; Horowitz and Mashford, 1979). These doses however, lead to toxicity in some patients and in 1981 it was reported that perhexiline is subject to saturable Mickaelis-Menton hepatic metabolism (Horowitz et al., 1981) via the cytochrome P450 2D6 enzyme system (Shah et al., 1982). Genetic polymorphism within this enzyme system, particularly within caucasian populations, determines the rate at which perhexiline is metabolised (hydroxylated), resulting in slow hydroxylators achieving toxic levels and rapid hydroxylators not reaching or maintaining adequate levels (Cooper et al., 1984). This necessitated the definition of the therapeutic range (Horowitz et al., 1986) of 0.15-0.6mg/L in plasma (tissue levels are higher due to the lipophilic structure (Amoah et al., 1986; Cooper et al., 1987) and a titration protocol involving rapid loading over three days at which time levels are measured and doses adjusted.

Perhexiline was originally developed as a coronary vasodilator (Hudak et al., 1970) and has been shown to dilate coronary vessels in some animal models at high concentrations; however this effect has not been demonstrated in humans. Perhexiline does have some L-type Ca2+ channel antagonist effects (Fleckenstein-Grun et al., 1978) but only at concentrations (EC_{50}: $8.3x10^{7}M$) greater than those utilised clinically. It is significantly less potent than other Ca2+ antagonists including verapamil, nifedipine and diltiazem (Barry et al., 1985) and has been shown to have no vasodilator or negative ionotropic effects at therapeutically relevant concentrations (Vaughan-williams, 1980). This observation has been supported by the observation that perhexiline is more clinically efficacious than other Ca2+ antagonists (Cole et al., 1990; Horowitz and Mashford, 1979).

Vaughan-Williams et al (Vaughan Williams, 1980) were the first to observe that perhexiline increased tissue phospholipids concentration, suggesting metabolic involvement in the mechanism of action. In theory this postulated mechanism appeared sound. As reviewed by Grynberg and Demaison (1996), in the heart, fatty acids oxidation accounts for approximately 60-100% of oxygen utilisation, with 0-20% of oxygen utilisation being accounted for by glucose/lactate metabolism. As glucose/lactate metabolism requires less oxygen to produce equivalent amounts of ATP (1 glucose molecule requires 12 oxygen molecules to produce 38 ATP, while 1 palmitate molecule requires 46 oxygen molecules to produce 130 ATP), then a theoretical improvement of 15-20% could be expected by a shift in metabolism. This mechanism of action for perhexiline was supported by work in isolated perfused rat heart,

demonstrating that perhexiline decreased palmitate utilisation and increased lactate utilisation and that this shift resulted in an increase in cardiac output with no change in oxygen utilisation (Jeffrey et al., 1995). Subsequent studies in hepatocytes and cardiomyocytes demonstrated that perhexiline alters substrate utilisation by direct inhibition of β-oxidation (Deschamps et al., 1994; Unger et al., 2005), via inhibition carnitine palmitoyl transferase–1 (CPT-1), which is responsible for uptake of fatty acids into mitochondria and therefore the rate limiting step in fatty acid oxidation in mitochondria (Kennedy et al., 1996).

1.6 Scope of the present study

The current studies seek firstly to examine the utility of pulse wave analysis for measurement of responses to GTN administered both orally and intravenously, in patients with coronary artery disease and in controls. The second part of the work seeks to investigate factors which may affect the efficacy of nitrate drugs. Oxidative stress is known to play an important role in the pathogenesis of coronary artery disease and has been postulated to be involved in impaired responsiveness to NO. These studies examined the effects of two drug treatments which may influence both NO responsiveness and oxidative stress on levels of oxidative stress markers and on NO responsiveness in various tissues. They also investigated whether levels of oxidative stress markers were elevated in patients with symptomatic coronary artery disease and in controls.

Chapter 2: Materials and Methods

2.1 Patient inclusion criteria

Three main categories of subjects were used in these studies: including: normal volunteers, patients with stable angina and unstable angina/non-Q wave myocardial infarction and patients who would have been eligible for the HOPE trial (Yusuf et al., 2000b).

All experimental protocols involving patients and normal subjects were approved by the Ethics of Human Research Committee of the Queen Elizabeth Hospital and informed consent was obtained prior to study entry in all subjects.

2.1.1 Normal subjects

These subjects had no known cardiovascular risk factors (except family history) and were not taking any regular medications. For some studies specific age groups were selected.

2.1.2 Stable angina patients

These patients were defined as having Canadian class II to III angina. Patients included in the studies described in this thesis were awaiting elective coronary angiography for investigation of chest pain. Stable angina patients receiving long-term prophylactic nitrate therapy were excluded.

2.1.3 Unstable angina/non-Q-wave myocardial infarction patients

Patients had a history of prolonged spontaneous chest pain with or without ST segment changes on ECG within the 24 hours prior to blood sampling. Criteria for diagnosis of non-Q-wave myocardial infarction were those of the European Society for Cardiology (Collinson et al., 2003).

2.1.4 HOPE type patients

Eligible patients were men and women aged ≥ 50 years who have a history of symptomatic coronary artery disease, stroke, peripheral vascular disease, or diabetes plus one other risk factor (hypertension, elevated total cholesterol level, low high density lipoprotein cholesterol levels, cigarette smoking or documented mircoalbuminuria). **Exclusion criteria:** Patients were excluded if they had heart failure, were receiving treatment including ACE inhibitor, angiotensin receptor antagonist or ADP receptor antagonist (as the major endpoint for the larger study involved platelet aggregation to ADP), had uncontrolled hypertension or overt renal dysfunction, had had a myocardial infarction or stroke within four weeks of study entry or had less than 4 ohms of ADP (1μM)-induced platelet aggregation.

2.2 Blood collection

All blood was collected after patients and volunteers had been rested quietly in a supine position for at least 20 minutes. Blood was then collected via venesection from an antecubital vein using a winged infusion set (to allow multiple syringes to be collected) or needles no smaller than 21 gauge. Blood was collected into plastic syringes, using minimal pressure in order to minimize activation of platelets and white blood cells. Blood was then transferred into tubes containing the appropriate anti-coagulant (either Na-EDTA or acid citrate as described in the protocols below) by gently expelling blood down the side of the tubes. Blood and anti-coagulant were then mixed by repeated gentle inversion.

2.3 Neutrophil superoxide release

2.3.1 Isolation of neutrophils

Whole blood was combined with Na-EDTA (4.5%, pH 7.4) in a ratio of 1 mL of anti-coagulant to 5mL of blood. Blood was then centrifuged at 150g for 10 minutes to separate platelet rich plasma from the red blood cell layer (RBC) and buffy coat. Plasma was aspirated and retained. RBC layer (including buffy coat) was then diluted with Hanks' balanced salt solution (HBSS, pH 7.38) to make up to the original blood volume and the solution gently mixed. Approximately 4 mL of Lymphoprep density gradient was then under laid beneath the RBC layer and different cells were separated by centrifugation (550g for 30 minutes). The upper layers (see figure 2.1) containing

remaining plasma, lymphocytes and monocytes were then removed, being careful not to disturb the buffy coat, which sits on top of the RBC layer and contains the neutrophils.

Neutrophils were then separated by lysis of RBC utilising salt lysis, with a buffer containing: 155mM NH_4Cl, 100μM Na_2EDTA, 10mM $NaHCO_3$. Following addition of lysis buffer tubes were repeatedly gently inverted until red blood cells were lysed, which is recognized by change of colour and appearance of cell suspension from opaque red to shiny, very dark red/black). The neutrophils were then pelleted by centrifugation at 500g for 10 minutes, and this step was repeated to ensure all RBC are removed. During the second lysis the colour of the cell suspension does not change so tubes were inverted for

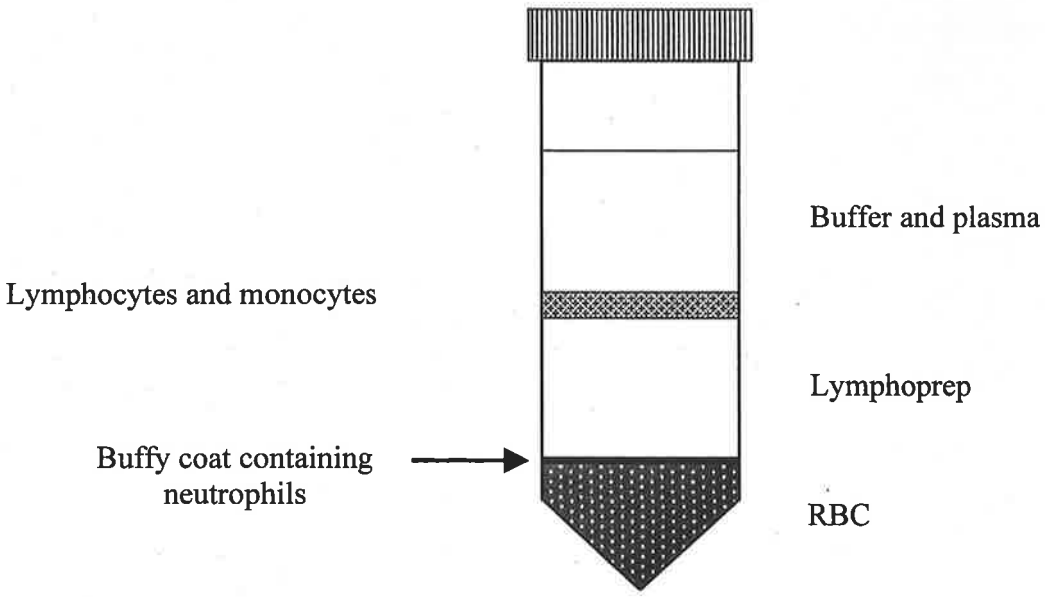


Figure 2.1: Cell layers as seen at the end of centrifugation across Lymphoprep density gradient.

approximately 2 minutes prior to centrifugation at 500g for 10 minutes to pellet the neutrophils. Neutrophils were then washed in HBSS to remove remaining lysis buffer by removing lysis buffer and then re-suspending cells in HBSS followed by pelleting of neutrophils. Neutrophil were then resuspended in 500uL of HBSS and cells counted using a counting chamber (Neubauer improved). Neutrophils were then diluted in the subjects' own platelet-free plasma (prepared by centrifugation of plasma from the first spin at 550g for 10 minutes to remove platelets) at a concentration of $1.7x10^6$ cells/mL.

2.3.2 Measurement of superoxide release

Superoxide release from neutrophils was measured using lucigenin (bis-N-methylacridinium nitrate, 10μM final) a chemiluminescent probe, which reacts with released superoxide to produce a luminescent product. Intensity of luminescence produced was measured using a Packard luminometer (6100 Pico-lite luminometer, Packard, Illinois). 294uL of cells and 3uL of 1mM lucigenin (to give final concentration of 10μM) were added to glass tubes and pre-warmed and dark adapted in the luminometer at 37°C for 15 minutes. Basal levels of superoxide were than measured for 1 minute at 20 second intervals using a Packard luminometer. Cells were then stimulated with the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP, range of concentrations from 0.125μM to 1μM) and chemiluminescence was then measured in arbitrary units at 20 second intervals for 5 minutes. Results were expressed as area under the curve x 10^6, calculated using GraphPad Prism Version 4. Reproducibility of the assay

was assessed by repeat sampling in 17 patients, 3 days apart, the mean change was -0.92±0.5 x10^6 units, a co-efficient of variation17.3%.

2.4 Platelet aggregation studies

2.4.1 Blood preparation

Platelet aggregation was measured using impedance aggregometry in whole blood (Gewirtz et al., 1985). Venous blood (9mL) was combined with acid citrate anticoagulant buffer (9mL of blood combined with 1mL of 2 parts 0.1M citric acid to 3 parts 0.1M trisodium citrate) and blood left to rest for 10 minutes prior to assay. Blood was then diluted 2 fold with 0.9% saline into plastic cuvettes to a final volume of 1 mL and diluted blood was pre-warmed at 37°C in a heating block for 7 minutes prior to assay.

2.4.2 Measurement of aggregation

Cuvettes containing pre-warmed, diluted blood were placed into a dual channel impedance aggregometer (Model 560, ChronoLog) and electrodes consisting of two wires between which electric current is passed were placed into the cuvettes. Samples were stirred using siliconised stir bars at 800 rpm. The nitric oxide donor, sodium nitroprusside (SNP 10μM, made up in 0.9% saline) or saline control were added to samples 1 minute prior to induction of aggregation. Aggregation was induced using

adenosine 5'-diphosphate (ADP, 1μM) and monitored by measurement of increases in electrical impedance caused by platelets aggregating between the two wires of the electrodes using a dual channel impedance aggregometer attached to a data acquisition system (Aggrolink version 4.71 model 810CA, Pennsylvania). Maximal aggregation within 7 minutes of induction was measured in ohms. For determination of inhibition of aggregation percentage inhibition by SNP was established by expressing the difference between maximal aggregation in the presence and absence of NO donor as a percentage of maximal aggregation.

2.5 Determination of Malondialdehyde

The MDA assay utilised in this study was the modified TBARS method previously developed by (Arstall et al., 1995) to minimise the potential for non-specific results. EDTA was used as the anti-coagulant, since as chelating agent it is able to limit auto oxidation. Two pre-treatment steps, aimed at minimizing the levels of potential sources of interference were included. The purity of TBA-MDA adduct produced by this modified protocol has been assessed using HPLC (as described in the PhD thesis of MA Arstall, Studies in myocardial ischaemia and infarction: effects of N-acetylcysteine on oxidative stress and myocardial salvage, submitted at the University of Adelaide, 1995).

2.5.1 Blood collection and storage

Blood was collected into 10mL polypropylene tubes containing 400μL of Na-EDTA (4.5% Na-EDTA, pH 7.4) and cooled on ice until centrifugation at 2800g for 10 minutes. Plasma was then aspirated into 1.5mL polypropylene tubes and stored at -80°C until assay.

2.5.2 Standard curve and quality control sample preparation

Blood was collected as above from several normal volunteers. Plasma was pooled and divided for preparation of quality controls and the remainder divided into 780μL aliquots and stored at -80°C until use. MDA stock (10μM) was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane by stirring in 0.15μM orthophosphoric acid for 1 hour. High (0.5μM) and low (0.1μM) quality controls were then made up and stored in 800uL aliquots at -80°C until assay.

2.5.3 Plasma extraction

Plasma samples, standards and quality controls were extracted to remove potential sources of products other than MDA that may react with thiobarbituric acid (TBA). Plasma samples, standards and quality controls were thawed at 4°C, centrifuged at 8000g to remove cryoprecipitate (not necessary for standards and quality controls as they had already been freeze thawed). An aliquot (800μL) was then taken from the supernatant for

assay. Following the removal of cryoprecipitate, samples, standards and quality controls were treated in an identical manner.

Acid precipitation was used to remove plasma proteins from the sample. Perchloric acid (200μL of 2.3M) was added and tubes mixed using a vortex mixer. Perchloric acid reacts with plasma protein to form an insoluble precipitate which was removed by centrifugation at 8000g for 5 minutes, followed by aspiration of supernatant. Supernatant was then combined with chloroform (500μL), which was used to extract lipids from plasma sample. Extracted plasma was then used for the TBA reaction as described below.

2.5.4 Thiobarbituric acid reaction

A 42mM solution of TBA was prepared and dissolved by sonication. Extracted plasma was divided into three 200μL aliquots and added to 10 mL polypropylene tubes containing 750μL of 0.15μM orthophosphoric acid, 250μL of TBA and 300μL of milli-Q water. The tubes were then boiled (with perforated caps) for 1 hour, after which tubes were cooled on ice. Once cool, 3.5mL of 70% chloroform/30% methanol was added to remove unbound TBA and the upper, non-chloroform layer transferred to disposable cuvettes for measurement of fluorescence intensity (excitation λ: 530nm, slit width 5, emission λ: 547nm, slit width 10, integration time: 5sec) using a Perkin Elmer LS 50 B luminescence spectrometer.

2.5.5 Calculation of MDA concentration

A standard curve was constructed between 0 and 1μM in normal plasma. Zero concentration was determined utilising a sample of the same plasma as the curve, spiked with water only. This reading was subtracted from all points in the standard curve to account for endogenous MDA. Background fluorescence from unbound TBA was measured utilising an aqueous blank, which was subtracted from all patient samples. MDA concentrations were then determined using adjusted fluorescence values from the linear regression line. Inter-assay variability was determined by examining the variation in MDA concentration calculated from QC's, the inter-assay CV was under 10% for both the high and low QC (9.8% and 8.2% respectively)

2.6 Pulse wave analysis via applanation tonometry

Pulse wave analysis is a non-invasive method for determination of arterial stiffness. The technique involves recording peripheral (in this work all readings were taken at the radial artery) arterial wave forms using a micromanometer tipped probe (where from). The probe is placed over the point at which the radial pulse can be felt on the dominant arm and gentle pressure is applied in order to flatten the artery against the bone (known as applanation tonometry) allowing the probe to record the pressure. Pressure waveforms are then recorded using a computer and software package (Sphygmocor Version 7, Atcor medical, Sydney). The software then takes an average of the waveforms recorded and calibrated using brachial artery blood pressure (recorded by sphygmomanometer). A validated mathematical transfer function (Karamanoglu et al., 1993; Segers et al., 2001)

is then used to derive a representation of the central waveform. From the derived wave form augmentation index is calculated by measuring the difference between the first and second systolic peaks, known as the augmentation pressure, which is then expressed as a percentage of total pulse pressure. At least three recordings were made at each time point. The reproducibility of the technique was tested by calculating the change in AIx over three days in patients with no changes in their medication, the mean change was 1.5±1.4 units, a 7% change with respect to baseline.

2.7 Materials

2.7.1 Drugs

Glyceryl trinitrate (GTN) for infusion was obtained from Mayne Pharma (Victoria, Australia). Vials contained 50mg of GTN/10mL (3 mL ethanol, 3mL propylene glycol and remainder water). Lower doses of GTN given sublingually during dose response experiments were made up from vials, and were then diluted in sterile distilled water. Dilutions were prepared immediately prior to use and stored on ice.

Perhexiline and glyceryl trinitrate sub-lingual tablets were manufactured by Sigma Pharmaceuticals (Melbourne, Australia) and ramipril by Aventis Pharmaceuticals (Macquarie Park, Australia).

2.7.2 Chemicals

2.7.2.1 Anticoagulants

Ethylenediamine tetra-acetic acid (disodium salt, Na-EDTA) was obtained from BDH chemicals and made up to 4.5% in glass distilled water and the pH adjusted to 7.4. Solution was then stored at 4°C until use.

Citrate anticoagulant was made up of two parts 0.1mM citric acid to three parts 0.1mM tri sodium citrate. Solution was stored at 4°C until use.

2.7.2.2 Neutrophil superoxide release

Isolation of neutrophils

Hanks' balanced salt solution was purchased from Gibco (NY, USA) as a 10 times solution which was then made up according to manufacturers instruction in glass distilled water and sodium bicarbonate (0.35g/L) added. The pH of the solution was then adjusted to 7.38. Once made up the solution was only used for two days and the pH was readjusted before use on the second day.

Lymphoprep: a sodium diatrizoate/polysucrose containing density gradient (density:1.007, osmolality: 290), was obtained from Axis-Shield, Oslo, Norway and was used without dilution. Unused portion was stored at 4°C until use.

Lysis buffer was made up of 155mM NH_4Cl, 100μM Na_2EDTA, 10mM $NaHCO_3$ all chemicals were purchased from BDH chemicals. Buffer was made up using glass distilled water and was only used for two days once made up.

Measurement of superoxide release

N-formyl-methionyl-leucyl-phenyl alanine (fMLP) was purchased from Sigma Chemical Company (St Louis, Mo, USA), and made up to 10mM in ethanol and stored at -20°C. Further dilutions were then made immediately prior to use in HBSS and stored on ice.

bis-N-methylacridinium nitrate (lucigenin) was purchased from Sigma Chemical Company (St Louis, Mo, USA) and made up in HBSS on the day of experiment.

2.7.2.3 Platelet aggregation

Adenosine 5'-diphosphate were obtained from Sigma Chemical Company (St Louis, Mo, USA) and was made up in 0.9% saline. Aliquots were then stored at -20°C and thawed immediately before use.

Sodium nitroprusside (sodium nitroferricyanide) from Sigma Chemical Company (St Louis, Mo, USA) and was made up in 0.9% saline on the day of each experiment. 0.9% saline from Baxter (Toongabbie, Australia).

2.7.2.4 Malondialdehyde

1,1,3,3-Tetraethoxypropane (TEP) was purchased from Sigma Chemical Company (St Louis, Mo, USA) and was used as the MDA standard. The standard was prepared by acid hydrolysis in 0.15M phosphoric acid for one hour (with stirring). Standard was made up on the day of use and then further diluted in milli-Q water immediately before use.

Orthophosphoric acid (85% pure stock) was obtained from BDH. It was made up to 0.15M in Milli-Q water and stored at room temperature.

Chloroform was obtained from Asia Pacific Specialty chemicals, NSW, Australia. Perchloric acid (60% pure stock) was obtained from BDH and made to 2.3M in milli-Q water and stored at room temperature until use.

2-Thiobarbituric acid was obtained from Sigma Chemical Company (St Louis, Mo, USA) and made up to 42mM in Milli-Q water. The suspension was dissolved using a sonicating bath, for no longer than 3 minutes.

Chapter 3: Effects of glyceryl trinitrate on apparent arterial stiffness in normal subjects and patients with angina: Transition from intravenous to oral therapy

3.1 Summary

The rate of reflection of the peripheral pulse wave is affected by compliance of small arteries (Yaginuma et al., 1986). Thus measures of arterial wave reflection may be utilised as surrogate estimates of "arterial stiffness", a parameter which may result not only from fixed structural changes but also from increased arterial tone. Peripheral arterial pressure waveforms have been studied for many years, however the ability to transform these waveforms mathematically to investigate central waveforms non-invasively is relatively new. Pulse wave analysis in its current form has been utilised to compare arterial stiffness in different patient groups, and a technique has been developed for measurement of endothelial function (Hayward et al., 2002; Wilkinson et al., 2002a). However the effects of endothelium-independent vasodilators such as GTN on this parameter have not been extensively studied. These studies aim to examine effects of GTN on augmentation index in normal subjects and in patients with stable angina pectoris (SAP) and to examine the impact of transition from intravenous GTN to oral ISDN as a means for circumvention of "rebound". We found that in normal subjects, single doses of GTN as low as 12μg induced substantial falls in AIx and that infusion of 5μg/min GTN in patients with SAP induced a 20±7% fall in AIx. The magnitude of vasodilator effect of low infusion rates of GTN is approximately equivalent to that of oral isosorbide dinitrate (ISDN, 10mg). Thus transition from low dose GTN infusion to oral ISDN in patients with ACS is not associated with significant "rebound" vasoconstriction as measured by changes in AIx.

3.2 Introduction

GTN infusion is often used as a component of treatment for the management of acute coronary syndromes such as unstable angina pectoris (UAP) or acute myocardial infarction (AMI). There is considerable evidence that intravenous infusion of GTN is associated with amelioration of ischaemic pain (Mikolich et al., 1980; Theroux et al., 2000).

Traditionally, nitrates were thought to resolve ischaemic pain mainly via dilation of veins and resultant reduction in pre-load with some selective dilation of large coronary arteries (Elkayam and Aronow, 1982). Some studies demonstrated that nitrates also dilated arterioles at high doses (LeWinter and Sobel, 1996; Parker and Parker, 1998). These conclusions were drawn primarily from studies demonstrating minimal changes in brachial or radial artery blood pressure. Studies utilising pressure measurements in the ascending aorta demonstrated that GTN had little effect on the magnitude of the first peak of the pressure waveform, as would be expected if venodilation and pre-load reduction was the primary mechanism, but showed a marked effect on the second systolic peak of the waveform, which represents a reduction in early wave reflection from conduit arteries (Kelly et al., 1990). More recent studies have also demonstrated measurable responses to GTN on wave reflection and hence on conduit arteries measured using augmentation index as a non-invasive parameter of pulse wave reflection (Jiang et al., 2002; Kelly et al., 2001; Pauca et al., 2005). An important limitation of these studies is that they utilise only a single large dose of GTN, for example, the early work of Kelly and colleagues (Kelly et al., 1990) utilised 300 and 600μg GTN doses. While most recent studies have

used doses lower than 600μg (typically 250-300μg), no study has investigated the dose response relationship for sublingual GTN.

In general responses to GTN observed in these studies are relatively large, as summarised in table 3.1. Thus it is clear that these results are not indicative of threshold GTN effects and are poor indications of GTN responsiveness. The only dose-ranging response relationship study utilised 2.5 and 15mg GTN patches (which deliver between 100 and 600μg/hr) and individuals with various cardiovascular conditions (Jiang et al., 2002). While the difference in delivery mode does not allow accurate extrapolation of these results to sub-lingual administration, this study demonstrates that even the lowest dose induces significant changes in augmentation index. This suggested that studies utilising single "high" doses may be unable to detect changes in sensitivity to GTN, which may have contributed to failure of some studies of endothelial dysfunction (Forstermann et al., 1988; Ludmer et al., 1986; McLenachan et al., 1991; Nabel et al., 1990; Zeiher et al., 1991) to detect significant hyporesponsiveness to GTN, irrespective of the methodology used for measurement of GTN response.

The question of the size of vessels dilated by GTN and the size of the vessels reflected by augmentation index measurement is of particular importance when considering responsiveness to GTN. Some studies have demonstrated that different vessels have differential susceptibility to the development of nitrate tolerance (Gori et al., 2004; Gori et al., 2003). If that is the case, this technique may have limited utility for detection of tolerance and/or pseudotolerance as regards "overall" vascular responsiveness to GTN.

There are many limitations to the utility of organic nitrates. Infusion rates of 10μg/min or greater of GTN for more than 24hrs have been shown to induce some degree of tolerance to the haemodynamic effects of GTN (Jugdutt and Warnica, 1988; Sage et al., 2000). In order to minimise the development of tolerance, it has been recommended that infusion rates of GTN be kept as low as possible in both UAP and AMI (Horowitz, 1992), especially as the tolerance-limiting option of intermittent nitrate administration is unsuitable for management of patients immediately after presentation with acute coronary syndromes (Thadani, 1997). The majority of patients in recent clinical studies have been treated with GTN infusion rates of approximately 5μg/min (Arstall et al., 1995; Beltrame et al., 2002; Horowitz and Henry, 1987).

Prolonged administration of organic nitrates may also induce pseudotolerance, defined as attenuation of response without diminution of underlying biochemical effect of released nitric oxide. Pseudotolerance appears to be mediated by both increased secretion of a variety of constrictor neurohormones (Pepine et al., 1997) and increased vascular responsiveness to vasoconstrictors (Hebert and Lam, 2000). The clinical manifestations of pseudotolerance during nitrate administration are confined to loss of therapeutic effect and are therefore indistinguishable from those of true tolerance. However, where nitrate therapy is interrupted, this leaves unopposed reactive vasoconstriction and pseudotolerance may lead to paradoxical worsening of ischaemia. This was first documented in the munitions industry in association with sudden cessation of exposure to large doses of GTN (Ben-David, 1989; Lund et al., 1968). "Rebound phenomena" have been documented after a sudden cessation of GTN infusion (Figueras

et al., 1991) and in patients receiving intermittent nitrate therapy who were found to be more prone to angina during "nitrate-free" periods (DeMots and Glasser, 1989; Ferratini et al., 1989). The same phenomenon also induced increases in frequency of asymptomatic ischaemia during nitrate-free periods (Freedman et al., 1995) and decreases in anginal threshold just prior to GTN patch re-application: termed the "zero hour phenomenon" (DeMots and Glasser, 1989).

The risk of rebound phenomena has led most clinicians to recommend transition from intravenous GTN to long-acting nitrate therapy after initial stabilisation of patients presenting with ACS. However, to date only one study (Lin and Flaherty, 1985) has evaluated the efficacy of such a transition in preventing clinically evident recurrence of ischaemia. In contrast, Figueras et al (1991) demonstrated that abrupt cessation of GTN infusion in a group of five patients with unstable angina pectoris, without immediate transition to cutaneous or oral long acting nitrate therapy was associated with immediate precipitation of ischaemia in a high proportion of patients.

The major objectives of the current studies were:

1) To examine the dose-response characteristics of GTN on augmentation index (AIx) in normal subjects (ie. those not expected to exhibit hyporesponsiveness to NO or NO donors)
2) To test the hypothesis that low infusion rates of GTN in common clinical use, induce significant falls in apparent arterial stiffness in patients with stable angina pectoris.

3) To examine the impact of transition from low dose intravenous GTN to oral ISDN as a means for circumvention of "rebound" measured by change in AIx.

3.3 Methods

3.3.1 Measurement of augmentation index (AIx)

Applanation tonometry was conducted as described in chapter 2.6. Apparent arterial stiffness was measured serially utilising applanation tonometry. Pulse pressure waveforms were recorded from the radial artery of the dominant arm and the corresponding central waves were derived via a validated transfer function (Karamanoglu et al., 1993; Segers et al., 2001). Augmentation index (AIx) was then recorded, with data corrected for heart rate variability. At least three recordings were made at each time point and brachial artery blood pressure was also recorded at each time point using the opposite arm to allow for calibration of the Sphygmocor software.

3.3.2 Experimental protocol

3.3.2.1 Measurement of vascular responses to GTN in normal subjects

A pilot study was conducted using 300μg (1/2 of a standard 'anginine' tablet). This dose induced substantial falls in AIx and moderate reductions in brachial systolic blood pressure (see table 3.2), and also induced severe headaches and symptomatic

hypotension in some subjects. The large change in AIx observed was consistent with the hypothesis that near-maximal responses were elicited.

Dose response curves were therefore constructed in 5 further normal subjects. Doses of GTN from 12μg to 150μg were administered (as 50μL of solution squirted under the tongue and held for at least 30 seconds without swallowing) in random order with both the volunteer and the person performing applanation tonometry blinded as to specific doses. Readings were taken at baseline, 1 minute, 2 minutes, 3 minutes, 5 minutes and 10 minutes. Due to differences in the time course of response between individuals (see figure 3.1), overall responses were measured using area under δ AIx response/time curve. A period of 20 minutes was allowed between doses to minimise the possibility of residual drug effect at the time of subsequent doses.

3.3.2.2 Assessment of responses to GTN infusion in patients with stable angina pectoris

Initial studies were performed to determine that infusion of GTN at normal clinical rates in the absence of tolerance/pseudotolerance induced reductions in AIx and to determine the magnitude of this effect. Patients with stable angina pectoris (SAP) undergoing diagnostic cardiac catheterisation plus/minus coronary angioplasty were evaluated in order to determine the magnitude of the blood pressure and AIx change induced by infusion of GTN (5μg/min). AIx was determined approximately 1 hour prior to initiation of GTN infusion and repeated after 2 hours of GTN administration

(5μg/min). Criteria for patient selection were stable sinus rhythm and no prophylactic nitrate therapy. GTN was infused intravenously utilising polyethylene non-adsorbant tubing.

3.3.2.4 Evaluation of effect of transition from intravenous GTN to oral isosorbide dinitrate

Patients with acute coronary syndromes (unstable angina or non-Q-wave acute myocardial infarction; ACS) in whom no further chest pain had occurred for at least 12 hours were evaluated during transition from GTN infusion (24-48 hours at 2.5-5μg/min mean 3.6μg/min) to oral isosorbide dinitrate (initial dose 10mg twice daily). No patient had received co-infusion of N-acetylcysteine, which potentiates GTN effect (Horowitz et al., 1983; Winniford et al., 1986). While it would have been ideal to establish the magnitude of acute haemodynamic response to GTN in the ACS cohort, this was in practice impossible due to sublingual GTN administration at presentation.

AIx was measured 30-60 minutes prior to cessation of GTN infusion, and repeated 2-4 hours post administration of first 10mg dose of oral ISDN.

3.3.3 Statistical methods

For normal subjects individual dose response curves for each sublingual GTN dose were constructed for each subject and the EC_{50} and maximal response determined

using Prism Graphpad software. Characteristics of the two patient groups studied were compared utilising non-paired t-tests for continuous data and Fisher's exact test for categorical data. Magnitude of changes in AIx was analysed utilising paired t-tests. Results are expressed as mean ± SD.

3.4 Results

3.4.1 Subject/patient characteristics

Normal subjects (n=5) were recruited on the basis that they were not taking any regular medications and had no known cardiac risk factors. The mean age of this group was 28±3 years and there were 2 males and 3 females. Patient characteristics for the SAP and ACS groups are shown in table 3.3. As would be expected, only the ACS group included patients treated with heparin: 44% of this group had NQAMI. All ACS patients were receiving either non-dihydropyridine calcium antagonists or β-adrenoceptor antagonists throughout the treatment period. In other respects, the two groups were similar, but mean age was greater in the UAP/NQAMI group. The ACS patients had been treated with intravenous GTN for 24-48 hours. The transition from GTN to ISDN was well tolerated with no patient experiencing further angina in the first 24 hours of ISDN therapy. In 2 of the patients with SAP receiving GTN, infusion rates were reduced to 2.5μg/min due to development of symptomatic hypotension.

3.4.2 Responses to GTN in normal volunteers

As shown on table 3.2, pilot studies demonstrated that 300μg of GTN, although a lower dose than those given in many previous studies (Greig et al., 2005; Waring et al., 2006; Westerbacka et al., 2004; Wilkinson et al., 2002b) induced large decreases in AIx (20±9%) and moderate falls in systolic blood pressure (7±3mmHg). Therefore, dose response curves were constructed using much lower doses of GTN (see figure3.2, table 3.4). From these data, the calculated ED_{50} for GTN was 27±22μg. Responses measured via AIx were detectable at much lower doses than those required to induce significant changes in brachial systolic artery blood pressure. Indeed changes in systolic blood pressure were minimal at lower GTN doses; 100μg doses induced a 16±4% fall in AIx but only 6±4 mmHg reduction in systolic BP.

3.4.3 Effects of GTN infusion on AIx in patients with SAP

Prior to initiation of GTN infusion heart rate-adjusted AIx was 23±10% in this group of patients, consistent with the greater age (Mitchell et al., 2004) of these patients than the normal subjects. Two hours after initiation of GTN infusion (5μg/min), AIx had fallen to 3±14% ($p<0.01$, fig 3.3) and systolic blood pressure fell by 20±19mmHg. In two patients infusion rate was reduced to 2.5μg/min before measurement of AIx due to development of hypotension with 5μg/min. Changes in AIx in these patients were 15 and 18% respectively.

3.4.4 Effects of transition from intravenous GTN to oral ISDN in patients with UAP/NQAMI

Prior to cessation of GTN infusion, the mean value of adjusted AIx in this group of patients was 8±4%. Transition to oral low dose ISDN therapy was associated with a further reduction in AIx to 5±6% (p=0.05, Fig 3.4).

3.5 Discussion

This study extends from evaluation of the dose-response characteristics of GTN in normal subjects, through to its use in patients with stable/unstable coronary artery disease. The common theme is the use of GTN induced reductions in Aix as a measure of GTN effect in vivo.

This is a development of considerable importance. There has always been great difficulty in deriving reproducible and non-invasive measures of GTN haemodynamic effect in humans. Measures such as changes in regional arterial flow are best addressed via local GTN infusion (Bloch et al., 1995; Fallen et al., 1995; Gross and Warltier, 1977) while GTN doses sufficient to induce measurable falls in systolic blood pressure often induce adverse symptoms (Fletcher et al., 1988; Hsi et al., 2005; Wainwright et al., 1993).

The current study has three main conclusions:

1) Demonstration that in normal subjects, single doses of GTN as low as 12μg induced substantial falls in AIx, while the ED_{50} is approximately 27μg. Furthermore, while 300μg of GTN induced a greater degree of hypotension, there was relatively little incremental effect on AIx.
2) Infusion of 5μg/min GTN in patients with SAP induced a 20±7% fall in AIx, without a significant fall in systolic blood pressure.
3) The magnitude of vasodilator effect of low infusion rates of GTN (5μg/min) is approximately equivalent to that of oral ISDN (10mg). Thus transition from low dose GTN infusion to oral ISDN in patients with ACS is not associated with significant "rebound" vasoconstriction as measured by changes in AIx.

Since the demonstration that afterload reduction potentially represents an important component of the therapeutic effects of nitrovasodilators (Kelly et al., 1990; Yaginuma et al., 1986), various techniques have been utilised for study of this effect. Until recently, the most common technique involved direct intra-arterial recording of waveforms utilising high fidelity transducers at the time of angiography (Fitchett et al., 1988; Gundel et al., 1981; Latson et al., 1988; Merillon et al., 1984; Pepine et al., 1979). Given the invasive nature of this technique it is not useful for studies in normal subjects or long term and/or multiple sampling studies. Since the introduction of pulse wave analysis, several studies (Chen et al., 1997; Kass et al., 2001) have demonstrated that effective afterload reduction in response to GTN, via delay of wave reflection can be

measured non-invasively by examination of AIx and that these findings agree with those from traces recorded invasively.

Evaluation of responsiveness of vessels to GTN has traditionally been confined to local responses of the brachial or coronary artery to single doses administered as part of protocols measuring endothelial function, to demonstrate that endothelium independent vasodilation is intact. However, some recent studies have demonstrated that this is not the case (Celermajer et al., 1992; Forstermann et al., 1988; Liao et al., 1991; Zhang et al., 2000). These findings along with the finding that impaired responsiveness to NO at both the vascular (Schachinger et al., 2000) and platelet (Willoughby et al., 2005) level are associated with poor prognostic outcomes, has generated increasing interest in whether there may be a change in sensitivity of vessels to NO rather than a change in the magnitude of maximal response.

At the time of commencement of these studies little was known about the ability of this technique to measure responsiveness to GTN. We have demonstrated that very low doses of GTN can produce measurable effects in both patients and in normal subjects. It has also recently been demonstrated by our group that this technique can be used to measure induction of nitrate tolerance (Holmes et al., 2005). However it is not known whether pseudotolerance can be detected via wave reflection. Given that pseudotolerance is caused by increased circulating levels of endogenous vasoconstrictors, the impact of this phenomenon on wave reflection is currently uncertain.

The components of the study performed in normal subjects raise several additional issues. First, ED_{50} values for sublingual GTN were of the order of 25μg,

which is far smaller than usual clinical doses. We did not perform comparable studies in patients with SAP (it would have been impracticable to do so in UAP patients). However, as it is likely that sublingual GTN dosing may contribute to the development of nitrate tolerance (Chirkov et al., 1997) it would be advisable for "usual" GTN doses to be closer to the minimal effective doses. The use of larger doses of GTN can be perhaps defended in the case of tablet formulations (ie uncertain loss of active principle) but there is clearly a case for reinvestigating the optimal dose of GTN in sublingual spray preparations. This sort of experiment would also help evaluate the relationship between changes in AIx with GTN and the relief of angina.

The other issue raised by the large decrease in AIx is the pharmacology of arterial vasodilatation resulting in delayed wave reflection. In the current study, both normal subjects and patients with SAP had very large reduction in AIx with GTN, implying a considerable vasomotor response to NO. Previous comparable studies in normal subjects and in patients with similarly elevated baseline AIx values are summarised in table 3.1

However the main implication of the response to GTN is that in many patients with "decreased compliance" of their peripheral arteries, the main problem is (nett) constrictor rather than fixed structural change. This therefore offers increased incentive for vasodilator therapy of conditions such as systolic hypertension of the elderly.

The studies in stable angina pectoris patients are also of interest. We have shown that infused GTN (5μg/min) induces a very large fall in AIx. In a recent study from our group, Holmes et al (2005) demonstrated that cutaneous GTN (15mg/24 hours) induced a mean fall of approximately 20% in AIx: thus the magnitude of effect

associated with 5μg/min GTN intravenously is similar to that of usual cutaneous prophylactic therapy. These data, like those in previous studies (Horowitz et al., 1983) of vasodilator effects of infused GTN, demonstrate the utility of this dosage regimen regarding haemodynamic effects, and argue against the routine usage of higher infusion rates (on the grounds of greater risk of tolerance (Henry et al., 1989)).

Ideally, the infusion rate of GTN in these patients might have been prolonged in order to document whether there was any measurable attenuation of GTN effect over 24 hours. This is of relevance if the AIx values (mean 3%) induced by GTN in the SAP patients are compared to those in the ACS patients (mean 8%) who received GTN infusion for greater than 24 hours. Unfortunately systematic evaluation of tolerance induction was logistically impossible in both groups.

The final part of the study investigated the effect of transition from low dose intravenous GTN to administration of oral ISDN in patients with UAP/NQAMI. This study demonstrated that this treatment regimen is associated with no loss of haemodynamic nitrate effect as measured by AIx fluctuation. This is an important finding because: firstly, there is a need to validate regimens suitable for transfer from GTN infusion which protect against the occurrence of "rebound" ischaemia, and secondly, haemodynamic data for ISDN have previously been obtained using larger doses, which are frequently poorly tolerated (Cohn et al., 1986).

The majority of cases of clinically documented "rebound" during nitrate therapy have not specified the prior GTN infusion regimen (for example Figueras et al(1991)). It is likely that "rebound" is particularly prominent after abrupt cessation

of high-dose nitrate infusion, just as rebound ischaemia observed in munitions workers also follows withdrawal from prolonged exposure to high levels of GTN (Ben-David, 1989; Lund et al., 1968). It is therefore routine clinical practice to reduce GTN infusion rates prior to transition to oral nitrate therapy. In the current study, the AIx values prior to transition were similar to those seen in SAP patients immediately after institution of GTN infusion. This suggests that there had been relatively little attenuation of nitrate effect during the infusion period. However this could not be ascertained definitely without estimation of pre-therapy AIx, a practical impossibility in ACS patients. The pre-GTN value of AIx is likely to have been somewhat higher in the ACS group than that in the SAP group, given that AIx increases with age (O'Rourke, 1990). While the precise value cannot be determined, it is clear that AIx would have been markedly reduced by GTN at the time of initial measurement.

The only previously reported detailed study of transition from intravenous GTN was conducted by Lin and Flaherty (1985). In a group of 10 patients with UAP, treated with 84±74μg/min (range 10-200μg/min) GTN for 36±26 hrs, transition to low dose cutaneous GTN (5 or 10mg/24hours) was not associated with recurrence of chest pain or major blood pressure fluctuation. The issue therefore is to achieve transition to cutaneous or oral nitrate therapy with similar haemodynamic effects to those of intravenous GTN.

Oral ISDN has been utilised extensively in the management of stable and unstable angina pectoris for many years. Despite the availability of 10mg tablets most patients have been treated with far higher doses (Parker et al., 1985). The inherent problem with ISDN therapy, as with all nitrates utilised in ACS treatment, is that the extent of tolerance

induction increases with dose and with lack of nitrate-free period. Hence 6-hourly administration of ISDN, a common practice in early studies (Parker et al., 1985), is no longer recommended (Parker et al., 1987).

A major limitation of the current study was that AIx was utilised as the only index of haemodynamic nitrate effect. Recent investigations have established the clinical importance of arterial dilation as a component of clinical efficacy of GTN, via reduced arterial stiffness (Kelly et al., 1990). However it remains possible that the relative potencies of GTN and ISDN might vary if other measures of nitrate effect (eg. coronary dilatation, venodilatation, inhibition of platelet aggregation) had been utilised. Some caution is also required when comparing the magnitude of effect of GTN in studies with different modes of administration, as blood levels of GTN in the circulation or more importantly in the vessels from which responsiveness is being measured have not been measured and absorption may differ according to administration. A further limitation of the study is that AIx was not measured during the interval following ISDN washout, however no patient developed rebound angina.

3.6 Conclusions

The current study establishes firstly, that previous investigations utilising doses of greater than 200μg of sublingual GTN to evaluate effects of this agent on apparent arterial stiffness were utilising near-maximal doses of the drug. Indeed, as 50% maximal

responses occur with approximately 25μg of GTN, evaluations of responsiveness should either use doses of this magnitude or, as here, construction of dose-response curves. The second conclusion from these studies is that infusion of GTN at the rate of 5μg/min, which is at the lower end of clinically utilised infusion rates, produces large falls in AIx, however, as dose response curves were not constructed it cannot be determined whether or not this response is maximal. Thirdly, we can conclude that in patients with ACS, transition from low dose intravenous GTN to oral ISDN (10mg) is associated with no loss of vasodilator effect, as measured by AIx. This suggests that there is no evidence of "rebound" phenomenon with this regimen.

Author	Agent/Regimen	Subjects	Baseline AIx	Response
Kelly et al, 1991	GTN/infusion at 3,30,300μg/min	Normal males, aged 25-45	5%	↓ with max dose was 15%
Pauca et al, 2001	GTN/infusion rate increased to reduce SBP to 100mmHg	Coronary artery disease	23%	↓ 15%
Hayward et al, 2002	GTN/250μg sublingual	Normal males mean age 25 and patients with CAD vs age matched controls	Young norm, 50%, older norm 84 vs CAD 63%	↓ 35% in young normal and 63% in older normals vs 23% in CAD
Wilkinson et al, 2002	GTN/500μg sublingual	Normals, mean age 32	Not given	↓ by 12%
Westerbacka et al, 2004	GTN/500μg sublingual	Normals mean age 26 and normals mean age 50	Young 0.5, older 25.2%	↓ by 16 in young group and 16 in older group
Greig et al, 2005	GTN/500μg sublingual	Normals, mean age 35	2%	↓ 12%
Warnig et al, 2006	GTN/500μg sublingual	Hypertensive vs controls	Not given	↓ 14%
Other vasoactive agents				
Stokes et al, 2003	ISMN/60mg single dose	Treated hypertensive divided into those with and without ACEi treatment	37 without ACEi and 30 with ACEi	↓15 in those with ACEi and 12 in those without
Wilkinson et al, 2001	L-NMMA/0.1, 0.3, 1.0 and 3.0mg/kg/min	Normal aged 21-42	0%	↑ by 25%
Wilkinson et al, 2002	Salbutamol 400μg ± L-NMMA 1.0mg/kg/min	Normals mean age 32	Not given	↓ 10, halved in the presence of L-NMMA
Hayward et al, 2002	Salbutamol/400μg	Normal males mean age 25 and patients with CAD vs age matched controls	Young norm 50%, older norm 84 vs CAD 63%	↓ 12 in young normals and by 13 in older normals vs 2% in CAD
Wilkinson et al, 2001	Angiotensin II/ 1-10ng/kn/min and noradrenoline100ng/kg/min	Normal, mean age 30	0%	AII ↑ by 9-33% and NAD by 0-16%, dose dependently
Kelly et al, 1991	Angiotensin II/ 75/150/300ng/min	Normal males aged 23-45	5%	↑ by 9%

Table 3.1. Utility of pulse-wave analysis for examination of augmentation index responses to GTN, modulators of endothelial NO release and vasoconstrictor agents. Summary of previously published studies.

Subject number	Max change in SBP (mmHg)	Max change in AIx
1	-10	-24
2	-4	-14
3	-9	-18
4	-8	-34
5	-5	-10
Average (SD)	-7(3)	-20(9)

Table 3.2. AIx and systolic blood pressure response of 6 normal individuals to a single 300μg dose of sub-lingual GTN.

Patient Cohort	UAP/NQAMI (n=16)	SAP (n=10)
Age	69±10	59±13*
Gender (M:F)	9:7	6:4
% NQAMI	44	0
Coronary Risk Factors (%)		
Smoking	12.5	20
Hypertension	56	50
Diabetes Mellitus	37.5	10
Hypercholesterolaemia	75	70
Concurrent Pharmacotherapy (%)		
Aspirin	100	100
Heparin	81	0
ACE inhibitor	40	40
ß-adrenoceptor antagonist	31	20
Calcium channel antagonist	81	70
Statin	50	70

Table 3.3. Characteristics of patients with unstable angina pectoris/non-Q-wave infarction (UAP/NQAMI) compared with those with stable angina pectoris * $p<0.05$

Subject	Age	Systolic BP	Baseline AIx	Max reduction in AIx Dose (μg)	Response	ED_{50} (μg)
1	31	122	0	12.5	9	15
				25	5	
				50	12	
				100	9	
				150	9	
2	30	117	14	12.5	4	27
				25	10	
				50	13	
				100	17	
				150	13	
3	30	87	-13	12.5	2	66
				25	10	
				50	9	
				100	15	
				150	12	
4	25	98	-16	12.5	14	14
				25	13	
				50	15	
				100	19	
				150	19	
5	26	104	7	12.5	15	11
				25	12	
				50	17	
				100	18	
				150	15	
Mean (SD)	28(3)	106(14)	-1.6(13)	12.5	8(6)	27(22)
				25	10(3)	
				50	13(3)	
				100	16(4)	
				150	14(4)	

Table 3.4. Dose response curve to sub-lingual GTN in normal subjects. Although maximal reductions in AIx are provided as absolute changes, calculation of ED50 was performed on the basis of area under the response-time curve.

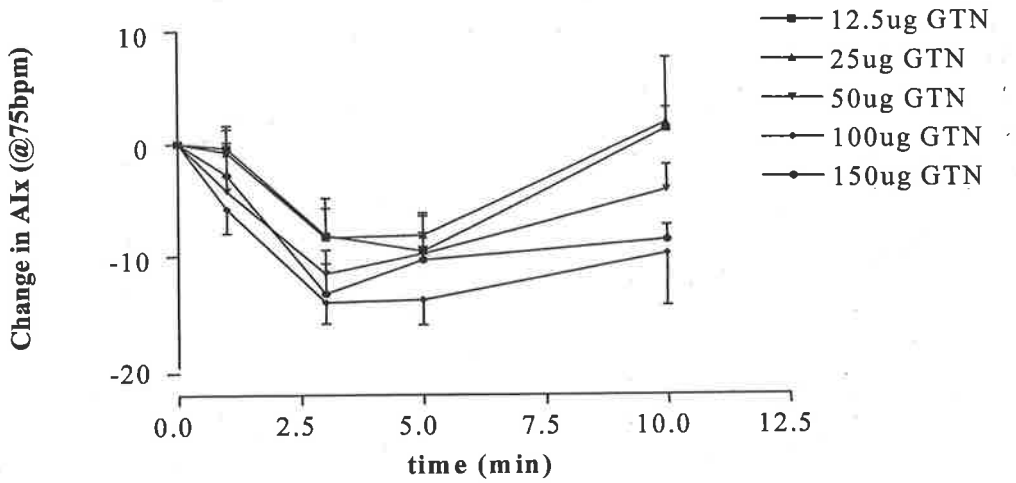


Figure 3.1. AIx time course in 5 normal following administration of doses of GTN ranging from 12.5-150µg

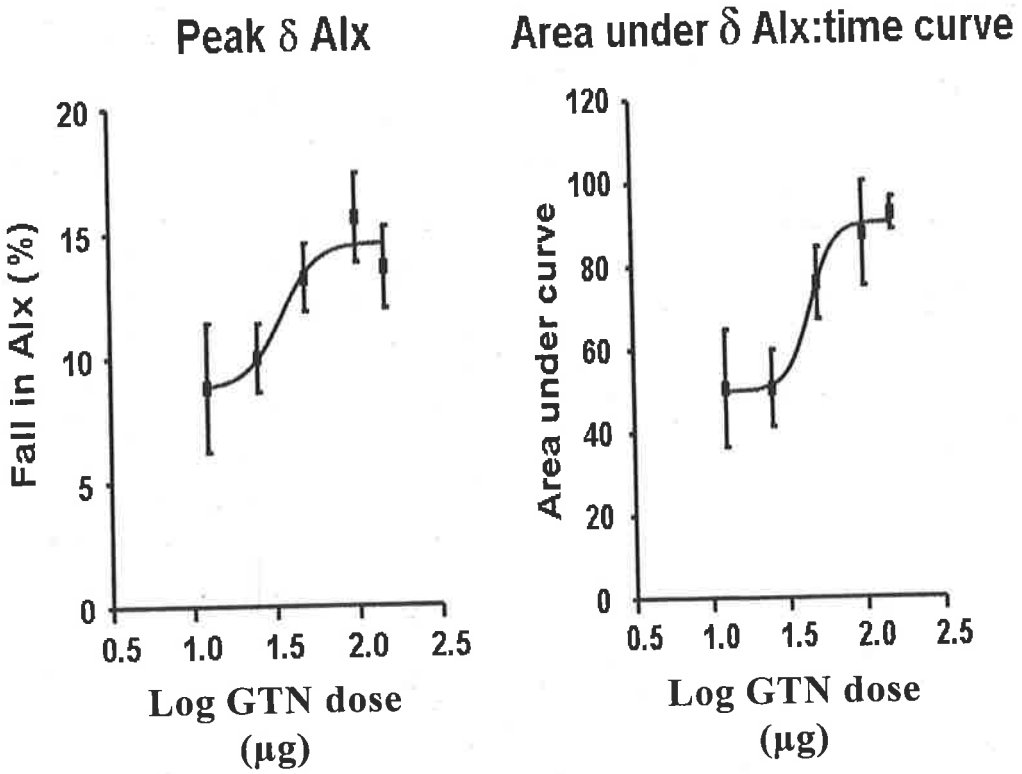


Figure 3.2. Dose response curves to GTN in normal subjects (n=5), shown as peak effect (left) and as area under the curve (right).

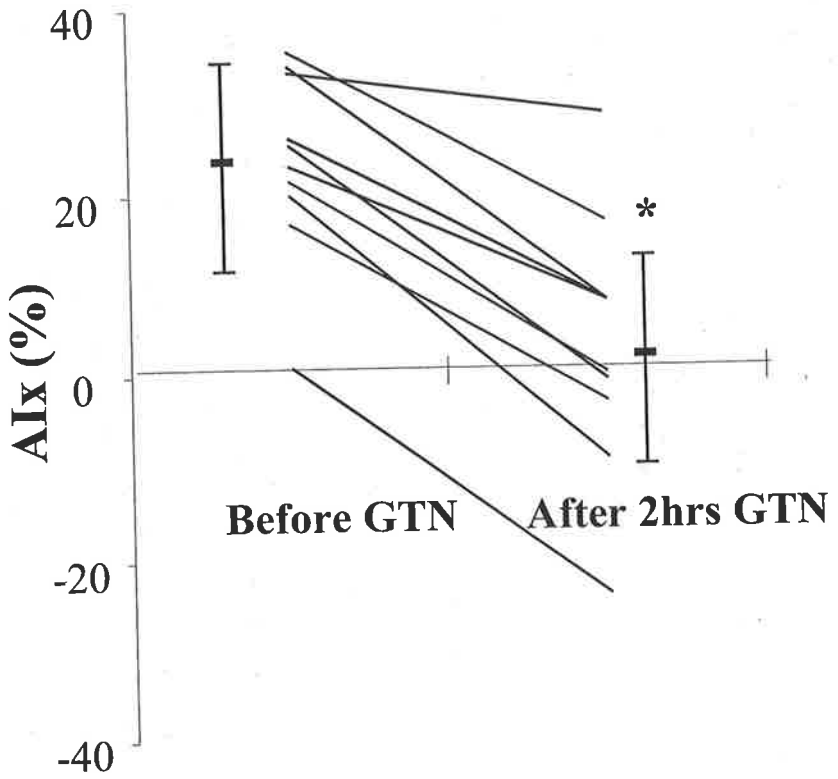


Figure 3.3. Effect of 2 hr GTN infusion (5μg/min) on augmentation index in patients with stable angina pectoris. * $p<0.05$ vs before GTN.

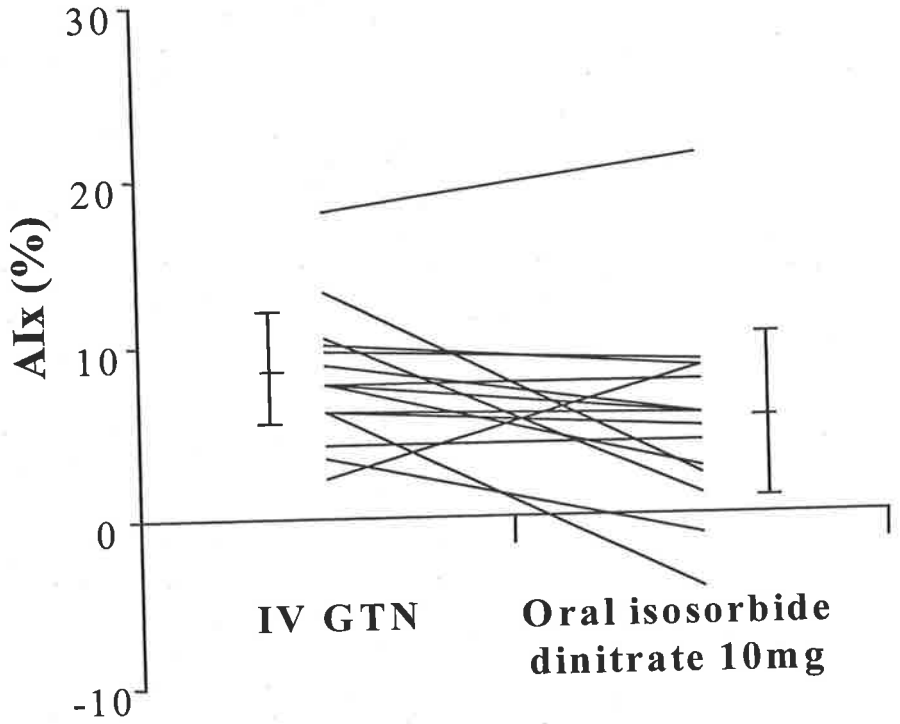


Figure 3.4. Augmentation index during transition from intravenous to oral nitrates in patients with acute coronary syndromes (p=0.05).

Chapter 4: Interactions between perhexiline and glyceryl trinitrate

4.1 Summary

Perhexiline, a "metabolic" anti-anginal agent currently under investigation in management of chronic heart failure and ACS improves platelet NO responsiveness in patients with impaired NO responsiveness. The current study investigated possible interactions between perhexiline and GTN on arterial stiffness, on neutrophil superoxide release (O_2^-) and on platelet NO responsiveness. Patients (n=39) with stable angina pectoris, awaiting cardiac catheterisation were randomised to blinded addition of perhexiline or unchanged drug therapy; all patients received GTN infusion for 2 hours. Vasomotor responses to perhexiline alone and in combination with GTN were examined using changes in augmentation index (AIx, via applanation tonometry). Neutrophil O_2^- release was measured ex vivo utilising lucigenin mediated chemiluminescence and effects of perhexiline on inhibition of platelet aggregation by the NO donor sodium nitroprusside (SNP) were also measured. Perhexiline alone did not affect AIx, O_2^- release or ex vivo platelet SNP response. GTN decreased AIx ($p<0.01$) and O_2^- release ($p<0.05$). The magnitude of inhibition of O_2^- release was significantly enhanced by perhexiline pre-treatment ($p<0.05$); while perhexiline had no effect on magnitude of vasomotor response to GTN. Therefore it can be concluded that perhexiline exerts no effects on arterial stiffness and does not potentiate GTN response and that in patients with normal platelet function perhexiline does not affect platelet NO responsiveness. We can also conclude that in vivo low dose GTN inhibits neutrophil O_2^- release; this effect is potentiated by pre-treatment with perhexiline. These "anti-inflammatory" effects of GTN may contribute to utility in ACS and CHF.

4.2 Introduction

Nitric oxide (NO) plays a fundamental role in vascular homeostasis including modulating vasomotor tone, platelet adhesion and aggregation. The majority of coronary risk factors (Clarkson et al., 1996; Creager et al., 1990; Duffy et al., 1999; Liao et al., 1991; McVeigh et al., 1994; Sorensen et al., 1994; Watts et al., 1996; Williams et al., 1996; Zeiher et al., 1995), as well as the presence of overt ischaemic heart disease (Celermajer et al., 1992; Forstermann et al., 1988) have been associated with anomalies of NO-mediated biological effect, including impaired endothelium mediated vasodilatation, impaired platelet responsiveness (Chirkov et al., 2001) and increased activation of neutrophils (Sugano et al., 2005). Although in many circumstances NO release is impaired, it is increasingly apparent that in most of these pathological states there is associated reduction in tissue responsiveness to NO (Chirkov et al., 2001; McVeigh et al., 1994). This impaired responsiveness may be mediated by either "scavenging" of released NO by free radicals such as the O_2^- (Laursen et al., 1997) or by dysfunction of the NO/soluble guanylate cyclase second messenger system (Mulsch et al., 1997). This phenomenon of "tissue resistance to NO", which occurs both in vasculature (Celermajer et al., 1992; Schachinger et al., 2000) and in platelets (Chirkov et al., 1999), carries independently adverse prognostic implications (Schachinger et al., 2000; Willoughby et al., 2005) and also implies reduction in the therapeutic efficacy of the organic nitrates, which act as NO donors.

A variety of agents have been shown to ameliorate NO resistance, including ACE inhibitors (Chirkov et al., 2004) and possibly statins (Stepien et al., 2003). More recently

we have shown that under some circumstances the "metabolic" anti-ischaemic agent perhexiline also potentiates NO responsiveness in platelets. In a study of patients with stable and unstable angina patients who were unresponsive to conventional therapy, perhexiline reversed platelet NO resistance, an effect which was correlated with anti-anginal effects. In the same study, perhexiline inhibited neutrophil O_2^- release in vitro (Willoughby et al., 2002).

These findings raise a number of important issues, related both to NO and to the role of perhexiline as a putative modulator of NO effects. Specifically, perhexiline has been considered as essentially hemodynamically inert (apart from very weak calcium antagonist properties (Barry et al., 1985; Fleckenstein-Grun et al., 1978)), and does not appear to increase incidence of headaches in organic nitrate-treated patients. It therefore appeared possible that the effects of perhexiline might include a platelet-selective interaction with NO. It also appeared that the effect of perhexiline on neutrophil O_2^- release might be subject to interactions with NO, given that high concentrations of NO donors also suppress O_2^- release in vitro (Wanikiat et al., 1997).

We therefore performed a clinically based blinded study to test, in a population of patients with stable angina pectoris, the following hypotheses:

1. That perhexiline exerts neither direct nor NO-potentiating effects on vasomotor tone.
2. That perhexiline inhibits neutrophil O_2^- release in vivo, and that this effect is potentiated by therapeutically infused NO donors.

3. That the effects of perhexiline include potentiation of platelet responsiveness to NO donors in a population with mild ischaemic symptoms.

4.3 Methods

4.3.1 Patient Selection

Patients were considered for entry on the basis of clinical diagnosis of either chest pain of uncertain aetiology or of stable angina pectoris (Canadian Class II or III) not requiring long-term prophylactic nitrate therapy. In all cases elective cardiac catheterisation was planned. Exclusion criteria (apart from long acting nitrate therapy) were treatment with perhexiline or other "metabolic" anti-anginal agents, utilisation of thienopyridine anti-aggregatory agents, previous adverse reactions to organic nitrates, clinically significant hepatocellular disease or findings of hemodynamically significant left main coronary stenosis.

4.3.2 Experimental protocol

The protocol is outlined in figure 4.1. Patients (n=39) were randomised to receive perhexiline (400mg/day) or no additional therapy 3 days prior to catheterisation, with blinding of all study personnel to treatment regimen; this 3 day pre-treatment facilitates attainment of therapeutic perhexiline levels (Horowitz et al., 1986). Intravenous infusion of GTN (5μg/min) utilizing non-adsorptive delivery tubing and glass reservoir was

commenced immediately prior to angiography and continued for at least 2 hours. Pulse wave analysis was performed and neutrophil O_2^- release measured prior to randomisation, immediately prior to catheterisation and after two hours of GTN infusion. Platelet studies were performed prior to randomisation and immediately prior to catheterisation. To investigate the possibility of an effect of the angiogram itself, a comparison group (n=6) underwent angiography with no additional treatment: neutrophil O_2^- release was measured before and two hours after angiography.

4.3.3 Applanation Tonometry

Apparent arterial stiffness was measured serially as described in chapter 2.6, utilising applanation tonometry, in which radial artery pressure wave forms are recorded. Pulse wave analysis (Sphygmocor version 7, Atcor Medical, Sydney, NSW, Australia) was then used to generate corresponding central waveforms via a validated transfer function. Augmentation index (AIx), a measure of systemic arterial stiffness (Vlachopoulos et al., 2001), was determined from the central waveform by expressing the difference between the first and second systolic peaks as a percentage on the total pulse pressure; measures of AIx were corrected for heart rate.

4.3.4 Neutrophil O_2^- production

This was assessed as described in chapter 2.3. Briefly, venous blood was anti-coagulated with EDTA. Plasma was separated by centrifugation and retained. Red blood

cell layer and buffy coat was diluted with Hanks' balanced salt solution (HBSS) and neutrophils were separated by centrifugation across Lymphoprep density gradient (Axis-Shield, Oslo, Norway). Hypotonic lysis (155mM NH_4Cl, 100μM Na_2EDTA, 10mM $NaHCO_3$) was used to remove red cells and isolated neutrophils were washed in HBSS and re-suspended in platelet free plasma at $1.7x10^6$ cells/mL. Neutrophils were stimulated with the chemotactic peptide 1μM fMLP. O_2^- release was measured at 37°C by lucigenin (10μM) mediated chemiluminescence at 20 second intervals for 5 minutes using a luminometer (6100 Pico-lite luminometer, Packard, Illinios) . Data are expressed as area under the curve (0-5 minutes).

4.3.5 Platelet Aggregation Studies

Platelet studies were conducted as described in chapter 2.4. Briefly, citrated venous blood was diluted 2 fold with 0.9% saline. Aggregation in response to adenosine 5'-diphosphate (ADP, 1μM) was measured using a dual channel impedance aggregometer (Model 560, ChronoLog) and recorded as maximal aggregation (in ohms). Inhibition of aggregation by sodium nitroprusside (SNP) was determined by measuring maximal aggregation to ADP after 1 minute pre incubation with SNP and results were expressed as percent inhibition of maximal aggregation by SNP.

4.3.6 Data Analysis

Normally distributed data were analysed by two-way ANOVA. Where raw data were not normally distributed data, changes over time (which were normally distributed) were compared between the two groups by t-tests, and changes within groups were analysed using 1-sample t-tests. Patient characteristics were compared using Fisher's exact test for categorical data or Student t-tests for continuous data.

4.5 Results

4.5.1 Patient Characteristics

As seen in table 4.1, patients in both groups were well matched for risk factor profile and drug treatment. The majority of patients were receiving one prophylactic anti-ischaemic agent (either β-adrenoceptor antagonist or non-dihydropyidine calcium antagonists). Most patients were also treated with statins and 49% were receiving either ACE inhibitors or AT1 antagonists. The majority of patients had limited coronary artery disease, with patients randomized to perhexiline having a higher ($p<0.05$) incidence of 3 vessel disease. All patients had well preserved left ventricular systolic function and normal pulmonary capillary wedge pressures.

Plasma perhexiline concentrations after 3 days of treatment were 0.32±0.01μg/mL (therapeutic range 0.15-0.6 μg/mL (Horowitz et al., 1986)). All patients tolerated both perhexiline and GTN without symptomatic side-effects over the study period.

4.5.2 Vascular studies

The effects of GTN infusion and its interaction with perhexiline therapy are summarised in figure 4.2. As previously described (see chapter 3) GTN, even at this low infusion rate, induced a marked fall in AIx ($p<0.01$, $F=251.8$). This was paralleled by a mean decrease in systolic blood pressure of 24±4mmHg. However there was no significant effect of perhexiline alone on AIx or on systolic blood pressure (mean change in perhexiline group: systolic BP, 3.3±1mmHg, AIx, 1.5±1.5%, p=NS), nor was there a significant interaction between AIx response to GTN and perhexiline therapy (see figure 4.2), consistent with the hypothesized "non-vasoactive" pharmacology of perhexiline. Furthermore, in patients with less than median responses to GTN (ie. relatively NO resistant at the vascular level) there was also no evidence of potentiation of GTN effect by perhexiline.

4.5.3 Neutrophil O_2^- Release

Changes in fMLP-stimulated neutrophil O_2^- release during GTN infusion are summarised in figure 4.3. Raw data are expressed as area under the O_2^- release: time curve from 0-5 minutes in arbitrary units x 10^6 and median data (inter-quartile range) are reported as data were not normally distributed. Perhexiline treatment for 3 days had no significant effect on O_2^- release (control: 5.08 (2.8-10.0) to 3.76 (2.7-6.8), perhexiline: 3.635 (2.54-7.28) to 4.510 (1.97-9.99)). However GTN induced a significant inhibition of O_2^- release (see figure 4.3, $p<0.05$, one sample t-test). The mean decrease in

neutrophil O_2^- release induced by GTN was approximately 3 times greater in the perhexiline group than in the control group. Furthermore (fig 4.4) there was a correlation of borderline significance (r^2=0.24, p=0.053) between the magnitude of GTN effect on neutrophil O_2^- release and plasma perhexiline level. Administration of contrast agents and heparin without GTN during angiography (n=6) did not affect neutrophil O_2^- release (mean delta of 0.92±0.55, p=NS).

4.5.4 Platelet Studies

Platelet responsiveness to NO was measured ex vivo as previously described (Chirkov et al., 1999) by measuring inhibition of ADP-induced aggregation by SNP, before and after three days of perhexiline treatment. Platelet responsiveness to SNP was unchanged during the three day study period in either the perhexiline treated or control group (2-way ANOVA, control: 47.0±5.8% to 52.1±7.1%, perhexiline: 57.8±5.8% to 62.8±5.8%, see figure 4.5). This result was not affected by changes in extent of aggregation as there was no significant change in the extent of aggregation in either group during the study period (control: 9±0.7 ohms to 8±0.7 ohms, perhexiline: 8.7±0.8 ohms to 8.0±1.0 ohms). Patients randomized to perhexiline exhibited relatively well preserved platelet responses to SNP (mean inhibition of aggregation by SNP in normal volunteers is 66±19% (Chirkov et al., 1999)) although SNP responsiveness tended to be lower than in patients not receiving perhexiline, this was not significant.

4.6 Discussion

These investigations were undertaken primarily to establish whether perhexiline, while exerting no vasomotor effects, potentiates non-vasomotor effects of GTN. Indeed the results revealed no evidence that perhexiline exerts clinically significant vasomotor effects, either alone or in combination with GTN. However, perhexiline, while not affecting neutrophil O_2^- release in monotherapy, markedly potentiated the effects of a clinically low infusion rate of GTN in suppressing O_2^- release. Perhexiline exerted no effects on platelet responsiveness to the NO donor SNP in the population tested. A further critically important finding was related to the effect of GTN on neutrophil O_2^- release: 5μg/min GTN, representing a very low clinical infusion rate (Arstall et al., 1995), suppressed O_2^- release by approximately 20%.

The finding that perhexiline lacks intrinsic or NO-potentiating effects on AIx was consistent with previous clinical experience with the drug: neither development of hypotension nor precipitation of headaches in nitrate treated patients are recognised side effects of the drug. Nevertheless, perhexiline does exert vasodilator effects in vitro in some animal models (Fleckenstein-Grun et al., 1978; Klaus and Guttler, 1978), probably via its weak L-type calcium antagonist effects. It is likely that these vasodilator and NO potentiating effects do not occur at the perhexiline concentrations attained in this study. However, it cannot be excluded that these effects were not seen in the current study due to the magnitude of effect of the GTN dose utilised on AIx (as discussed in chapter 3). This is of particular importance given that the platelet data demonstrate that this cohort have relatively intact responsiveness to NO donors.

The lack of significant potentiation of SNP-induced inhibition of platelet aggregation by perhexiline was unexpected. Willoughby et al (2002) have previously shown that perhexiline potentiates SNP and GTN effects on platelet aggregation in cohorts of patients with both stable and unstable angina. The lack of significant effect in the current study probably reflects the relatively normal baseline platelet function in the current study group, whereas the previous cohort was markedly hypo-responsive to NO donors prior to initiation of perhexiline therapy. The lack of platelet NO resistance may also suggest that vascular responses to NO were intact. This would not only affect ability to detect potentiation of platelet NO responsiveness, but may also impair our ability to detect potentiation of responses to endogenous NO in vasculature via changes in AIx.

This is the first in vivo human study to document the effects of GTN or any other organic nitrate in suppressing O_2^- release from neutrophils. This is potentially a most important finding, which may improve understanding of the therapeutic potential of all agents releasing or potentiating NO, not just organic nitrates. The observed suppression of O_2^- release was detected ex vivo, and cannot be attributed to "scavenging" of released O_2^- by the $NO^{\cdot}$ radical, given the very short half life of GTN. While inhibition of superoxide release has previously been demonstrated in arterial segments taken from rabbits fed a high-cholesterol diet and treated with isosorbide mononitrate (Muller et al., 2004), the biochemical mechanism(s) of this effect were not evaluated. Previous investigators have raised the possibility that NO may exert "anti-inflammatory" effects, and it has been shown that high concentrations of NO inhibit NAD(P)H oxidase, a major source of O_2^- release (Klaus and Guttler, 1978). It remains to be determined whether the

currently observed NO effect is modulated by activation of soluble guanylate cyclase (although there is some evidence that activation of soluble guanylate cyclase inhibits activation and O_2^- release from neutrophils (Wang et al., 2002)), and whether this acute GTN effect is potentially subject to tolerance induction. Clinically, the finding is of particular interest as regards the management of acute coronary syndromes, in which inflammatory response plays a pivotal role (Buffon et al., 2002). Disturbance of coronary artery plaques (during angioplasty) has been shown to result in activation of neutrophils in vivo (van der Wal et al., 1994) and neutrophil infiltrate has been found at the site of ruptured plaques (Naruko et al., 2002). Activation of inflammatory cells has also been shown to contribute to impairment of endothelial function (Sugano et al., 2005). Interestingly, low dose isosorbide mononitrate, which has no beneficial effect on 35 day mortality post myocardial infarction in the ISIS4 study, markedly reduced day 1 mortality (1995) via an unknown mechanism. Furthermore, these findings may be relevant in explaining the efficacy of combination of isosorbide dinitrate with the NAD(P)H oxidase inhibitor hydralazine in patients with chronic heart failure(Taylor et al., 2004), a condition characterised by activation of inflammatory processes (Ellis et al., 2000).

As regards potentiation of effects of GTN on neutrophil O_2^- release by perhexiline, only fragmentary mechanistic data are currently available. Perhexiline inhibits O_2^- release from neutrophils in vitro (Kennedy et al., 2006; Willoughby et al., 2002), but the interaction with GTN /NO has not been investigated in this system. Irrespective of the underlying mechanism(s) of this interaction, these findings are very

relevant to the evolving role of this agent in management of acute myocardial ischaemia (Philpott et al., 2004) and possibly heart failure (Lee et al., 2005a).

4.7 Limitations and future directions

A number of important issues have not been addressed in the current study. Neutrophil studies did not examine mechanisms of responses to GTN or perhexiline, concentration response relationships or potential attenuation of GTN effect due to nitrate tolerance. It would also be of interest to compare the interaction between perhexiline and GTN in a population with a high prevalence of platelet NO resistance (such as patients with severely symptomatic stable angina or unstable angina (Willoughby et al., 2002)) in order to evaluate the relative extents of the perhexiline-GTN interactions in platelets and neutrophils. Furthermore, it remains possible that perhexiline might exert vasomotor effects at the level of the large arteries, venous circulation and /or other vessels not represented by changes in AIx. Vascular studies may also have been affected by lack of NO resistance, which was not measured in this study. Given the findings in chapter 3, the dose of GTN used may have been too high (although we have not examined dose response to intravenous GTN) to detect changes in sensitivity and only changes in maxima would have been detectable.

4.8 Conclusions

The current study has demonstrated that perhexiline is vasoactively inert both as monotherapy and when combined with intravenous GTN, and that in this patient cohort with normal platelet function that perhexiline has no effect on platelet NO responsiveness. However the study also demonstrates for the first time that in vivo administration of low infusion rates of GTN inhibits neutrophil O_2^- release, and that this effect is potentiated by pre-treatment with perhexiline.

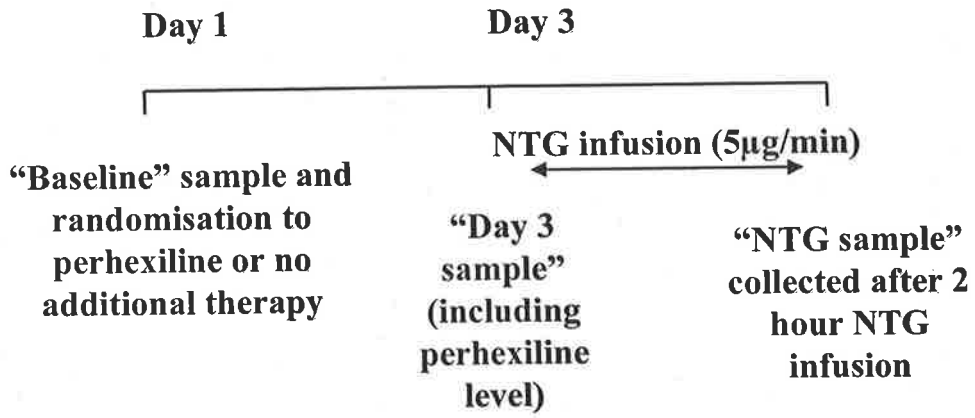


Figure 4.1. Schema: Experimental protocol.

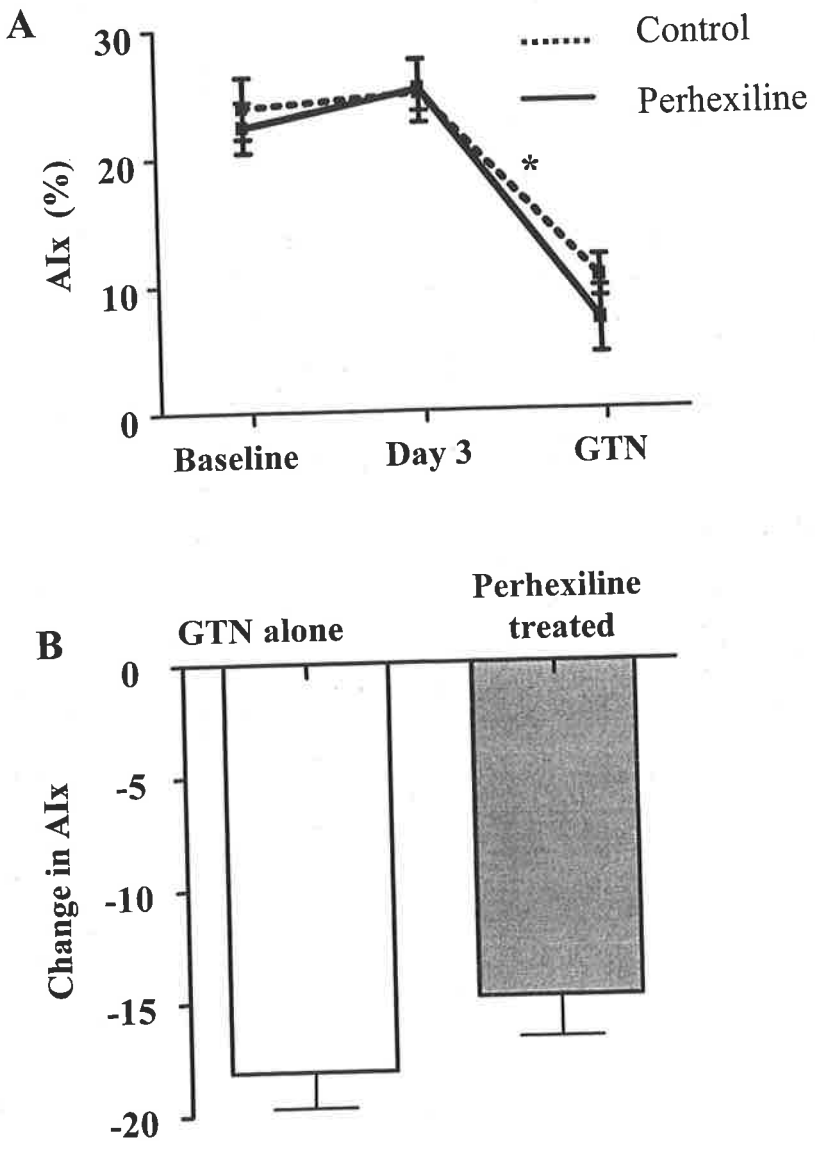


Figure 4.2. A. Effect of perhexiline alone and in combination with GTN on augmentation index (AIx). Perhexiline alone had no significant effect on AIx as indicated by no change from baseline to day 3. * GTN infusion significantly reduced AIx in both treated and untreated patients ($p<0.01$, $F=251.8$, ANOVA). B. There was no significant difference in the magnitude of this change between groups (unpaired t-test).

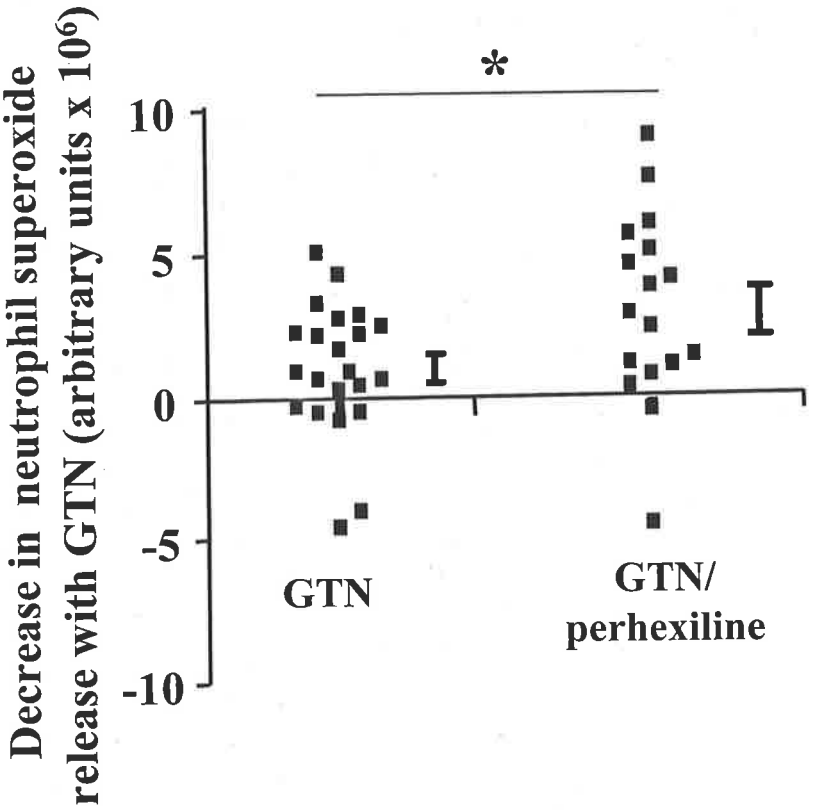


Figure 4.3. Comparative effects of GTN alone and GTN in combination with perhexiline in suppressing neutrophil O_2^- release. GTN alone decreased neutrophil O_2^- release ($p<0.01$, one-way t-test) and the magnitude of this effect was significantly greater in the perhexiline treated group ($p<0.05$, unpaired t-test).

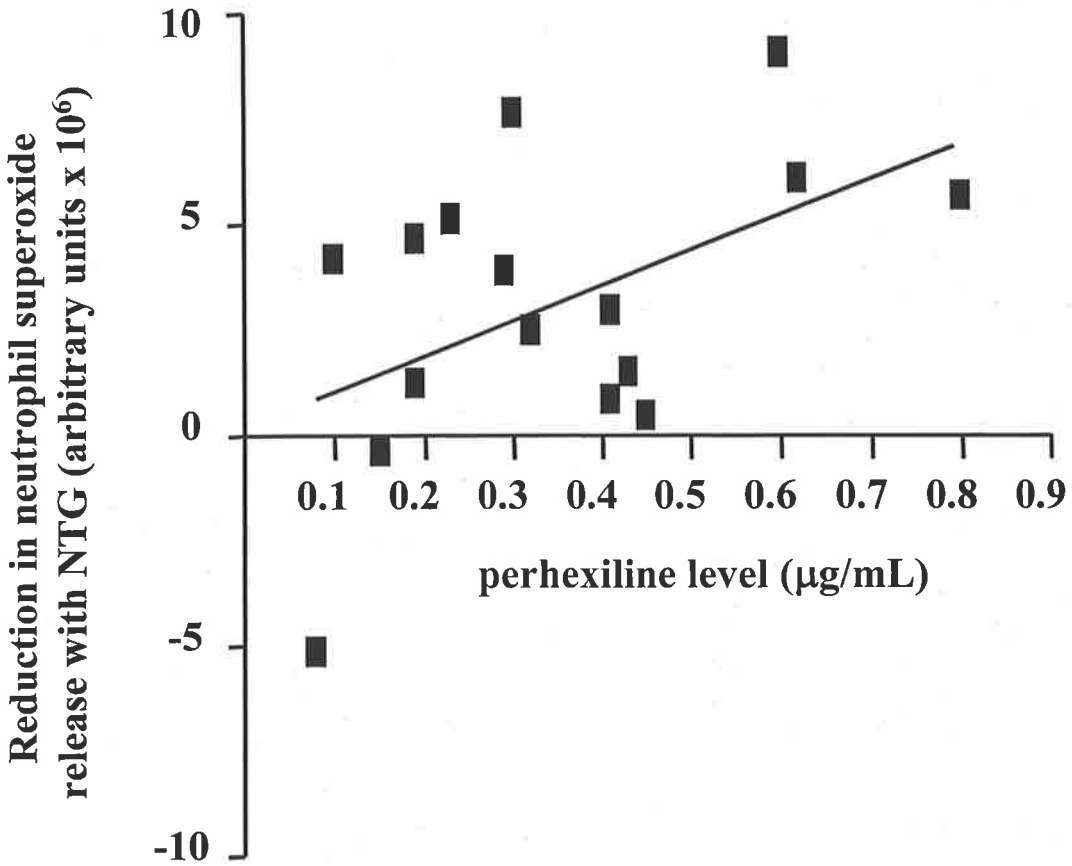


Figure 4.4 Relationship between plasma perhexiline concentration and reduction in neutrophil O_2^- release during perhexiline treatment r^2=0.24, p=0.053.

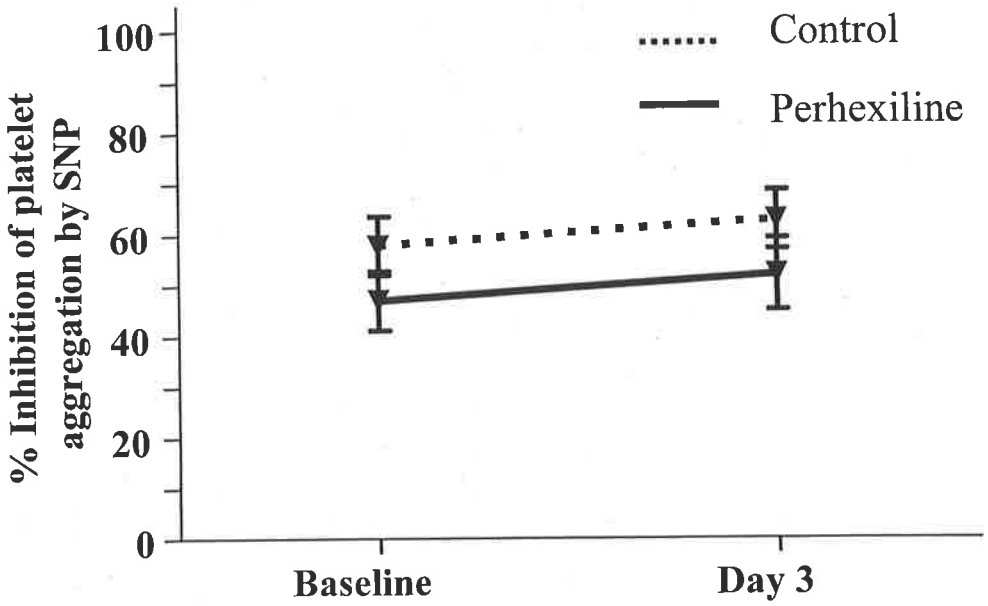


Figure 4.5 Effect of perhexiline on platelet NO responsiveness. There was no change in response to the NO donor SNP in either the control group (dotted line) or the perhexiline group (solid line) over the 3 day study period (p=ns, F=1.441, ANOVA).

Table 4.1: Characteristics of patients in control and perhexiline groups. †: significant stenosis = <50% stenosis in a major epicardial vessel, *: $p<0.05$

	Control Group n=22	**Perhexiline Group n=17**
Risk Factors		
Age(±SEM)	57±0.6	62±0.6
Male gender	13 (59%)	10 (59%)
Current smoking	5 (23%)	5 (29%)
Hypertension	12 (55%)	10 (59%)
Hypercholesterolemia	18 (82%)	13 (76%)
Diabetes Mellitus	4 (18%)	3 (18%)
Drug Treatment		
Statin	16 (73%)	11 (65%)
β-Adrenoceptor antagonist	8 (36%)	3 (18%)
Aspirin	21 (95%)	17 (100%)
ACE inhibitor/AT1 receptor antagonist	10 (46%)	9 (53%)
Ca antagonist	14 (64%)	13 (76%)
Number of Stenosed/occluded vessels†		
0	3 (14%)	0
1	9 (41%)	6 (35%)
2	10 (45%)	5 (29%)
3	0	6 (35%)*

Chapter 5: Elevated oxidative stress markers are associated with symptomatic status in patients with coronary artery disease.

5.1 Summary

Increased oxidative stress both predisposes and contributes to acute myocardial ischaemia. However, only a limited number of studies have examined whether various biochemical parameters of oxidative stress are abnormal in plasma of patients with symptomatic ischaemia. The current study investigated whether acute coronary syndromes (ACS, unstable angina/non-Q-wave myocardial infarction, are associated with increased stimulated neutrophil superoxide (O_2^-) release and/or elevation of plasma malondialdehyde (MDA) concentrationss compared to stable angina (SAP) or to absence of ischaemia, and whether there is a correlation between changes in these two markers of oxidative stress.

14 normal subjects, 30 SAP and 29 ACS patients were studied. FMLP-stimulated neutrophil O_2^- release was measured in neutrophils isolated from whole blood by centrifugation across a density gradient and expressed as area under the lucigenin enhanced chemiluminescence: time curve. Plasma MDA was measured using a modified TBARS assay.

Neutrophil O_2^- release did not vary significantly between groups, although elevation beyond the normal range occurred somewhat more frequently in the SAP and ACS patients. After correcting for nitrate effects, O_2^- release was significantly greater ($p<0.01$) in ACS that in SAP.

MDA levels were significantly greater in both ACS and SAP patients ($p<0.05$ and $p<0.01$ respectively) than in normal subjects. However there was no significant correlation between O_2^- release and MDA concentration within any patient group.

Within the ACS group, only number of coronary risk factors (β=0.41, p=0.03) was significantly associated with increased O_2^- release on backwards stepwise multiple logistic regression analysis.

In conclusion, while this study demonstrates that ACS (and marginally SAP) are associated with incremental oxidative stress, various markers of such stress show poor instantaneous correlation either with each other or with clinical parameters in individual patients.

5.2 Introduction

A large number of recent investigations have drawn attention to the relationship between inflammatory change and both active (Buffon et al., 2002; Naruko et al., 2002; van der Wal et al., 1994) and potential (John et al., 2000) myocardial ischaemia. Implicit in the putative role of inflammation in modulating ischaemic events is its association with incremental oxidative stress (see Griendling et al (2000) for review). Oxidative stress results in depletion of endogenous antioxidant molecules and also in formation of complexes formed by interaction between oxidant species and proteins, lipids, carbohydrates and DNA.

Generation of oxidant stress occurs locally, within ruptured plaques and aggregating platelets. However there is increasing evidence that a generalized state of both inflammation (for example, demonstrated by increases in concentration of CRP and Amyloid A (Ehlers et al., 2002; Liuzzo et al., 1994)) and oxidative stress (for example, depletion of glutathione peroxidase (Ceconi et al., 1988; Kratnov et al., 2005)) is associated with incremental risk of acute coronary events. A major component in the induction of inflammatory response may be activation and release of superoxide (O_2^-) from neutrophils mainly via NAD(P)H oxidase. While increased neutrophil activation and O_2^- release has been reported widely as a marker of oxidative stress (Biasucci et al., 1996; Dinerman et al., 1990; Mehta et al., 1989) there are many other potential markers, and it is not clear to what extent this parameter of free radical release correlates with markers of the release or effect of free radicals, such as the generation of modified compounds.

Various products of free radical interaction with biological compounds can be utilised to assess levels of oxidative stress. Assay of modified lipids has frequently been utilised, given the relative stability of many of these compounds. We have previously developed an assay which measures plasma concentrations of malondialdehyde (MDA), via a refinement of the relatively non-specific TBARS reaction (Arstall et al., 1995), and demonstrated a transient increase in plasma MDA concentrations after acute ST elevation myocardial infarction, which was attenuated by therapy with N-acetylcysteine.

The objectives of the current study were firstly to examine whether the presence of stable or unstable symptomatic myocardial ischaemia (excluding S-T elevation AMI) is associated with either increased neutrophil O_2^- generating capacity or elevated plasma levels of MDA relative to normal values, and also to identify the clinical determinants of levels of these two markers of oxidative stress. Secondly, we sought to determine whether there is a correlation between extent of neutrophil O_2^- generation and plasma MDA levels.

5.3 Methods

5.3.1 Patient inclusion criteria

Three groups of subjects were compared:

1) Normal volunteers over the age of 40 years, with no known cardiovascular risk factors, not receiving any regular medication within the 2 weeks prior to recruitment.

2) Patients with stable angina pectoris (SAP): Canadian class I-II angina, undergoing diagnostic coronary angiography for investigation of chest pain. Patients requiring prophylactic nitrate therapy were excluded, as the major objective of evaluation of the cohort was evaluation of the haemodynamic, anti-aggregatory and neutrophil function effects of short-term administration of nitroglycerine (GTN, see chapter 4).
3) Patients with unstable angina/non-Q-wave myocardial infarction (acute coronary syndromes, ACS): Admitted to the coronary care unit for ACS and studied within 24hrs of index chest pain.

Patients with SAP continued previously prescribed non-nitrate anti-ischaemic medication. Patients with ACS received intravenous GTN infusion, together with heparin/aspirin.

5.3.2 Neutrophil O_2^- release

O_2^- release was assessed via lucigenin mediated chemilumiscence as described in chapter 2.6. Briefly, venous blood was anti-coagulated with Na-EDTA. Plasma was separated by centrifugation and retained. Red blood cell layer and buffy coat were diluted with Hanks' balanced salt solution (HBSS) and neutrophils were separated by centrifugation across Lymphoprep density gradient (Axis-Shield, Oslo, Norway). Hypotonic lysis (155mM NH_4Cl, 100μM Na_2EDTA, 10mM $NaHCO_3$) was used to remove red blood cells and isolated neutrophils were washed in HBSS and re-suspended

in platelet free plasma at $1.7x10^6$ cells/mL. Neutrophils were stimulated with the chemotactic peptide fMLP (1μM). O_2^- release was measured at 37°C by lucigenin (bis-N-methylacridinium nitrate, 10μM) mediated chemiluminescence at 20 second intervals for 5 minutes using a luminometer (6100 Pico-lite luminometer, Packard, Illinios). Data are expressed as area under the curve (0-5 minutes).

5.3.3 Malondialdehyde assay (MDA)

This method was conducted as described in chapter 2.5. Briefly, blood was anti-coagulated with Na-EDTA. Blood was centrifuged and plasma aspirated and stored at -70°C until assay. Plasma was extracted firstly by precipitation of proteins with perchloric acid followed by extraction with chloroform (to remove lipids). Extracted plasma was added to tubes containing thiobarbituric acid (TBA, 42mM) and orthophosphoric acid (0.15M). Samples were then boiled for 1 hour, after which unbound TBA was removed by extraction with 70:30 chloroform: methanol. Fluorescence intensity was measured at excitation λ of 530nm and emission λ of 547nm using a Perkin Elmer LS 50B luminescence spectrometer. Readings were corrected for an aqueous blank and concentration of MDA determined from a standard curve constructed in normal plasma spiked with standard (1,1,3-tetraethoxypropane hydrolysed in 0.15M orthophosphoric acid to form MDA).

5.3.4 Statistical Analysis

Categorical data were analysed using Fischer's exact test and continuous data by 1-way ANOVA (or Kruskall-Wallis test for non-normally distributed data, normality was determined using the D'Agostino & Pearson omnibus normality test). These statistics were calculated using Graphpad Prism V 4. Correlation between levels of the two markers of oxidative stress was analyzed using linear regression and determinants of MDA levels and neutrophil O_2^- release were evaluated by backwards stepwise multiple logistic regression analysis using SPSS. Data are expressed as mean ± SEM unless otherwise stated and the limit of statistical significance was set at $p<0.05$.

5.4 Results

5.4.1 Subject characteristics

The individuals evaluated were: 14 normal subjects, 30 patients with SAP and 32 patients with ACS (Table 5.1). These three groups were well matched for age and gender distribution. Patients with ACS were evaluated 12±1 hours after index episode of chest pain and 50% had non-Q-wave myocardial infarction as the index diagnosis. Baseline pharmacotherapy was similar between the SAP and the ACS groups with the exception of intravenously infused GTN and N-acetylcysteine (NAC).

5.4.2 Neutrophil O_2^- release

At baseline, levels of neutrophil O_2^- release did not differ significantly between groups (figure 5.1 ANOVA: K-W 2.35, p=0.31). However, there was a trend for mean higher values in ACS patients than in normals (normal: 3.8, SAP: 3.8 (median) and ACS: 7.06).

If the proportion of patients with elevation of neutrophil O_2^- release beyond the normal range (that is, two standard deviations above the mean for normal subjects (ie >7.63units) is considered, there was an excess of these elevated levels among patients with SAP (20%) and ACS (28).

In view of the results of the experiments outlined in chapter 4, it was appreciated that the differences between patients with SAP and ACS might result in part from infusion of GTN in the latter group. Therefore a secondary comparison was undertaken between these two groups, examining O_2^- release in the presence of GTN infusion in both cases (see figure 5.2). Data are summarised in figure 5.2, in which SAP patient who received perhexiline were excluded (given the actions of perhexiline in potentiating GTN effect: see chapter 4). Neutrophil O_2^- release was significantly greater in the ACS than the SAP group (unpaired t-test: means: ACS 7.06 vs SAP 3.08, p<0.001).

5.4.3 MDA

MDA levels within the ACS group were not normally distributed. Therefore between group analysis was performed using the non-parametric ANOVA (Kruskal-Wallis test). There was a significant difference between the groups (K-W=11.46,

p=0.003) and Dunn's post tests demonstrated that MDA levels were significantly greater in both the SAP and the ACS groups than in normal subjects ($p>0.05$ and 0.01 respectively, figure 5.3).

5.4.4 Correlations between O_2^- release and MDA levels

There was no correlation between levels of the two markers of oxidative stress in ant subject group. Data for the ACS patients are shown figure 5.4.

5.4.5 Determinants of elevation of markers in individual patients

Given the findings that MDA levels were elevated in both SAP and ACS patients, while O_2^- release was increased only in ACS patients (taking into account the GTN effect (see figure 5.2). The ACS cohort was utilised to attempt to identify possible determinants of increases of oxidative stress in individual patients, via backwards stepwise multiple logistic regression. Variables entered into the model were: interval between index pain and sampling, presence/absence of biomarkers of infarction (CK/troponin rise), treatment with NAC, treatment with ACE inhibitor/AII receptor antagonist and number of coronary risk factors. Only the number of coronary risk factors ($\beta=0.41$, $p=0.03$) was significantly (and directly) correlated with neutrophil O_2^- release. None of these factors were significant correlates of MDA levels.

5.5 Discussion

Oxidative stress, detected in either the SAP or ACS group in the current study, might have represented both a precipitant and a result of the ischaemic process. Analogous with inflammation, extent of oxidative stress in individual patients might be postulated as a marker of short or medium term outcome. However, for this to be the case, it would be necessary for incremental stress to be easily detectable. The current study demonstrates that:

1. SAP is associated with only borderline increases in oxidative stress markers.
2. ACS is associated with more obvious increases in markers of oxidative stress, but the extent of increase is not associated with the presence/absence of infarction.
3. There was no correlation between values of the two markers examined in individual patients.

Neutrophil O_2^- release did not differ significantly between groups at baseline. However all patients with ACS were receiving GTN, which suppresses neutrophil O_2^- release (see chapter 4) and possibly MDA formation. In the presence of GTN, O_2^- release was significantly greater in the ACS that the SAP patient group.

The differences between groups were somewhat similar utilising MDA as a marker: although there was a trend for the highest levels to occur in the ACS patients (figure 5.3), statistical analysis showed significant elevation in both the ACS and SAP groups, relative to normal subjects.

The utilisation of a patient group with relatively mild angina (class II and III with 42% having only 1 major coronary vessel with greater than 50% stenosis) and high proportion on optimal medical treatment may be of relevance as regards the O_2^- data. Thus, these results may not reflect the entire clinical spectrum of SAP, especially in untreated patients, but do suggest that systemic oxidative stress is limited. This point is further supported by studies which included both coronary sinus sampling and peripheral sampling and found that activation of neutrophils and increased O_2^- release can only be demonstrated in coronary sinus samples (De Servi et al., 1995; Kowalski et al., 1997).

While patients with ACS had evidence of incremental oxidative stress, there was no significant correlation between neutrophil O_2^- release and MDA levels. Furthermore, only the total number of coronary risk factors was predictive of neutrophil O_2^- release and none of the putative modulators of MDA levels were significant predictors of this parameter. A similar study was reported by Huraux et al (1999), utilising ex vivo O_2^- release from internal mammary arteries. In that study, total number of coronary risk factors and hyperlipidaemia were correlates of incremental O_2^- release. However in the current study, the majority of patients with hyperlipidaemia had been treated with statins, which may exert anti-oxidant effects (Wassmann et al., 2002). It therefore appears that within the spectrum of ACS patients receiving nitrate therapy, systematic increases in markers of oxidative stress will be limited and show little relationship to either activity of ischaemia or pharmacotherapy.

50% of the patients in the ACS group were receiving NAC, which reduces O_2^- release from neutrophils (Kharazmi et al., 1988; Sadowska et al., 2006), and which we

have previously shown to suppress MDA in STEMI patients (Arstall et al., 1995). However, the administration of NAC in ACS patients in the current study was not randomized: it was utilised for management of patients with recurrent or prolonged chest pain within the group. Therefore any potential effects of NAC on either O_2^- release or MDA may have been obscured by selection bias. This may also have affected results by limiting oxidative stress in the most severe patients.

The major limitations of the current study were: firstly, the inability to study ACS patients prior to the administration of nitrates, which was a virtually inevitable problem and secondly, the lack of methodology for comparing oxidative stress within the coronary circulation with systemic values. For example, it is possible that sampling of coronary sinus blood would have provided greater evidence of the heterogeneity between groups and individual patients, as it has for markers of platelet activation (van den Berg et al., 1989) and neutrophil myeloperoxidase content (Buffon et al., 2002).

5.6 Conclusions

The study demonstrates that both ACS and (marginally) SAP are associated with incremental oxidative stress. However, different measures of oxidative stress provide markedly different results as regards magnitude of disturbance in individual patients, to the extent that no single estimate of either O_2^- release nor of MDA, a product of free radical effect, would prove of value as a correlate of extent of ongoing ischaemia in individual patients.

	Normal n=14	SAP n=30	ACS n=32
Age	**53±3**	**60±2**	**60±1**
Gender (% male)	**57**	**63**	**60**
Coronary Risk Factors (%)			
Smoking	**0**	**30**	**47**
Hypertension	**0**	**47**	**50**
Diabetes Mellitus	**0**	**17**	**31**
Hypercholesterolemia	**0**	**77**	**75**
Concurrent Pharmacotherapy (%)*			
Aspirin	**0**	**100**	**100**
ACE inhibitor/AII receptor antagonist	**0**	**43**	**38**
ß-adrenoceptor antagonist	**0**	**30**	**25**
Calcium channel antagonist	**0**	**60**	**69**
Statin	**0**	**63**	**41**

Table 5.1. Patient characteristics * All patients in the ACS group also received intravenously infused GTN (2.5-10µg/min). Patients with SAP were studied before and after infusion of GTN. 38% of ACS patients also received intravenous N-acetylcysteine.

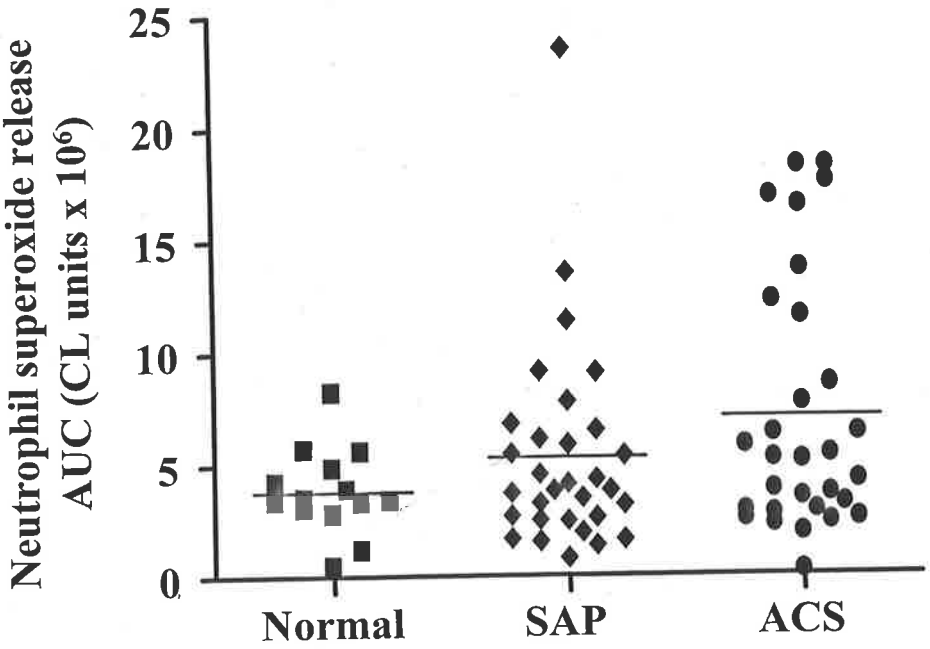


Figure 5.1. Neutrophil O_2^- release as area under the chemiluminescence time curve in: normal subjects, patients with stable angina (SAP), prior to administration of nitroglycerine and patients with acute coronary syndromes (ACS). There was no significant difference between any groups. Patients with ACS were all receiving intravenous GTN±NAC (K-W 2.35, p=0.31).

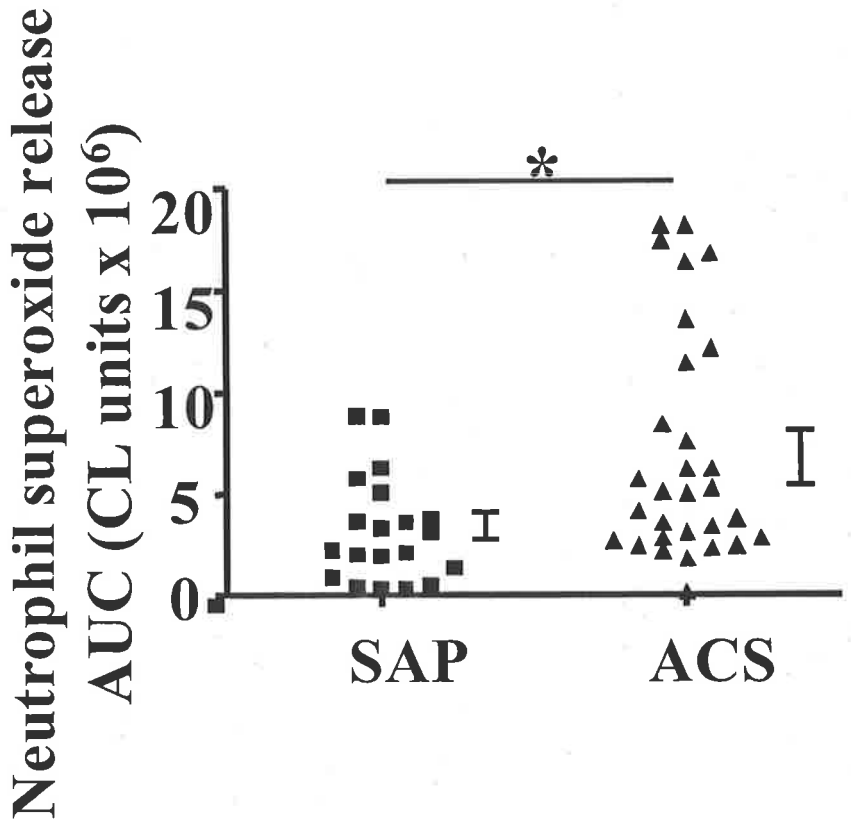


Figure 5.2. Comparison of neutrophil O_2^- release in the presence of infused GTN. Among SAP, only those treated with GTN alone (ie those without perhexiline) are considered, $p<0.001$, unpaired t-test.

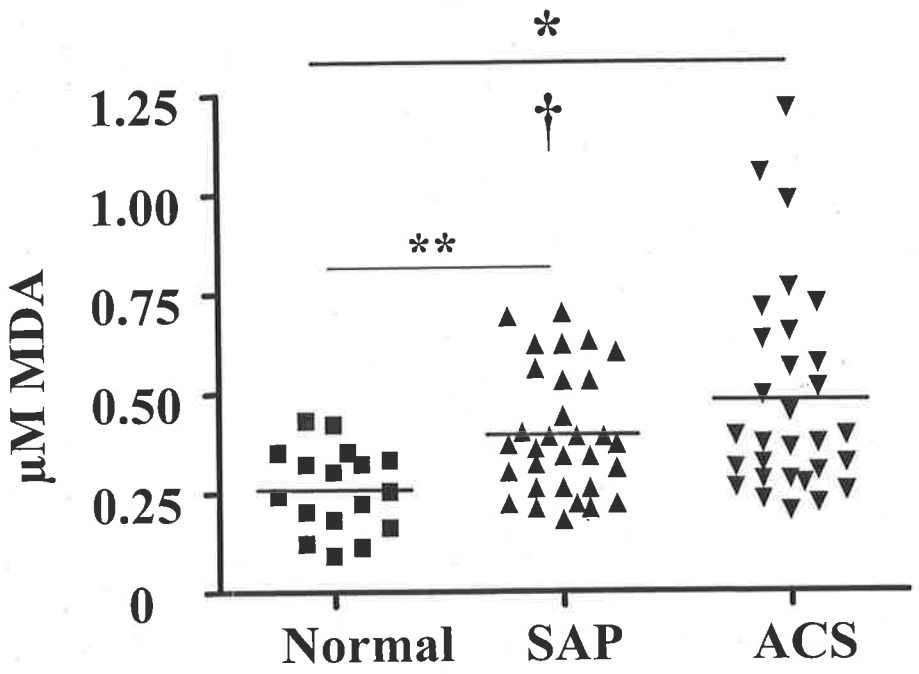


Figure 5.3. MDA levels in normal subjects, patients with stable angina (SAP) and acute coronary syndromes (ACS). There was a significant difference between groups (Kruskall-Wallis: K-W=11.46, p=0.003*) and post-hoc analysis demonstrated that MDA levels were significantly greater in SAP and ACS groups than in normal subjects (Dunn's post tests, p<0.05** and 0.01† respectively).

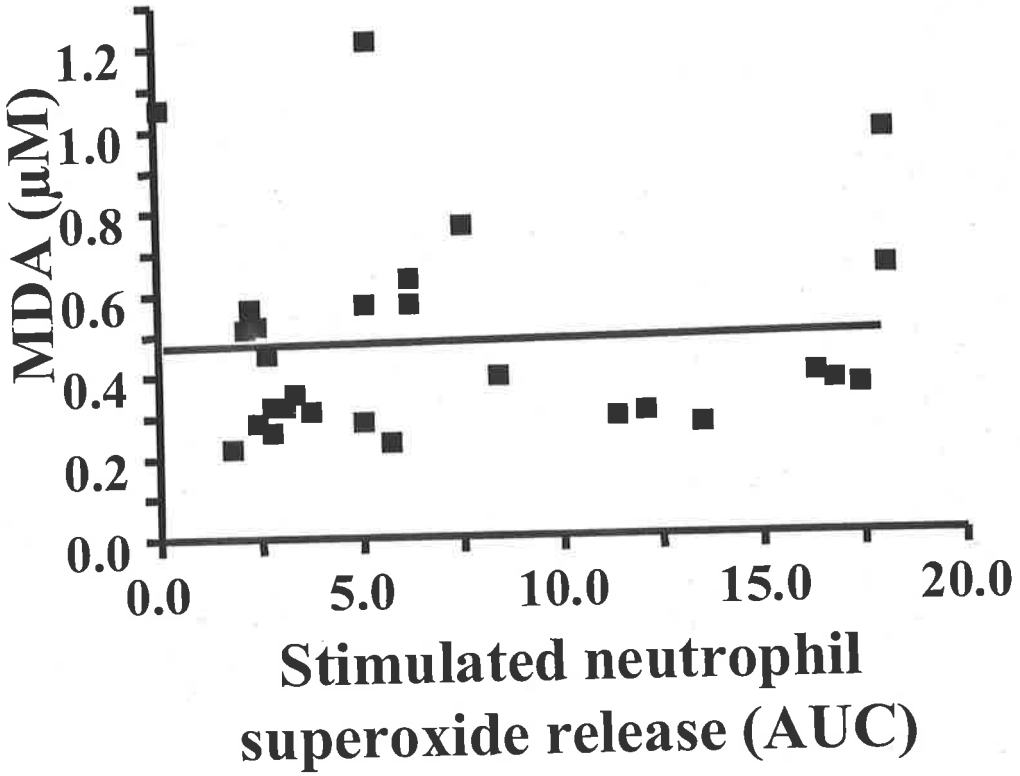


Figure 5.4 Relationship between neutrophil O_2^- release and MDA in patients with acute coronary syndromes (r2=0.01, p=0.4).

Chapter 6: Effects of therapy with ramipril on platelet responsiveness to nitric oxide: implication of oxidative stress

6.1 Summary

In general, trials designed to reduce incidence of cardiovascular events via redox active medications have yielded disappointing results. In the HOPE trial, ramipril, which inhibits angiotensin converting enzyme (ACE) and thus potentially obviates the NAD(P)H oxidase stimulating effects of angiotensin II (AII), improved cardiovascular outcomes, while vitamin E supplementation was without beneficial effect.

In the PLATELET-HOPE study, we sought to determine whether the effects of ramipril might reflect improvements in platelet responsiveness to nitric oxide (NO), and whether these changes might be related to reductions in redox stress and endothelial dysfunction. One hundred and nineteen patients whose characteristics were compatible with the HOPE study entry criteria were treated with ramipril or placebo in a double-blind trial of three months duration. Platelet responsiveness to the NO donor sodium nitroprusside (SNP), corrected for extent of aggregation, was utilised as the primary endpoint. Asymmetric dimethyl arginine (ADMA) was measured as a marker of endothelial dysfunction, while pulse-wave analysis was used to quantitate changes in apparent arterial stiffness (measured as augmentation index: AIx). Plasma malondialdehyde (MDA) concentrations were utilised as indices of oxidative stress.

Ramipril significantly ($p<0.001$) improved aggregation-corrected SNP response, while also reducing AIx ($p=0.02$) and ADMA ($p=0.05$). There was no significant difference in MDA concentration. Prospectively defined stratification of the patients on the basis of pre-ramipril responsiveness to SNP revealed platelet function improved only

in those who were initially NO resistant. Within the sub-population there was no effect of ramipril on either ADMA or MDA levels.

It is concluded that ramipril sensitises platelets to NO, but that this effect is essentially limited to the NO resistant (ie high risk) subset of the population. Despite the theoretical effect of ramipril in inhibiting O_2^- release and thus reducing redox stress; this effect was not documented in the current population.

6.2 Introduction

Angiotensin converting enzyme (ACE) inhibitors are well established in the treatment of heart failure, arterial hypertension, post-acute myocardial infarction and high risk coronary artery disease. Various studies have demonstrated ACE inhibitors also reduce the incidence of fatal and non-fatal atherothrombotic-related complications, including reductions in mortality in patients with either chronic heart failure (CONSENSUS (Swedberg and Kjekshus, 1988), SOLVD (1992)) or evidence of heart failure after acute myocardial infarction (SAVE (Rutherford et al., 1994), AIRE(1993)).

The ramipril arm of the recent Heart Outcomes Prevention Evaluation (HOPE) study (Yusuf et al., 2000b) demonstrated that high dose ramipril (10mg/day) reduced the rates of death from cardiovascular events in a "high risk" patient population (at least 55 years old, with a history of coronary artery disease, stroke, peripheral vascular disease, or diabetes plus at least one other cardiovascular risk factor (including hypertension, elevated total cholesterol levels, low high-density lipoprotein cholesterol levels, cigarette smoking or documented microalbuminuria). The HOPE study also demonstrated that rates of myocardial infarction, stroke, cardiac arrest, heart failure and complications relating to diabetes were significantly reduced with ramipril therapy. Furthermore, the EUROPA study (Fox, 2003) reported a 20% reduction in risk of the combined endpoint of cardiac arrest, myocardial infarction or death from cardiovascular disease in perindopril-treated patients.

However, not all ACE inhibitor trials have demonstrated beneficial effects. The recently reported Prevention of Events with Angiotensin Converting Enzyme Inhibition

(PEACE) study showed no beneficial effects of trandipril: the reason(s) for this negative result remain speculative (Pfeffer et al., 2001).

Irrespective of results, none of these studies were designed to provide mechanistic data regarding potential beneficial effects of ACE inhibitors. However, many potentially beneficial effects of ACE inhibitors have been demonstrated, and may contribute to improved outcomes. There are two main categories of demonstrated effects which may contribute to the beneficial effects of ACE inhibitors, these can be divided into: haemodynamic improvement, such as blood pressure reduction and improvement of endothelial function, and non-haemodynamic effects including inhibition of cellular growth factors (Soejima et al., 1999; We et al., 2002; Youn et al., 1999), ventricular and vascular remodeling (Ali et al., 1998; Beckwith and Munger, 1993; Sihm et al., 1995) and superoxide release via NAD(P)H oxidase (Berry et al., 2000; Wingler et al., 2001).

NAD(P)H oxidase inhibition is an indirect effect of ACE inhibition, via reversal of AII induced upregulation of the NAD(P)H oxidase subunits. While upregulation of NOX-1, NOX-4 (Wingler et al., 2001) and p22Phox (Fukui et al., 1997) have been demonstrated in animal models and in human smooth muscle cell preparations (Touyz et al., 2002; Touyz et al., 2005), at present there is no evidence of direct in vivo effects on activity or expression of NAD(P)H oxidase of either angiotensin II or of ACE inhibitors. The role of anti-oxidant mechanisms is also brought in to question by the failure of several large trials utilising anti-oxidant therapy, notably the vitamin E arm of the HOPE trial to demonstrate any beneficial effect on outcomes (Yusuf et al., 2000a).

The question of mechanisms of risk reduction is also complicated by the finding that while ACE inhibitors treatment does reduce AII levels acutely (Johnston et al., 1981), that this effect is often not sustained presumably due to induction of other pathways such as the chymase pathway (Chen et al., 2005). Despite this, the beneficial effects of ACE inhibitor therapy on cardiovascular end-points persist.

Chirkov et al (1999) have previously demonstrated that patients with either stable angina or acute coronary syndromes exhibit increased platelet aggregability and decreased platelet responsiveness to the anti-aggregatory effects of nitric oxide donors ("NO resistance"), a phenomenon which is in part due to increased oxidative stress. Specifically, in patients with stable angina, increased whole blood superoxide content has been causally implicated in NO resistance. Platelet NO resistance is an independent predictor of cardiac death or cardiovascular-related readmission during long-term follow-up (Willoughby et al., 2005). Furthermore, in patients with congestive heart failure (NYHA class II-IV) platelet NO responsiveness was markedly increased by short-term perindopril therapy (Chirkov et al., 2004). These data suggest that improvement in platelet function via limitation of NO resistance may contribute to the beneficial effect of ACE inhibitors in preventing acute thrombotic events (eg as in the HOPE and EUROPA studies).

We conducted a randomised, double-blind, parallel group trial to examine the efficacy of ramipril (10mg/day) versus matched placebo in modulating platelet NO responsiveness in patients at high cardiovascular risk. In addition we examined the effects of ramipril on apparent arterial stiffness, endothelial function as measured via

plasma concentrations of asymmetric dimethyl arginine (ADMA) a marker of endothelial dysfunction (Mittermayer et al., 2005; Perticone et al., 2005) and oxidative stress measured via plasma MDA concentrations (Arstall et al., 1995). The study investigated the effect of ramipril over a 3 month period.

6.3 Methods

6.3.1 Subjects

A total of one hundred and nineteen patients (mean age 67±8 (SD) years) who were at high cardiovascular risk were enrolled in the study. The study was approved by the Ethics of Research Committee of the Queen Elizabeth Hospital and written informed consent was obtained prior to study entry.

6.3.1.1 Inclusion criteria

Inclusion criteria were similar to that of the HOPE study (Yusuf et al., 2000b). Eligible patients included men and women aged ≥ 50 years who had a history of symptomatic coronary artery disease, stroke, peripheral vascular disease, and/or diabetes plus one other risk factor (hypertension, elevated total cholesterol level, low high density lipoprotein cholesterol levels, cigarette smoking or documented mircoalbuminuria).

6.3.1.2 Exclusion criteria

Patients were excluded if they had heart failure, were taking an ACE inhibitor, angiotensin receptor antagonist or ADP receptor antagonist (the latter due to resultant problems with assessment of platelet function), had uncontrolled hypertension or overt nephrology, had suffered a myocardial infarction or stroke within four weeks prior to study entry. Furthermore, all patients who had initial ADP-induced aggregation responses of less than 4 ohms were excluded due to resultant difficulty in measuring inhibition of aggregation.

6.3.2 Study design

As shown in figure 6.1, potentially eligible patients (n=202) attended a screening session where blood samples were collected for the measurement of platelet aggregation. Patients who had more than 4 ohms of ADP-induced aggregation (n=119) at screening were randomised to receive ramipril or matched placebo. Ramipril was initiated at 5mg/day. After 1 week of therapy all patients had a return visit where ramipril was up-titrated to 10mg unless contraindicated (ie. significant cough, symptomatic hypotension). All investigations performed at baseline were repeated after 4 and 12 weeks.

6.3.3 Criteria for withdrawal

Criteria were firstly, inability to tolerate doses of ramipril of 5mg or greater, and occurrence of a major cardiovascular event during therapy. Patients were withdrawn

from the study due to: 2 new cardiovascular events, 5 withdrew at their own volition, 2 due to cough, 1 due to dizziness and 1 did not disclose that they were receiving ACE inhibitor therapy. There were more withdrawals in the ramipril group (9) than in the placebo group (2: 1 opted out and 1 new cardiovascular event).

6.3.4 Endpoints

The prospectively defined primary endpoint of the study was platelet responsiveness to the NO donor sodium nitroprusside, expressed alone or corrected for changes in ADP-induced aggregation if necessary after 12 weeks therapy.
Secondary endpoints were:

1. Changes in ADP-induced aggregation
2. Changes in augmentation index (AIx), a measure of apparent arterial stiffness which is responsiveness to NO bioavailability (Wilkinson et al., 2002a).
3. Plasma ADMA concentrations.
4. Plasma MDA concentrations.
5. Changes in intra-platelet cyclic guanosine monophosphate (cGMP).
6. Changes in all of the above parameters in the subset of the population who exhibited NO resistance at the platelet level at baseline.

6.3.3 Platelet Function

Platelet studies were conducted as described in chapter 2.4. Briefly, citrated venous blood was diluted 2 fold with 0.9% saline. Aggregation in response to adenosine 5'-diphosphate (ADP, 1μM) was measured using a dual channel impedance aggregometer (Model 560, ChronoLog) and recorded as maximal aggregation (in ohms).

Inhibition of aggregation by sodium nitroprusside (SNP, 10μM) was determined by measuring maximal aggregation to ADP after 1 minute pre incubation with SNP and results were expressed as percent inhibition of maximal aggregation by SNP.

6.3.4 Pulse wave analysis

Pulse wave analysis was used to determine AIx (SyphymoCor software) as described in chapter 2.6. Briefly, a micromanometer probe (SPT-301B; Millar Instruments, Texas, USA) was used to obtain recordings of the peripheral pressure waveforms by flattening, the radial artery of the dominant arm. Data were collected directly into the SyphymoCor system and after 20 sequential waveforms had been acquired, an average peripheral waveform was generated. The waveform was then scaled from brachial artery blood pressure. The corresponding central (ascending aortic) waveform was derived from the radial artery waveform using a validated transfer function; from this augmentation and AIx was then derived.

6.3.5 Asymmetric Dimethyl Arginine

Plasma concentrations of the endogenous nitric oxide (NO) synthase inhibitor ADMA, its inactive stereo-isomer $N^G,N^{G'}$-dimethyl-L-arginine (SDMA) and of arginine, the substrate of NO synthase, were measured by T. Heresztyn via a previously published HPLC method (Heresztyn et al., 2004).

6.3.6 Malondialdehyde (MDA)

This method was conducted as described in chapter 2.5. Briefly, blood was anti-coagulated with Na-EDTA. Blood was centrifuged and plasma aspirated and stored at -70°C until assay. Plasma was extracted firstly by precipitation of proteins with perchloric acid followed by extraction with chloroform (to remove lipids). Extracted plasma was combined with thiobarbituric acid (TBA, 42mM) and orthophosphoric acid (0.15M). Samples were then boiled for 1 hour, after which a further extraction with 70:30 chloroform: methanol was undertaken in order to minimise interferance by unbound TBA. Fluorescence intensity was measured at excitation λ of 530nm and emission λ of 547nm using a Perkin Elmer LS 50B luminescence spectrometer. Readings were corrected for an aqueous blank and concentration of MDA determined from a standard curve.

6.3.7 Intra-platelet cGMP determination

Platelet-rich plasma (0.5 ml) was incubated at 37°C with SNP (10 μmol/L) for 1 min. Intraplatelet cGMP content was assayed as described previously (Chirkov et al., 1999).

Briefly, after incubation plasma was filtered through GF/C Glass Microfibre Filters (Whatman, UK) for harvesting the platelets. Filters with absorbed platelets were rinsed with physiological saline and placed into 0.5 ml of 4 mM EDTA for further extraction of cGMP in a boiling water bath for 5 min. After centrifugation of samples at 3000 *g* for 10 min, cGMP concentration in supernatant was estimated using "cGMP [^{125}I] assay system" (Amersham).

6.3.5 Statistical analysis

All data were analysed according to intention to treat, and the primary hypothesis was analysed via analysis of covariance of the ADP:SNP response relationship. Baseline characteristics of the placebo and ramipril groups were compared utilizing non-paired t-test (all parameters examined being normally distributed).

The change in AIx, MDA, ADMA and cGMP from baseline to 3 months was compared between placebo and ramipril groups using non-paired t-tests.

As regards the issue of subset analysis:

1. "NO resistant" patients: The presence of baseline NO resistance was defined as a baseline SNP response of less than 32% representing a response greater than two standard deviations below the mean expected in normal volunteers (as described in (Willoughby et al., 2005)). Data from the subset of patients were analysed in the same way as for the entire cohort, and compared with data from the remainder of the patient population.

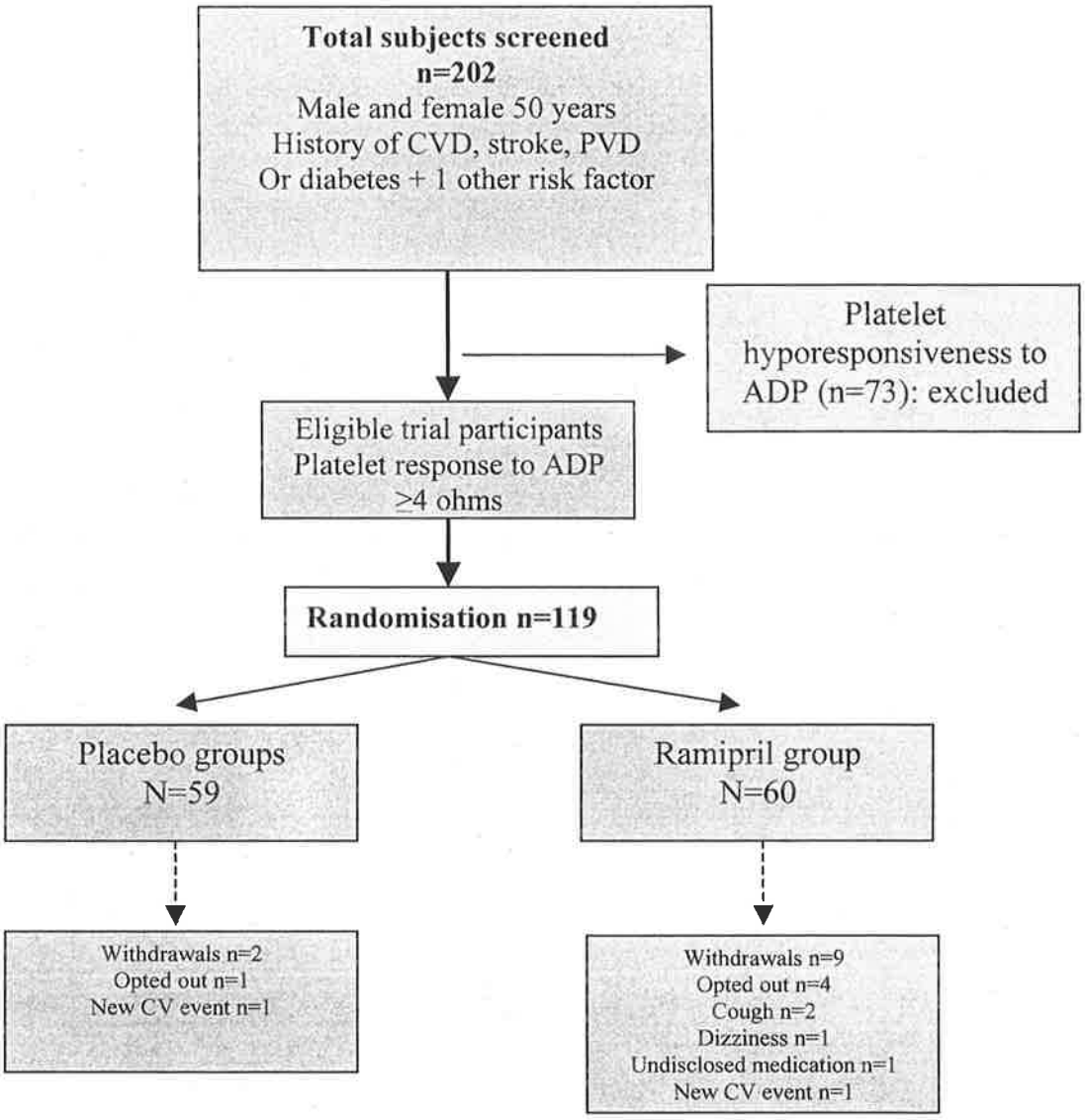


Figure 6.1: Trial Schematic

2. Data after 4 weeks treatment: In view of the prospective decision to utilise only 12 week data for the primary end-point analysis, we utilised week 4 data only for the secondary end-point of examination of time-course of all significant effects of ramipril. Analysis was via repeat measures ANOVA with post-hoc Dunnett's test for multiple comparisons.

All statistical analysis was performed utilizing SPSS (Version 12). Data are expressed as mean ± SEM, unless otherwise stated.

6.4 Results

6.4.1 Subject characteristics

Baseline demographic characteristics of those patients eligible for study entry are summarised in Table 6.1. Placebo and Ramipril groups were generally well matched with regards these parameters. As regards baseline pharmacotherapy (table 6.2), there was agreater use of statin therapy within the placebo groups (p=0.02 vs ramipril group). Study medication was generally well tolerated.

6.4.2 Platelet aggregation

Baseline platelet responsiveness to ADP (1 μM) was similar between groups (7.6±2.6 vs 7.4±2.4 Ohms, placebo vs ramipril groups respectively p=0.66). There was a

progressive minor reduction in the extent of aggregation to ADP over the 3 month period. This reduction tended to be greater (p=0.2) in the placebo, than in the treated individuals (figure 6.2).

6.4.3 Platelet NO responsiveness

Mean platelet responsiveness to SNP for the total cohort was 44.8±2.6 % inhibition of aggregation. There was no significant difference in NO responsiveness between groups at study entry (47.3±3.7% vs 42.4±3.7 % inhibition of aggregation, placebo vs ramipril group, p=0.35). Using response of less than 32% as criteria for platelet resistance, a total of 49 patients (28 in the ramipril group and 21 in the placebo group) were NO resistant.

Due previous studies demonstrating that SNP response is dependant on ADP response (Chirkov et al., 2001) and to the reduction in ADP response with time, which tended to be greater in the ramipril group (see figure 6.2), all SNP data for the entire patient group were analysed relative to ADP response.

The change in SNP: change in ADP response relationship over three months of ramipril vs placebo therapy is depicted in figure 6.3. For placebo-treated patients, mean SNP response did not vary significantly irrespective of ADP response. However, in ramipril-treated patients, there was a markedly inverse relationship between SNP and ADP responses. This SNP-ADP response relationship was significantly different

($p<0.001$) for ramipril vs placebo treated groups, with sensitisation to SNP by ramipril per unit ADP response.

In order to investigate the time course of this effect, we also performed an ANCOVA analysis on the change in SNP: change in ADP response relationship at 4 weeks (figure 6.3), which demonstrated a similar relationship at this time point ($p<0.001$), suggesting that the platelet effects of ramipril occur in the first 4 weeks and are then maintained through to the week 12 time-point.

6.4.4 Blood pressure and pulse wave analysis

Three months of ramipril therapy marginally reduced systolic blood pressure (-5.2±2.3 vs -11.7±3.0 mmHg, placebo vs ramipril group, $p=0.07$). Diastolic blood pressure was significantly reduced by ramipril therapy (-1.8±1.3 vs -6.2±1.3 mmHg, placebo vs ramipril group, $p=0.03$). Mean arterial pressure was also significantly reduced (Table 6.2). There was no significant change in heart rate in either the placebo and ramipril groups. We also examined the effect of ramipril therapy on arterial stiffness, as measured by AIx. At study entry there was no difference in AIx between the placebo and ramipril groups (23.7±1.2% vs 24.9±1.2% respectively). After 3 months therapy, there was no change in AIx in the placebo group (+0.7±1.3%), and AIx was significantly decreased in the ramipril group (-3.9±1.6, $p=0.02$, table 6.3).

6.4.5 ADMA

ADMA levels were elevated in the ramipril group (0.59±0.01μM) compared to the placebo group (0.55±0.01 μM, $p<0.01$) at baseline. Changes in plasma concentrations of ADMA, utilised as a marker of endothelial dysfunction, are summarised in figure 6.4. ADMA concentrations were differentially reduced in patients receiving ramipril therapy at 12 weeks ($P=0.05$, figure 6.4). However the difference in change in ADMA concentrations between groups was small: approximately 7% of mean values for the patient group studied. In order to establish to time course for this effect on ADMA, we performed a secondary analysis of changes in ADMA concentrations in the first four weeks in the placebo and ramipril groups. There was no significant difference in the magnitude of the change between baseline and week 4 between groups (see figure 6.4). This suggests that the change in ADMA, while only marginally significant, requires more than 4 weeks of therapy.

6.4.5 MDA

Baseline MDA concentrations were not different between groups at baseline (placebo: 0.36±0.03μM vs ramipril: 0.39±0.04μM). On univariate analysis, the following parameters were examined as possible correlates of differential MDA concentration: age, gender, cholesterol, body mass index, baseline aggregation response, and baseline platelet SNP response. None of these were associated with significant deviation of concentrations from those of the group mean.

Treatment with ramipril was associated with a small mean reduction in MDA concentrations of the order of 10%, but did not reach statistical significance relative to placebo (figure 6.5, Mann-Whitney test, p= 0.25).

6.4.6 Platelet cGMP

There was no difference between cGMP levels in placebo and ramipril groups at baseline. There was also no significant change over 12 weeks in either group and therefore there was no difference in the magnitude of change between groups (table 6.3).

6.4.7 ADP-induced aggregation subgroups

There was no difference in the extent of ADP-induced aggregation between treatment groups amongst normal NO responders groups or impaired NO responder groups at baseline. ADP-induced aggregation was reduced after 3 months of therapy in those with normal responders in both treatment groups but not in the impaired responder subgroups (see figure 6.6). In normal NO responders change in ADP response was -1.7±0.5 vs -0.2±0.6 Ohms (placebo vs ramipril group, p=0.05), while for impaired NO responders change in ADP response was -0.6±0.4 vs -1.7±0.5 Ohms (placebo vs ramipril group, p=0.1).

6.4.8 Impaired vs normal platelet NO responsiveness

Subject groups were divided into those patients who had a normal, greater than 32% inhibition of aggregation by SNP or impaired NO response ≤32 % inhibition by SNP at study entry. Utilising these criteria, 38 subjects in the placebo group were classified as having a normal response, and 21 as having an impaired response. In the ramipril group 32 subjects had a normal response while 28 had impaired responses. There was no significant difference in the NO responsiveness between ramipril and control groups in either subgroup at baseline (see figure 6.7). However at week 12, NO responsiveness was increased compared to placebo in the poor responders ($p<0.002$), while NO responsiveness was unchanged in the normal responders ($p=0.4$). As described above, extent of aggregation is a determinant of SNP responsiveness; therefore we performed 2 separate ANCOVAs in order to examine the effect of ramipril on the relationship between change in ADP and change in SNP response in both the impaired responder and normal NO responder groups (Figure 6.8). There was a significant change in ADP: change in SNP relationship between the placebo and ramipril groups for subjects with an impaired NO responsiveness at study entry ($P<0.001$), but no difference in the normal responder group. This result demonstrates that improvement in NO responsiveness is dependant on impaired responsiveness at baseline.

6.4.9 Biochemical marker in normal and impaired NO responders.

At baseline there was no difference between any parameters, in any group, with the exception of plasma ADMA levels being higher in the impaired ramipril group than in the impaired placebo group. There were no differential effects of ramipril on ADMA or MDA in either the normal or impaired NO responder groups (see table 6.4).

6.5 Discussion

The primary endpoint of the current study was the effect of ramipril on platelet responsiveness to NO. Given the dependence of NO responsiveness on initial aggregation response, the study utilised the relationship between change in SNP response and change in ADP response to demonstrate by analysis of co-variance that treatment with ramipril causes a non-parallel upward shift in this relationship. The secondary endpoints of the study utilised biochemical markers (markers of oxidative stress, endothelial function and soluble guanylate cyclase activity) and subset analysis (impaired vs normal NO responders) to investigate mechanisms that may modulate this effect.

Apparent arterial stiffness, a NO sensitive parameter measured by AIx, was decreased after 12 weeks in the ramipril group and ADMA, a marker of endothelial dysfunction, was marginally reduced by 12 weeks of ramipril therapy. The potential impact of oxidative stress on changes in NO responsiveness was also examined by evaluation of MDA, a measurement of lipid peroxidation. The current study showed no effect of ramipril on MDA levels. Intra-platelet cGMP, utilised as a measure of potential sensitisation of soluble guanylate cyclase, was also unaffected by ramipril therapy.

Investigation of changes in SNP responsiveness in those who had the lowest responses at baseline ("NO resistant"cohort) revealed that the greatest ramipril induced sensitisation response occurred in this group, indeed, in those with normal responses at baseline, the relationship between change in aggregation and change in SNP was not significantly different from that in the placebo group.

This study adds to current knowledge by demonstrating that ramipril alters the relationship between ADP response and SNP response, and that it sensitises platelets from those with poor responsiveness to the anti-aggregatory effects of NO. Currently there are no analogous studies in this population although it has been demonstrated in a similar group that captopril and ramipril inhibit platelet aggregation induced by thrombin and captopril also inhibited ADP induced aggregation (Skowasch et al., 2006). A small, short term study in patients with congestive heart failure during treatment with perindopril demonstrated improvement in platelet NO responsiveness (Chirkov et al., 2004). While this study utilised patients who were commencing perindopril as a component of therapy, and as such was not randomised or blinded and there was no control group it did utilise a group with poor baseline responsiveness to NO donors. Another important feature of the latter group was the low rate of statin use, while in the current cohort more than 80% of the subject were treated with statins, which may sensitise platelets to NO (Chirkov et al., 1999). In another study, addition of ramipril to simvastatin therapy did not produce significant additional benefit over simvastatin alone on endothelial function, MDA or inflammatory markers (Koh et al., 2004). This may

have been a factor in lack of improvement of platelet NO responses in the current cohort given the high rate of statin utilisation.

Apparent arterial stiffness, measured via AIx, was significantly reduced by ramipril therapy in the current study. This result was as expected given that ACE inhibition reduces the concentration of angiotensin II, a potent vasoconstrictor which has well established effects on AIx (Kelly et al., 2001; Wilkinson et al., 2001), and both ramipril (Ahimastos et al., 2005) and the angiotensin II receptor blocker valsartan (Klingbeil et al., 2002) have been shown to decrease AIx. While it is likely that this result reflects reduced vasoconstriction, it is also possible that ramipril treatment improves vasodilator responses to endothelium derived NO. While AIx is not a direct measure of endothelial function, there is evidence that AIx is sensitive to changes in NO release (Wilkinson et al., 2002b). In this regard there was also a marginal differential reduction in levels of ADMA, a marker of endothelial dysfunction. It has been previously demonstrated that ACE inhibition reduces ADMA levels (Chen et al., 2002a; Delles et al., 2002; Napoli et al., 2004). While ADMA is commonly known as an endogenous NOS inhibitor, there is some evidence that ADMA concentrations may also be affected by oxidative stress via redox sensitivity of dimethylarginine dimethylaminohydrolase, the enzyme responsible for ADMA clearance (Jia et al., 2005).

However, the direct measure of oxidative stress utilised in this study, MDA, was unaffected by ramipril treatment. Previous studies have demonstrated reduction in MDA during treatment with ACE inhibitors. However these studies were conducted in patients with hypertension, who were receiving treatment with only one or two anti-hypertensive

agents (Mak et al., 1990; Napoli et al., 2004), as mentioned above, it is likely that background treatment of the current cohort my have contributed to the underestimation of ramipril effect. Despite the widely held belief that reduction in oxidative stress is a major component of the beneficial effect of ACE inhibitors, it must be emphasised that there are a multitude of large trials utilising anti-oxidant therapy, including the vitamin E component of the HOPE trial (Yusuf et al., 2000a), which show no benefit with anti-oxidant therapy. It may be that measures of "systemic oxidative stress" are missing more important and possibly specific localised effects. In vitro studies, have demonstrated that angiotensin II exerts direct actions on NAD(P)H oxidase (Berry et al., 2000; Wingler et al., 2001). NAD(P)H oxidase is a major source of superoxide anion production within the vasculature and is found in both the vessel wall and in phagocytes. It may have therefore, been beneficial to measure NAD(P)H oxidase activity rather than MDA. However direct measurement of NAD(P)H oxidase activity in vessels requires access to tissue samples, and while given the magnitude on the current study it was impractical to measure neutrophil superoxide release in all patients.

Overall, the results of the current study suggest that therapy with ramipril induces a relatively small increase in platelet NO responsiveness, which is most likely of little clinical relevance. However, given the much greater magnitude of the effect in patients with poor initial responses to NO, addition of ramipril therapy may be of benefit in this group.

6.6 Limitations

The major limitation of the current study is the lack of measurement of angiotensin II levels, particularly as there is some evidence that the angiotensin lowering effect of ACE inhibitors may be diminished after 3 months (Farquharson and Struthers, 2002; van de Wal et al., 2006). Another important limitation is the lack of a more direct measure of superoxide production or NAD(P)H oxidase activity or expression.

6.7 Conclusions and clinical implications

The major conclusion of the current study is that ramipril sensitises platelets to the anti-aggregatory effects of NO but that this effect occurs predominantly in patients with poor initial responses to NO. As regards mechanisms of this effect, we have failed to demonstrate a decrease in oxidative stress during ramipril treatment, utilising MDA as a marker. In terms of clinical implications, this finding suggests that ramipril therapy may be of greater benefit in the NO resistant subset of patients. This could be tested by dividing the HOPE cohort on the basis of clinical parameters associated with platelet "NO resistance" and examining differential effectivness.

The effect of ramipril on platelet function also suggests that ACE inhibitor therapy may have additional utility in the prevention of thrombus formation, for example, as adjunct therapy following angioplasty or stenting.

	Placebo (n=59)	Ramipril (n=60)	p-value
Male (%)	**38 (64)**	**35 (58)**	**NS**
Age (SD)	**66.4 (9.7)**	**67.2 (10.6)**	**NS**
Height (SD)	**166.8 (12.0)**	**165.1 (18.1)**	**NS**
Weight (SD)	**81.5 (18.7)**	**81.3 (19.5)**	**NS**
BMI (SD)	**29.6 (9.0)**	**30.2 (9.4)**	**NS**
SBP (SD)	**144.7 (23.2)**	**145.3 (24.5)**	**NS**
DBP (SD)	**82.6 (8.9)**	**80.6 (12.7)**	**NS**
HR (SD)	**68 (8.9)**	**66 (11.0)**	**NS**
Past MI (%)	**28 (48)**	**20 (33)**	**NS**
CABG (%)	**14 (24)**	**23 (38)**	**NS**
PCI (%)	**31 (53)**	**22 (37)**	**0.09**
Diabetes (%)	**9 (15)**	**15 (25)**	**NS**
Hypertension (%)	**26 (44)**	**21 (35)**	**NS**
Cholesterol level (SD)	**4.2 (0.9)**	**4.5 (0.9)**	**0.05**
Smoker (%)	**19 (32)**	**12 (20)**	**0.15**
Creatinine level (SD)	**0.092 (0.015)**	**0.094 (0.019)**	**NS**

Table 6.1. Patient Demographics at study entry: clinical parameters and coronary risk factors. All p-values greater than 0.10 have been designated as non-significant (NS).

Abbreviations: BMI, body mass index, SBP, systolic blood pressure, DBP, diastolic blood pressure, HR, heart rate, CABG, coronary artery bypass surgery, PCI, percutaneous coronary intervention

	Placebo (n=59)	Ramipril (n=60)	p-value
Aspirin (%)	**51 (86)**	**54 (90)**	**NS**
Statin (%)	**57 (97)**	**49 (82)**	**0.02***
B-adrenoreceptor antagonist (%)	**9 (15)**	**16 (27)**	**NS**
Calcium antagonist (%)	**41 (69)**	**31 (52)**	**0.06**
Nitrate (%)	**22 (37)**	**21 (35)**	**NS**
Warfarin (%)	**5 (8)**	**2 (3)**	**NS**
Perhexiline (%)	**1 (2)**	**4 (7)**	**NS**

Table 6.2. Pharmacotherapy at baseline (%). All p-values greater than 0.10 have been designated as non-significant (NS).

Parameter	Δ Placebo group N=57	Δ Ramipril Group N=51	p-value
MAP (mmHg)	-0.71±2.4	-8.1±1.8	0.02
SBP (mmHg)	-5.2±2.3	-11.7±3.0	0.07
DBP (mmHg)	-1.8±1.3	-6.2±1.3	0.03
HR (bpm)	1.1±2.0	2.2±1.2	NS
AIx (%)	0.7±1.3	-3.9±1.6	0.02
MDA (μM)	0.006±0.021	-0.042±0.036	NS
ADMA (μM)	0.007±0.001	-0.016±0.001	0.05
Intraplatelet cGMP (pM/10^9 platelets)	-3.1±5.7	-3.0±5.8	NS

Table 6.3. Effect of ramipril on measured parameters

	Normal responders		Impaired responders	
	placebo	**ramipril**	**placebo**	**ramipril**
ADMA (μM)	0.012±0.011	-0.013±0.012	0.004±0.011	-0.002±0.011
MDA (μM)	-0.008±0.02	-0.027±0.05	-0.04±0.04	-0.06±0.05

Table 6.4. Change in levels of biochemical markers in subjects who had normal (>32%) or impaired (≤32%) SNP response at baseline with 12 weeks of ramipril or placebo.

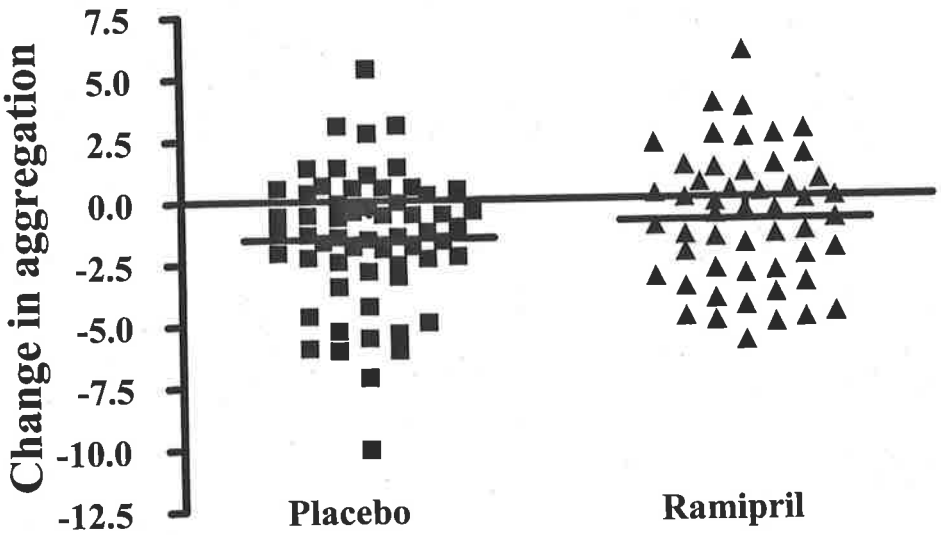


Figure 6.2. Effect of 12 weeks of ramipril or placebo on ADP induced aggregation. Unpaired t-test, p=0.22.

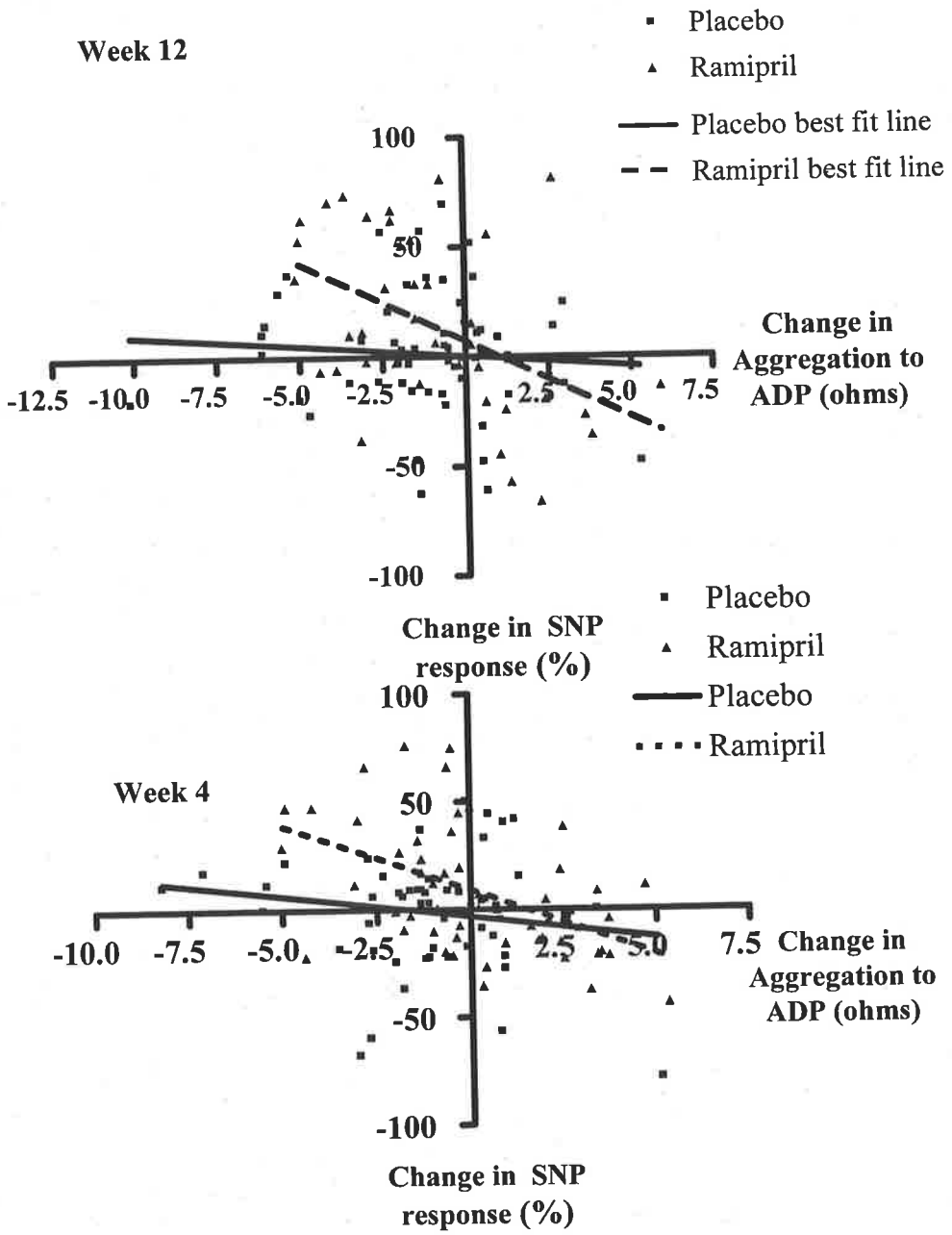


Figure 6.3. Effects of 12 weeks (upper) and 4 weeks (lower) of ramipril or placebo on the relationship between ADP and SNP responses. ANCOVA, $p<0.001$ both time points.

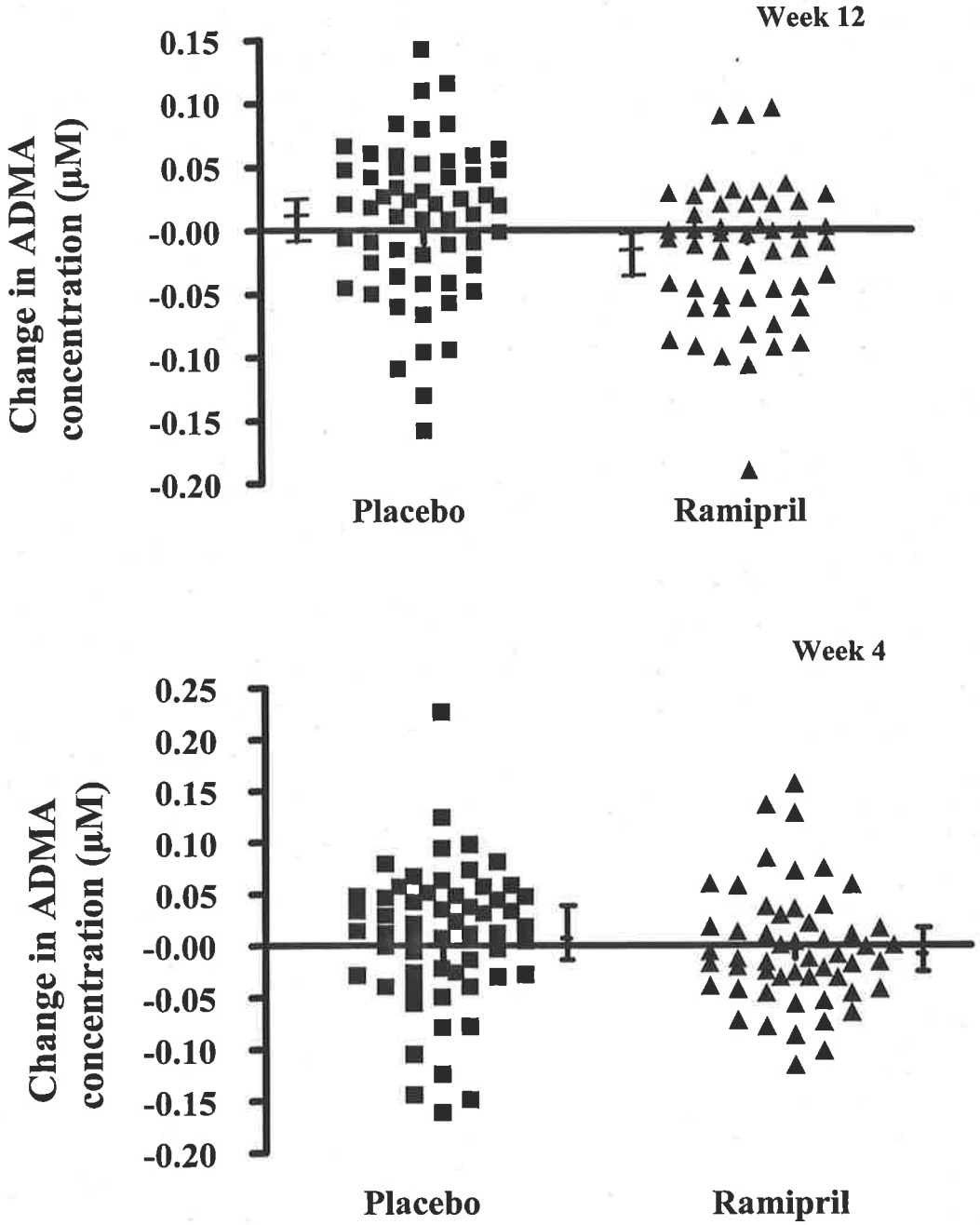


Figure 6.4. Effect of 12 weeks (upper) and 4 weeks (lower) of ramipril and placebo on plasma ADMA concentrations. Unpaired t-test, p=0.05 at week 12, p=0.4 at week 4.

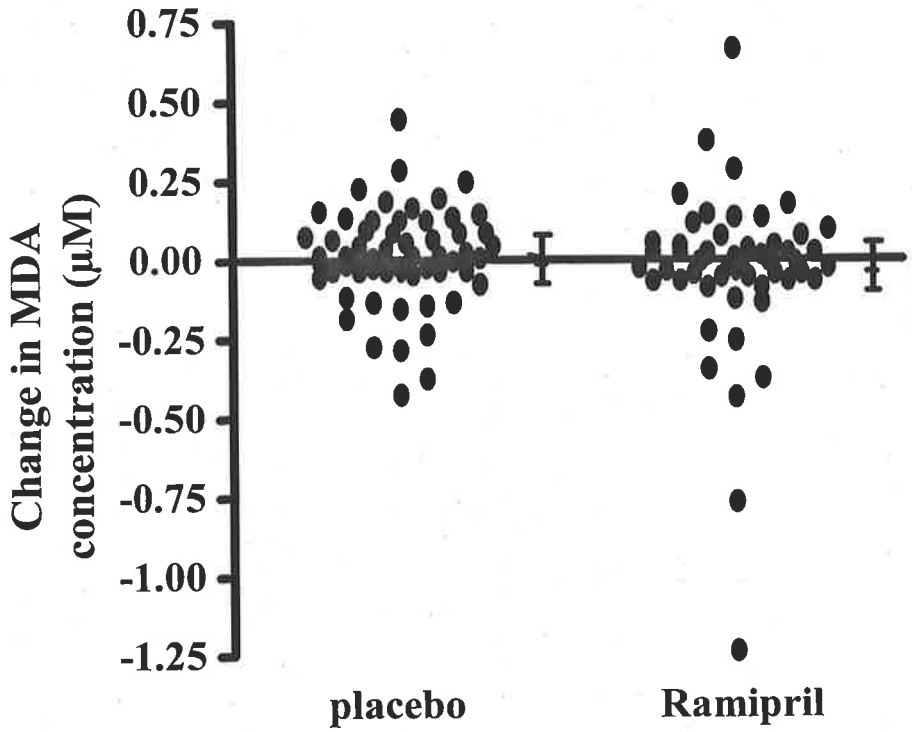


Figure 6.5. Effect of 12 weeks of ramipril or placebo on MDA concentration. Median ±interquartile range, MannWhitney test, p=NS

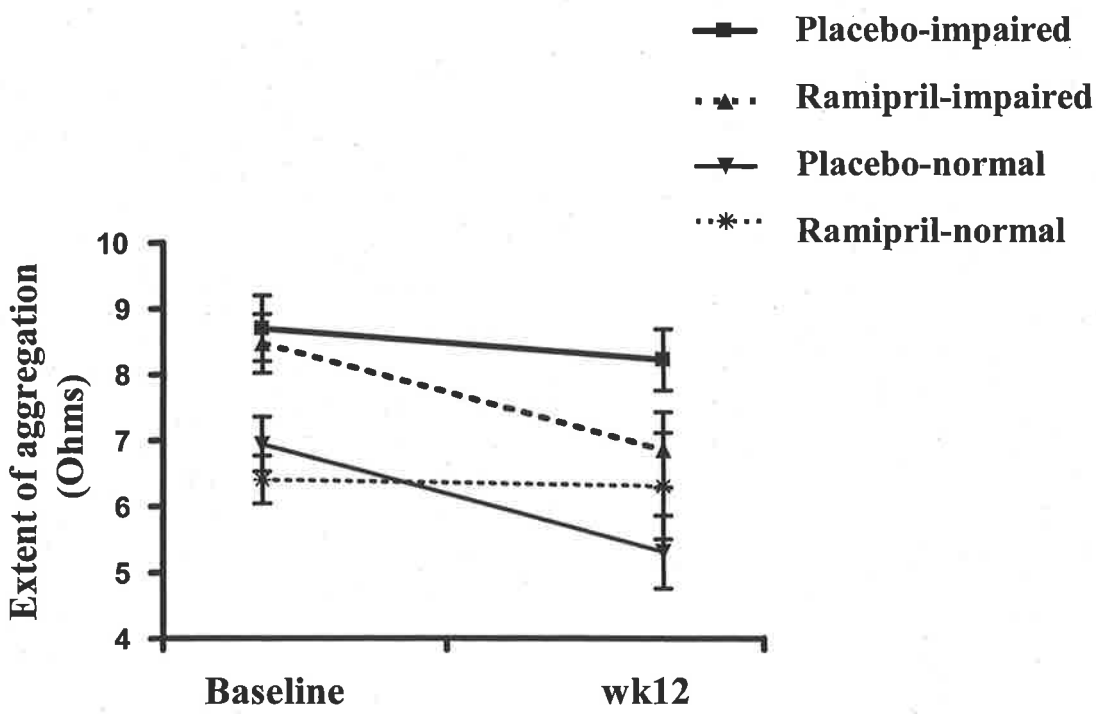


Figure 6.6 Change in aggregation in the impaired vs normal NO responders

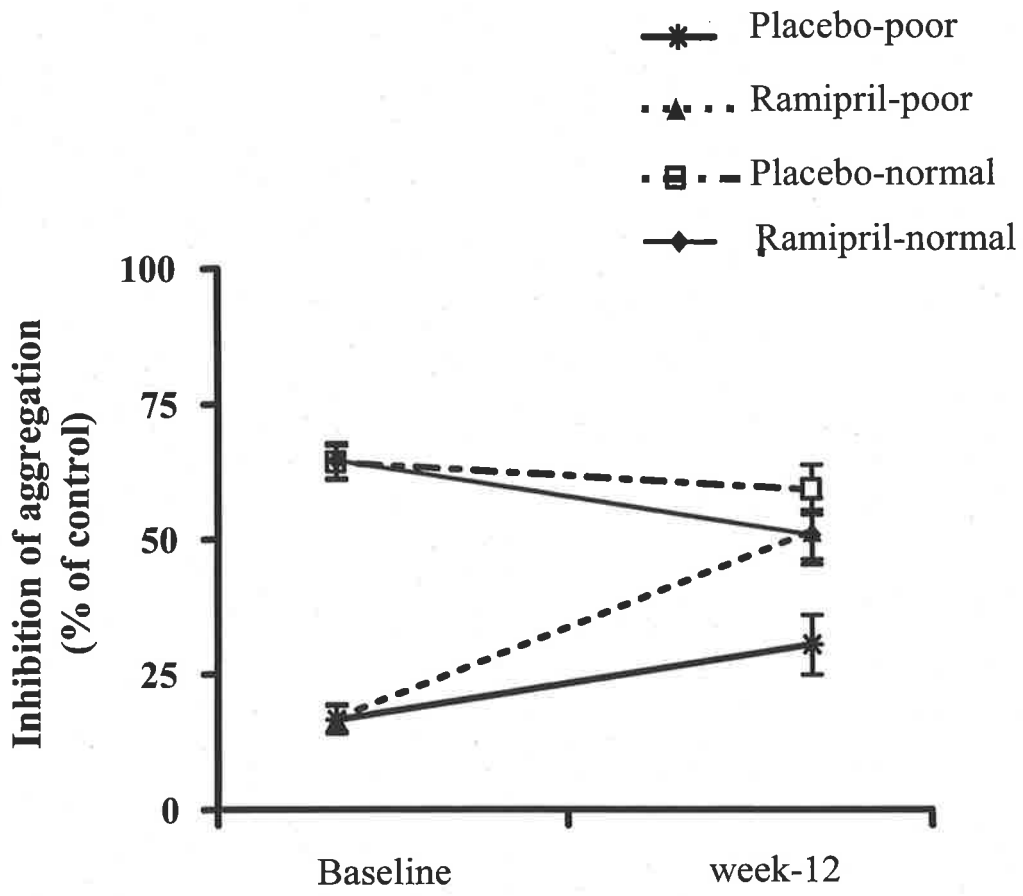


Figure 6.7 Differential responsiveness to SNP after 12 weeks of ramipril or placebo in patients with "normal" SNP responses at baseline vs those who have less than 32% responsiveness at baseline "impaired responsiveness"

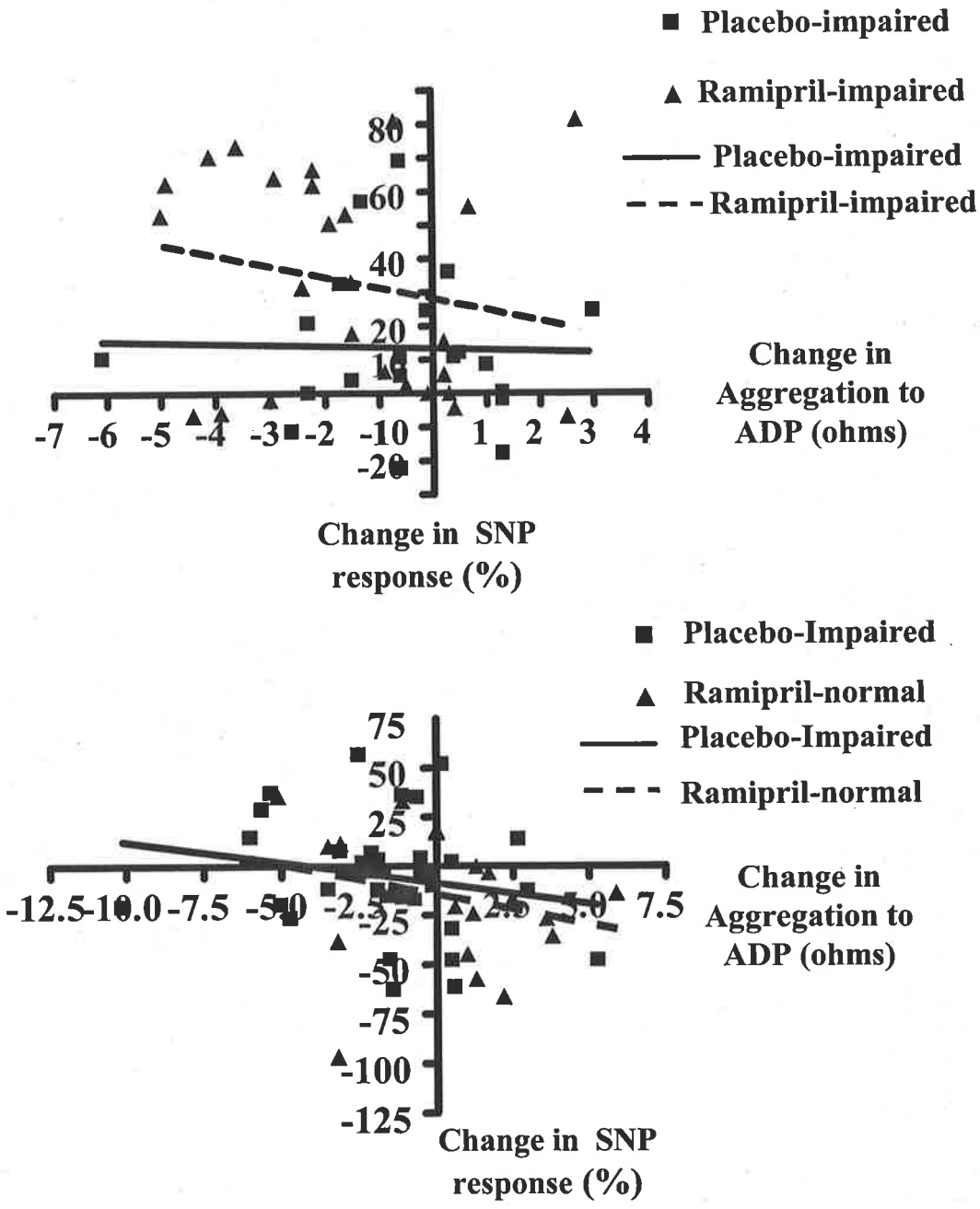


Figure 6.8. Effect of ramipril on the relationship between change in ADP and change in SNP responsiveness in patients with impaired (upper graph, ANCOVA $p<0.001$) vs normal (lower graph, ANCOVA p=NS) responsiveness to SNP at baseline in the ramipril and placebo groups.

Chapter 7: General discussion and future directions

7.1 Summary of results

The studies in this thesis examine aspects of nitrate pharmacology and NO physiology in subjects ranging from normal volunteers to patients with acute coronary syndromes. The major findings of the studies were as follows:-

1. In normal subjects, utilisation of sublingual GTN doses greater than 200μg for evaluation of effects of GTN on apparent arterial stiffness induced near-maximal responses, which may obscure the detection of changes in sensitivity. Indeed, as 50% maximal responses occured with approximately 25μg of GTN, future evaluations of responsiveness should either use doses of this magnitude or ideally, construction of dose-response curves. Investigation of responses to intravenously infused GTN at the rate of 5μg/min, an infusion rate at the lower end of clinically utilised regimens, produced large falls in AIx in patients with stable angina pectoris. Again this suggests near-maximal responses, as the magnitude of this effect approached that of the higher sublingual doses. While initial responsiveness to GTN could not be measured in patients with acute coronary syndromes due to pre admission use of sublingual GTN, we can conclude that in patients with ACS, transition from low dose intravenous GTN to oral ISDN (10mg) is associated with no loss of vasodilator effect, as measured by AIx. This suggests that there is little likelihood of occurrence of "rebound" phenomena with this regimen.

2. Investigation of the possible potentiation of NO responsiveness by perhexiline was undertaken on the basis of a previous study from our group in which treatment with perhexiline significantly improved NO responses in platelets from patients with refractory angina. The current study demonstrated that perhexiline has no effect on

apparent arterial stiffness either alone or in combination with GTN. In this patient cohort with normal platelet function perhexiline also had no significant effect on platelet NO responsiveness. However the study also demonstrated that in vivo administration of low infusion rates of GTN inhibits neutrophil O_2^- release, and that this effect is potentiated by pre-treatment with perhexiline.

3. In order to assess oxidative stress as a potential source of differential NO responsiveness between patient groups, two different markers of oxidative stress were utilised for comparison between three patient groups. The study demonstrated that both ACS and, to a marginal extent, SAP are associated with incremental oxidative stress. However, the study also demonstrated that different measures of oxidative stress provided markedly different results as regards magnitude of disturbance in individual patients. This led to the conclusion that no single estimate of either superoxide release or of MDA, a product of free radical effect, would prove of value as a correlate of extent of ongoing ischaemia in individual patients.

4. In contrast to the perhexiline study, the ACE inhibitor ramipril improved platelet NO responsiveness in patients at high risk of cardiovascular events, such as those recruited in the HOPE trial. Ramipril is a pharmacological agent which has been associated with both improved function of the NO system in vasculature and reduction of oxidative stress, but has not been tested previously with respect to its effects on platelet NO response. In particular, the current study demonstrated that ramipril sensitises platelets to the anti-aggregatory effects of NO but that this effect occurs predominantly in patients with poor initial responses to NO. With respect to mechanisms of this effect, this study

failed to demonstrate either a decrease in oxidative stress (utilising MDA as a marker) or sensitisation of the soluble guanylate cyclase system during ramipril treatment. The delineation of protective effect of ramipril on platelet function however, raises the possibility of future novel roles for this ACE inhibitor therapy in short term prevention of thrombus formation such as following angioplasty or stent insertion.

These studies have examined two agents, perhexiline and ramipril, with apparently complementary effects in heart failure and increasingly common utilisation in myocardial ischaemia. The effects of these agents on tissue function suggest that limitation of oxidative stress may be region selective (for example in neutrophils for perhexiline and in platelets for ramipril). More importantly, a previous study of the effects of perhexiline on platelet responsiveness to NO showed reversal of NO resistance in patients with refractory angina, and lower baseline responses to NO than those seen in chapter 4 (Willoughby et al., 2002). These data, combined with the current studies suggest that the improvement of NO responsiveness may be related to the baseline responsiveness, and therefore platelet studies may represent a potential method for assessment of patients who may benefit most from therapy with these drugs.

7.2 Future directions

1. Demonstration that perhexiline is a non-vasoactive agent that reduces superoxide release from neutrophils, suggests that it may have additional clinical utility in conditions associated with increased inflammatory cell activation and oxidative stress such as

myocardial infarction, diabetes and unstable coronary artery disease and chronic heart failure. In fact, a recent study has demonstrated therapeutic benefit of perhexiline in chronic heart failure (Lee et al., 2005) including improvement in VO_2 max, ejection fraction and quality of life. While these changes might be attributed to the metabolic effect of perhexiline (Kennedy et al., 1996), currently there are no studies of the relationship between the "metabolic" effects of perhexiline and inhibition of superoxide release. However another inhibitor of fatty acid oxidation, trimetazidine, has been demonstrated to have anti-inflammatory effects (Tritto et al., 2005; Williams et al., 1993). While the mechanism by which perhexiline affects superoxide release has not been investigated, the intriguing possibility is that perhexiline may selectively interfere with activity of the phagocytic NAD(P)H oxidase (Kennedy et al., 2006). This could be tested by investigation in knockout animal models.

2. These studies have contributed to the understanding of another potential mechanism of action of ACE inhibitors. The finding that ramipril therapy improves NO responsiveness has relevance to both primary and secondary prevention in groups at high risk of thrombotic events. At present, ramipril or the other ACE inhibitors tend to be utilised in all patients perceived to be at high risk for occurance of acute myocardial ischaemia. However, if platelet responsiveness to NO is a major therapeutic target, treatment might be individualised, and response to treatment categorised individually. Furthermore, information is currently lacking as to whether angiotensin receptor antagonists also protect against the development of acute ischaemia. This could be addressed in two possible ways: firstly by performing an analogous study with an

angiotensin receptor antagonist or by ex vivo administration a of bradykinin receptor antagonist such as HOE140 (Alla et al., 1993; Wirth et al., 1991) to determine the contribution of bradykinin to reversal of NO resistance by ramipril.

3. The relationship between vascular NO bio-availability and oxidative stress is complicated, particularly with respect to the interplay between the sources and effects of both agents. NO acts as a protective agent in the vasculature, by dilating arteries to minimise damage resulting from high intra-arterial pressures and by inhibiting inflammatory cell/platelet adhesion. In the presence of endothelial dysfunction, particularly when accompanied by increased oxidative stress, this defence system is impaired. GTN has traditionally been used as a vasodilator: evidence from this thesis and other studies has demonstrated that the beneficial effects of therapy with GTN may extend beyond vasodilation. Effects on the relationship between platelets, inflammatory cells and the endothelium have all been shown to be affected by supplementation of NO via GTN therapy.

There is also emerging evidence that NO may be involved in production and mobilisation of endothelial progenitor cells (EPC) which are involved in angiogenesis (Ozuyaman et al., 2005) and appear to be important in functional improvement and infarct size limitation following coronary occlusion (Massa et al., 2005; Numaguchi et al., 2006). This area of therapeutics is more unexplored than novel: it has already been demonstrated that day one mortality is reduced by organic nitrates in the ISIS-4 trial (ISIS-4, 1995) and that the largely unexplained effects of combined therapy with ISDN and hydralazine improve survival in African-American patients with chronic heart failure

(Taylor et al., 2004). However there is a need to investigate whether the "anti-inflammatory" effects of organic nitrates are also relevant outside neutrophils and whether they are subject to tachyphylaxis (ie tolerance) and whether these effects mediate the abovementioned clinical benefits.

4. While the importance of increased levels of superoxide in the vasculature, particularly with respect to vessels affected by atherosclerosis is rarely doubted, the relative importance of different markers of oxidative stress in the absence of a direct measure is debatable. In these studies, it has been demonstrated that there is no correlation between the two markers utilised and as discussed in chapter 5, this is most likely due to either measurement of different aspects of the oxidative stress or a difference in the time course of elevation of these markers (neutrophil O_2^- release is a source and MDA a product of oxidative stress), particularly in the systemic circulation. However this does raise the issue of how to measure oxidative stress for assessment of patients who will benefit from therapies aimed at reducing oxidative stress. At present, it appears that no single measure is of use for oxidative stress measurement in risk assessment in individual patients. At present this is not a clinical issue. However, it is possible that precise characterisation of oxidative stress in individual patients might facilitate development of therapeutics with redox-active agents. At present, this seems a remote possibility.

Finally, it should be pointed out that the critical experiments in chapters 4 and 6 evaluated novel aspects of the pharmacology of "old" drugs (GTN, perhexiline and ramipril), shedding new light on their clinical efficacy. It is a sad reality of

pharmacological investigation that critically important drug effects are often discovered late, so that neither the spectrum of clinical utility nor of potential toxicity is appreciated when the drug is first released. These studies provide a compelling basis for extension of initial evaluation of all cardioactive drugs to include assessment of drug effects on O_2^- release and on oxidative stress.

References

1992. Effect of enalapril on mortality and the development of heart failure in asymptomatic patients with reduced left ventricular ejection fractions. The SOLVD Investigattors. *N Engl J Med.* 327:685-91.

1993. Effect of ramipril on mortality and morbidity of survivors of acute myocardial infarction with clinical evidence of heart failure. The Acute Infarction Ramipril Efficacy (AIRE) Study Investigators. *Lancet.* 342:821-8.

Abrams, J. 1980. Nitrate tolerance and dependence. *Am Heart J.* 99:113-23.

Abrams, J. 1991. The mystery of nitrate resistance. *Am J Cardiol.* 68:1393-6.

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.

Ago, T., T. Kitazono, H. Ooboshi, T. Iyama, Y.H. Han, J. Takada, M. Wakisaka, S. Ibayashi, H. Utsumi, and M. Iida. 2004. Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circulation.* 109:227-33.

Ahimastos, A.A., A.K. Natoli, A. Lawler, P.A. Blombery, and B.A. Kingwell. 2005. Ramipril reduces large-artery stiffness in peripheral arterial disease and promotes elastogenic remodeling in cell culture. *Hypertension.* 45:1194-9.

Ali, S.M., E.J. Brown, Jr., S.R. Nallapati, and I.A. Alhaddad. 1998. Early angiotensin converting enzyme inhibitor therapy after experimental myocardial infarction prevents left ventricular dilation by reducing infarct expansion: a possible mechanism of clinical benefits. *Coron Artery Dis.* 9:815-21.

Alioua, A., J.P. Huggins, and E. Rousseau. 1995. PKG-I alpha phosphorylates the alpha-subunit and upregulates reconstituted GKCa channels from tracheal smooth muscle. *Am J Physiol.* 268:L1057-63.

Alla, S.A., J. Buschko, U. Quitterer, A. Maidhof, M. Haasemann, G. Breipohl, J. Knolle, and W. Muller-Esterl. 1993. Structural features of the human bradykinin B2 receptor probed by agonists, antagonists, and anti-idiotypic antibodies. *J Biol Chem.* 268:17277-85.

Amoah, A.G., B.J. Gould, D.V. Parke, and J.D. Lockhart. 1986. Further studies on the pharmacokinetics of perhexiline maleate in humans. *Xenobiotica.* 16:63-8.

Anderson, T.J., A. Uehata, M.D. Gerhard, I.T. Meredith, S. Knab, D. Delagrange, E.H. Lieberman, P. Ganz, M.A. Creager, A.C. Yeung, and et al. 1995. Close relation of endothelial function in the human coronary and peripheral circulations. *J Am Coll Cardiol.* 26:1235-41.

Angdin, M., G. Settergren, J. Liska, and R. Astudillo. 2001. No effect of L-arginine supplementation on pulmonary endothelial dysfunction after cardiopulmonary bypass. *Acta Anaesthesiol Scand.* 45:441-8.

Archer, S.L., J.M. Huang, V. Hampl, D.P. Nelson, P.J. Shultz, and E.K. Weir. 1994. Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc Natl Acad Sci U S A.* 91:7583-7.

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.

Arndt, H., J.B. Russell, I. Kurose, P. Kubes, and D.N. Granger. 1993. Mediators of leukocyte adhesion in rat mesenteric venules elicited by inhibition of nitric oxide synthesis. *Gastroenterology.* 105:675-80.

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.

Assanelli, D., A. Bonanome, A. Pezzini, F. Albertini, P. Maccalli, M. Grassi, S. Archetti, R. Negrini, and F. Visioli. 2004. Folic acid and vitamin E supplementation effects on homocysteinemia, endothelial function and plasma antioxidant capacity in young myocardial-infarction patients. *Pharmacol Res.* 49:79-84.

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.

Bauersachs, J., A. Bouloumie, A. Mulsch, G. Wiemer, I. Fleming, and R. Busse. 1998. Vasodilator dysfunction in aged spontaneously hypertensive rats: changes in NO synthase III and soluble guanylyl cyclase expression, and in superoxide anion production. *Cardiovasc Res.* 37:772-9.

Beall, A.C., K. Kato, J.R. Goldenring, H. Rasmussen, and C.M. Brophy. 1997. Cyclic nucleotide-dependent vasorelaxation is associated with the phosphorylation of a small heat shock-related protein. *J Biol Chem.* 272:11283-7.

Beckwith, C., and M.A. Munger. 1993. Effect of angiotensin-converting enzyme inhibitors on ventricular remodeling and survival following myocardial infarction. *Ann Pharmacother.* 27:755-66.

Beltrame, J.F., S. Stewart, S. Leslie, S. Poropat, and J.D. Horowitz. 2002. Resolution of ST-segment elevation following intravenous administration of nitroglycerin and verapamil. *Am J Cardiol.* 89:452-5.

Ben-David, A. 1989. Cardiac arrest in an explosives factory worker due to withdrawal from nitroglycerin exposure. *Am J Ind Med.* 15:719-22.

Benetos, A., M. Safar, A. Rudnichi, H. Smulyan, J.L. Richard, P. Ducimetieere, and L. Guize. 1997. Pulse pressure: a predictor of long-term cardiovascular mortality in a French male population. *Hypertension.* 30:1410-5.

Bennett, B.M., B.J. McDonald, R. Nigam, P.G. Long, and W.C. Simon. 1992a. Inhibition of nitrovasodilator- and acetylcholine-induced relaxation and cyclic GMP accumulation by the cytochrome P-450 substrate, 7-ethoxyresorufin. *Can J Physiol Pharmacol.* 70:1297-303.

Bennett, B.M., B.J. McDonald, and M.J. St James. 1992b. Hepatic cytochrome P-450-mediated activation of rat aortic guanylyl cyclase by glyceryl trinitrate. *J Pharmacol Exp Ther.* 261:716-23.

Bennett-Richards, K., M. Kattenhorn, A. Donald, G. Oakley, Z. Varghese, L. Rees, and J.E. Deanfield. 2002. Does oral folic acid lower total homocysteine levels and improve endothelial function in children with chronic renal failure? *Circulation.* 105:1810-5.

Berliner, S., O. Rogowski, R. Rotstein, R. Fusman, I. Shapira, N.M. Bornstein, V. Prochorov, A. Roth, G. Keren, A. Eldor, and D. Zeltser. 2000. Activated polymorphonuclear leukocytes and monocytes in the peripheral blood of patients with ischemic heart and brain conditions correspond to the presence of multiple risk factors for atherothrombosis. *Cardiology.* 94:19-25.

Berry, C., C.A. Hamilton, M.J. Brosnan, F.G. Magill, G.A. Berg, J.J. McMurray, and A.F. Dominiczak. 2000. Investigation into the sources of superoxide in human blood vessels: angiotensin II increases superoxide production in human internal mammary arteries. *Circulation.* 101:2206-12.

Biasucci, L.M., G. D'Onofrio, G. Liuzzo, G. Zini, C. Monaco, G. Caligiuri, M. Tommasi, A.G. Rebuzzi, and A. Maseri. 1996. Intracellular neutrophil myeloperoxidase is reduced in unstable angina and acute myocardial infarction, but its reduction is not related to ischemia. *J Am Coll Cardiol.* 27:611-6.

Bloch, W., D. Hoever, D. Reitze, L. Kopalek, and K. Addicks. 1995. Exogenously supplied nitric oxide influences the dilation of the capillary microvasculature in vivo. *Agents Actions Suppl.* 45:151-6.

Boyd, D.G., and R.B. Davis. 1988. Observations on human platelet aggregation in native whole blood: synergism and sensitivity to aggregating agents in vitro. *Thromb Res.* 50:429-36.

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.

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.

Byington, R.P., J.W. Jukema, J.T. Salonen, B. Pitt, A.V. Bruschke, H. Hoen, C.D. Furberg, and G.B. Mancini. 1995. Reduction in cardiovascular events during pravastatin therapy. Pooled analysis of clinical events of the Pravastatin Atherosclerosis Intervention Program. *Circulation.* 92:2419-25.

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.

Cashin-Hemphill, L., G. Holmvang, R.C. Chan, B. Pitt, R.E. Dinsmore, and R.S. Lees. 1999. Angiotensin-converting enzyme inhibition as antiatherosclerotic therapy: no answer yet. QUIET Investigators. QUinapril Ischemic Event Trial. *Am J Cardiol.* 83:43-7.

Ceconi, C., S. Curello, A. Cargnoni, R. Ferrari, A. Albertini, and O. Visioli. 1988. The role of glutathione status in the protection against ischaemic and reperfusion damage: effects of N-acetyl cysteine. *J Mol Cell Cardiol.* 20:5-13.

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.* 340:1111-5.

Chello, M., P. Mastroroberto, F. Perticone, V. Celi, and A. Colonna. 1998. Nitric oxide modulation of neutrophil-endothelium interaction: difference between arterial and venous coronary bypass grafts. *J Am Coll Cardiol.* 31:823-6.

Chen, C.H., E. Nevo, B. Fetics, P.H. Pak, F.C. Yin, W.L. Maughan, and D.A. Kass. 1997. Estimation of central aortic pressure waveform by mathematical transformation of radial tonometry pressure. Validation of generalized transfer function. *Circulation.* 95:1827-36.

Chen, J.W., N.W. Hsu, T.C. Wu, S.J. Lin, and M.S. Chang. 2002a. Long-term angiotensin-converting enzyme inhibition reduces plasma asymmetric dimethylarginine and improves endothelial nitric oxide bioavailability and coronary microvascular function in patients with syndrome X. *Am J Cardiol.* 90:974-82.

Chen, P.M., X.G. Leng, L.L. Fan, J. Ma, Y.F. Wang, and L.Y. Chen. 2005. Changes of chymase, angiotensin converting enzyme and angiotensin II type 1 receptor expressions in the hamster heart during the development of heart failure. *Chin Med J (Engl).* 118:1886-92.

Chen, Z., J. Zhang, and J.S. Stamler. 2002b. From the Cover: Identification of the enzymatic mechanism of nitroglycerin bioactivation. *Proc Natl Acad Sci U S A.* 99:8306-11.

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., 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, J.D. Martelli, and J.D. Horowitz. 2004. Effect of perindopril on platelet nitric oxide resistance in patients with chronic heart failure secondary to ischemic left ventricular dysfunction. *Am J Cardiol.* 93:1438-40, A10.

Chirkov, Y.Y., A.S. Holmes, J.M. Stepien, R.A. Anderson, M.P. Frenneaux, and J.D. Horowitz. 2002. Platelet nitric oxide resistance in patients with congestive heart failure is ameliorated with ACE inhibition (abstr). *J Am Coll Cardiol.* 39:201B-202B.

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. *J Am Coll Cardiol.* 37:1851-7.

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.

Chung, S.J., and H.L. Fung. 1990. Identification of the subcellular site for nitroglycerin metabolism to nitric oxide in bovine coronary smooth muscle cells. *J Pharmacol Exp Ther.* 253:614-9.

Clancy, R.M., J. Leszczynska-Piziak, and S.B. Abramson. 1992. Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *J Clin Invest.* 90:1116-21.

Clapp, B.R., A.D. Hingorani, R.K. Kharbanda, V. Mohamed-Ali, J.W. Stephens, P. Vallance, and R.J. MacAllister. 2004. Inflammation-induced endothelial dysfunction involves reduced nitric oxide bioavailability and increased oxidant stress. *Cardiovasc Res.* 64:172-8.

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.

Cohn, J.N., D.G. Archibald, S. Ziesche, J.A. Franciosa, W.E. Harston, F.E. Tristani, W.B. Dunkman, W. Jacobs, G.S. Francis, K.H. Flohr, and et al. 1986. Effect of vasodilator therapy on mortality in

chronic congestive heart failure. Results of a Veterans Administration Cooperative Study. *N Engl J Med.* 314:1547-52.

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.

Collinson, P.O., A.C. Rao, R. Canepa-Anson, and S. Joseph. 2003. Impact of European Society of Cardiology/American College of Cardiology guidelines on diagnostic classification of patients with suspected acute coronary syndromes. *Ann Clin Biochem.* 40:156-60.

Cooke, J.P., J. Dzau, and A. Creager. 1991. Endothelial dysfunction in hypercholesterolemia is corrected by L-arginine. *Basic Res Cardiol.* 86:173-81.

Cooper, R.G., D.A. Evans, and A.H. Price. 1987. Studies on the metabolism of perhexiline in man. *Eur J Clin Pharmacol.* 32:569-76.

Cooper, R.G., D.A. Evans, and E.J. Whibley. 1984. Polymorphic hydroxylation of perhexiline maleate in man. *J Med Genet.* 21:27-33.

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.

Cross, J.M., A.E. Donald, R. Kharbanda, J.E. Deanfield, R.G. Woolfson, and R.J. MacAllister. 2001. Acute administration of L-arginine does not improve arterial endothelial function in chronic renal failure. *Kidney Int.* 60:2318-23.

Cucoranu, I., R. Clempus, A. Dikalova, P.J. Phelan, S. Ariyan, S. Dikalov, and D. Sorescu. 2005. NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. *Circ Res.* 97:900-7.

Curello, S., C. Ceconi, A. Cargnoni, A. Cornacchiari, R. Ferrari, and A. Albertini. 1987. Improved procedure for determining glutathione in plasma as an index of myocardial oxidative stress. *Clin Chem.* 33:1448-9.

Czapski, G.A., D. Avram, D.V. Sakharov, K.W. Wirtz, J.B. Strosznajder, and E.H. Pap. 2002. Activated neutrophils oxidize extracellular proteins of endothelial cells in culture: effect of nitric oxide donors. *Biochem J.* 365:897-902.

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 Servi, S., A. Mazzone, G. Ricevuti, I. Mazzucchelli, G. Fossati, D. Gritti, L. Angoli, and G. Specchia. 1995. Clinical and angiographic correlates of leukocyte activation in unstable angina. *J Am Coll Cardiol.* 26:1146-50.

Delles, C., M.P. Schneider, S. John, M. Gekle, and R.E. Schmieder. 2002. Angiotensin converting enzyme inhibition and angiotensin II AT1-receptor blockade reduce the levels of asymmetrical N(G), N(G)-dimethylarginine in human essential hypertension. *Am J Hypertens.* 15:590-3.

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.

Dernier, J., and A. Colen. 1977. [Influence of perhexiline maleate on the electrocardiographic signs of myocardial ischemia. Ergometric study]. *Acta Cardiol.* 32:41-53.

Deschamps, D., V. DeBeco, C. Fisch, B. Fromenty, A. Guillouzo, and D. Pessayre. 1994. Inhibition by perhexiline of oxidative phosphorylation and the beta-oxidation of fatty acids: possible role in pseudoalcoholic liver lesions. *Hepatology.* 19:948-61.

DiFabio, J., Y. Ji, V. Vasiliou, G.R. Thatcher, and B.M. Bennett. 2003. Role of mitochondrial aldehyde dehydrogenase in nitrate tolerance. *Mol Pharmacol.* 64:1109-16.

Dikshit, M., and P. Sharma. 2002. Nitric oxide mediated modulation of free radical generation response in the rat polymorphonuclear leukocytes: a flowcytometric study. *Methods Cell Sci.* 24:69-76.

Dinerman, J.L., and J.L. Mehta. 1990. Endothelial, platelet and leukocyte interactions in ischemic heart disease: insights into potential mechanisms and their clinical relevance. *J Am Coll Cardiol.* 16:207-22.

Dinerman, J.L., J.L. Mehta, T.G. Saldeen, S. Emerson, R. Wallin, R. Davda, and A. Davidson. 1990. Increased neutrophil elastase release in unstable angina pectoris and acute myocardial infarction. *J Am Coll Cardiol.* 15:1559-63.

Doshi, S., I. McDowell, S. Moat, M. Lewis, and J. Goodfellow. 2003. Folate improves endothelial function in patients with coronary heart disease. *Clin Chem Lab Med.* 41:1505-12.

Doshi, S.N., I.F. McDowell, S.J. Moat, N. Payne, H.J. Durrant, M.J. Lewis, and J. Goodfellow. 2002. Folic acid improves endothelial function in coronary artery disease via mechanisms largely independent of homocysteine lowering. *Circulation.* 105:22-6.

Drexler, H., A.M. Zeiher, K. Meinzer, and H. Just. 1991. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet.* 338:1546-50.

Duffy, S.J., G. New, R.W. Harper, and I.T. Meredith. 1999. Metabolic vasodilation in the human forearm is preserved in hypercholesterolemia despite impairment of endothelium-dependent and independent vasodilation. *Cardiovasc Res.* 43:721-30.

Ehlers, R., E. Buttcher, H.K. Eltzschig, S. Kazmaier, S. Szabo, U. Helber, and H.M. Hoffmeister. 2002. Correlation between ST-T-segment changes with markers of hemostasis in patients with acute coronary syndromes. *Cardiology.* 98:40-5.

Elkayam, U., and W.S. Aronow. 1982. Glyceryl trinitrate (nitroglycerin) ointment and isosorbide dinitrate: a review of their pharmacological properties and therapeutic use. *Drugs.* 23:165-94.

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.

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.

Fallen, E.L., C. Nahmias, A. Scheffel, G. Coates, R. Beanlands, and E.S. Garnett. 1995. Redistribution of myocardial blood flow with topical nitroglycerin in patients with coronary artery disease. *Circulation.* 91:1381-8.

Farquharson, C.A., and A.D. Struthers. 2002. Gradual reactivation over time of vascular tissue angiotensin I to angiotensin II conversion during chronic lisinopril therapy in chronic heart failure. *J Am Coll Cardiol.* 39:767-75.

Feelisch, M., and M. Kelm. 1991. Biotransformation of organic nitrates to nitric oxide by vascular smooth muscle and endothelial cells. *Biochem Biophys Res Commun.* 180:286-93.

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.

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.

Fitchett, D.H., G.J. Simkus, J.P. Beaudry, and D.G. Marpole. 1988. Reflected pressure waves in the ascending aorta: effect of glyceryl trinitrate. *Cardiovasc Res.* 22:494-500.

Fleckenstein-Grun, G., A. Fleckenstein, Y. Byron, and K. Kim. 1978. Mechanisms of action of calcium antagonists in the treatment of CAD with special reference to perhexiline maleate. In: Perhexiline Maleate. Proceedings of a symposium. Exerpta Medica, Amsterdam. pp. 1-22.

Fletcher, A., P. McLoone, and C. Bulpitt. 1988. Quality of life on angina therapy: a randomised controlled trial of transdermal glyceryl trinitrate against placebo. *Lancet.* 2:4-8.

Forster, S., I. Woditsch, H. Schroder, and K. Schror. 1991. Reduced nitric oxide release causes nitrate tolerance in the intact coronary circulation. *J Cardiovasc Pharmacol.* 17:867-72.

Forstermann, U., A. Mugge, U. Alheid, S.M. Bode, and J.C. Frolich. 1989. Endothelium-derived relaxing factor (EDRF): a defence mechanism against platelet aggregation and vasospasm in human coronary arteries. *Eur Heart J.* 10:36-43.

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.

Fox, K.M. 2003. Efficacy of perindopril in reduction of cardiovascular events among patients with stable coronary artery disease: randomised, double-blind, placebo-controlled, multicentre trial (the EUROPA study). *Lancet*. 362:782-8.

Franklin, S.S., S.A. Khan, N.D. Wong, M.G. Larson, and D. Levy. 1999. Is pulse pressure useful in predicting risk for coronary heart Disease? The Framingham heart study. *Circulation*. 100:354-60.

Freedman, S.B., B.V. Daxini, D. Noyce, and D.T. Kelly. 1995. Intermittent transdermal nitrates do not improve ischemia in patients taking beta-blockers or calcium antagonists: potential role of rebound ischemia during the nitrate-free period. *J Am Coll Cardiol*. 25:349-55.

Fukao, M., H.S. Mason, F.C. Britton, J.L. Kenyon, B. Horowitz, and K.D. Keef. 1999. Cyclic GMP-dependent protein kinase activates cloned BKCa channels expressed in mammalian cells by direct phosphorylation at serine 1072. *J Biol Chem*. 274:10927-35.

Fukuda, S., T. Yasu, D.N. Predescu, and G.W. Schmid-Schonbein. 2000. Mechanisms for regulation of fluid shear stress response in circulating leukocytes. *Circ Res*. 86:E13-8.

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. p22phox mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats. *Circ Res*. 80:45-51.

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.

Galle, J., J. Bauersachs, R. Busse, and E. Bassenge. 1992. Inhibition of cyclic AMP- and cyclic GMP-mediated dilations in isolated arteries by oxidized low density lipoproteins. *Arterioscler Thromb*. 12:180-6.

Gewirtz, H., M. Steiner, H. Sasken, and A.S. Most. 1985. Measurement by electrical impedance aggregometry of porcine platelets response to selected physiological agonists. *Proc Soc Exp Biol Med*. 179:324-30.

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.

Goldberg, R.B., M.J. Mellies, F.M. Sacks, L.A. Moye, B.V. Howard, W.J. Howard, B.R. Davis, T.G. Cole, M.A. Pfeffer, and E. Braunwald. 1998. Cardiovascular events and their reduction with pravastatin in diabetic and glucose-intolerant myocardial infarction survivors with average cholesterol levels: subgroup analyses in the cholesterol and recurrent events (CARE) trial. The Care Investigators. *Circulation*. 98:2513-9.

Gori, T., P. Harvey, J.S. Floras, and J.D. Parker. 2004. Continuous therapy with nitroglycerin impairs endothelium-dependent vasodilation but does not cause tolerance in conductance arteries: a human in vivo study. *J Cardiovasc Pharmacol*. 44:601-6.

Gori, T., L. Saunders, S. Ahmed, and J.D. Parker. 2003. Effect of folic acid on nitrate tolerance in healthy volunteers: differences between arterial and venous circulation. *J Cardiovasc Pharmacol*. 41:185-90.

Granger, D.N., T. Vowinkel, and T. Petnehazy. 2004. Modulation of the inflammatory response in cardiovascular disease. *Hypertension*. 43:924-31.

Greig, L.D., S.J. Leslie, F.W. Gibb, S. Tan, D.E. Newby, and D.J. Webb. 2005. Comparative effects of glyceryl trinitrate and amyl nitrite on pulse wave reflection and augmentation index. *Br J Clin Pharmacol*. 59:265-70.

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.

Gross, G.J., and D.C. Warltier. 1977. Intracoronary versus intravenous nitroglycerin on the transmural distribution of coronary blood flow. *Cardiovasc Res*. 11:499-506.

Gruhn, N., J. Aldershvile, and S. Boesgaard. 2001. Tetrahydrobiopterin improves endothelium-dependent vasodilation in nitroglycerin-tolerant rats. *Eur J Pharmacol*. 416:245-9.

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.

Grynberg, A., and L. Demaison. 1996. Fatty acid oxidation in the heart. *J Cardiovasc Pharmacol.* 28 Suppl 1:S11-7.

Gundel, W., G. Cherry, B. Rajagopalan, L.B. Tan, G. Lee, and D. Schultz. 1981. Aortic input impedance in man: acute response to vasodilator drugs. *Circulation.* 63:1305-14.

Guzik, T.J., N.E. West, E. Black, D. McDonald, C. Ratnatunga, R. Pillai, and K.M. Channon. 2000. Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. *Circ Res.* 86:E85-90.

Haefeli, W.E., N. Srivastava, K.T. Kelsey, J.K. Wiencke, B.B. Hoffman, and T.F. Blaschke. 1993. Glutathione S-transferase mu polymorphism does not explain variation in nitroglycerin responsiveness. *Clin Pharmacol Ther.* 53:463-8.

Hayward, C.S., M. Kraidly, C.M. Webb, and P. Collins. 2002. Assessment of endothelial function using peripheral waveform analysis: a clinical application. *J Am Coll Cardiol.* 40:521-8.

Hebert, D., and J.Y. Lam. 2000. Nitroglycerin rebound associated with vascular, rather than platelet, hypersensitivity. *J Am Coll Cardiol.* 36:2311-6.

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.

Heitzer, T., H. Just, and T. Munzel. 1996. Antioxidant vitamin C improves endothelial dysfunction in chronic smokers. *Circulation.* 94:6-9.

Heitzer, T., T. Schlinzig, K. Krohn, T. Meinertz, and T. Munzel. 2001. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation.* 104:2673-8.

Henry, P.J., J.D. Horowitz, and W.J. Louis. 1989. Determinants of in vitro nitroglycerin tolerance induction and reversal: influence of dose regimen, nitrate-free period, and sulfhydryl supplementation. *J Cardiovasc Pharmacol.* 14:31-7.

Heresztyn, T., M.I. Worthley, and J.D. Horowitz. 2004. Determination of l-arginine and NG, NG - and NG, NG' -dimethyl-L-arginine in plasma by liquid chromatography as AccQ-Fluor fluorescent derivatives. *J Chromatogr B Analyt Technol Biomed Life Sci.* 805:325-9.

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.

Hitchcock, P.J., I.J. Rutovitz, R.V. Dando, A.J. Eathorne, and G. Weiss. 1977. A multicentre trial of perhexiline maleate in the treatment of angina pectoris. *Curr Ther Res Clin Exp.* 21:36-42.

Holmes, A.S., Y.Y. Chirkov, S.R. Willoughby, S. Poropat, J. Pereira, and J.D. Horowitz. 2005. Preservation of platelet responsiveness to nitroglycerine despite development of vascular nitrate tolerance. *Br J Clin Pharmacol.* 60:355-63.

Hong, H.J., G. Hsiao, T.H. Cheng, and M.H. Yen. 2001. Supplemention with tetrahydrobiopterin suppresses the development of hypertension in spontaneously hypertensive rats. *Hypertension.* 38:1044-8.

Horowitz, J.D. 1992. Role of nitrates in unstable angina pectoris. *Am J Cardiol.* 70:64B-71B.

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., and P.J. Henry. 1987. Recent developments in nitrate therapy of ischaemic heart disease. *Med J Aust.* 146:93-6.

Horowitz, J.D., and M.L. Mashford. 1979. Perhexiline maleate in the treatment of severe angina pectoris. *Med J Aust.* 1:485-8.

Horowitz, J.D., P.M. Morris, O.H. Drummer, A.J. Goble, and W.J. Louis. 1981. High-performance liquid chromatographic assay of perhexiline maleate in plasma. *J Pharm Sci.* 70:320-2.

Horowitz, J.D., S.T. Sia, P.S. Macdonald, A.J. Goble, and W.J. Louis. 1986. Perhexiline maleate treatment for severe angina pectoris--correlations with pharmacokinetics. *Int J Cardiol.* 13:219-29.

Hsi, D.H., A. Roshandel, N. Singh, T. Szombathy, and Z.S. Meszaros. 2005. Headache response to glyceryl trinitrate in patients with and without obstructive coronary artery disease. *Heart.* 91:1164-6.

Hudak, W.J., R.E. Lewis, and W.L. Kuhn. 1970. Cardiovascular pharmacology of perhexiline. *J Pharmacol Exp Ther.* 173:371-82.

Huraux, C., T. Makita, S. Kurz, K. Yamaguchi, F. Szlam, M. Tarpey, J. Wilcox, D. Harrison, and J. Levy. 1999. Superoxide production, risk factors, and endothelium-dependant relaxations in human internal mammary arteries. *Circulation.* 99:53-59.

Ignarro, L.J., G.M. Buga, and G. Chaudhuri. 1988a. EDRF generation and release from perfused bovine pulmonary artery and vein. *Eur J Pharmacol.* 149:79-88.

Ignarro, L.J., R.E. Byrns, G.M. Buga, K.S. Wood, and G. Chaudhuri. 1988b. Pharmacological evidence that endothelium-derived relaxing factor is nitric oxide: use of pyrogallol and superoxide dismutase to study endothelium-dependent and nitric oxide-elicited vascular smooth muscle relaxation. *J Pharmacol Exp Ther.* 244:181-9.

Ignarro, L.J., H. Lippton, J.C. Edwards, W.H. Baricos, A.L. Hyman, P.J. Kadowitz, and C.A. Gruetter. 1981. Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J Pharmacol Exp Ther.* 218:739-49.

Ingerman-Wojenski, C., J.B. Smith, and M.J. Silver. 1983. Evaluation of electrical aggregometry: comparison with optical aggregometry, secretion of ATP, and accumulation of radiolabeled platelets. *J Lab Clin Med.* 101:44-52.

ISIS-4, i. 1995. ISIS-4: 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 (Fourth International Study of Infarct Survival) Collaborative Group. *Lancet.* 345:669-85.

Jayakody, L., M. Senaratne, A. Thomson, and T. Kappagoda. 1987. Endothelium-dependent relaxation in experimental atherosclerosis in the rabbit. *Circ Res.* 60:251-64.

Jeffrey, F.M., L. Alvarez, V. Diczku, A.D. Sherry, and C.R. Malloy. 1995. Direct evidence that perhexiline modifies myocardial substrate utilization from fatty acids to lactate. *J Cardiovasc Pharmacol.* 25:469-72.

Jia, S.J., D.J. Jiang, C.P. Hu, X.H. Zhang, H.W. Deng, and Y.J. Li. 2005. Lysophosphatidylcholine-induced elevation of asymmetric dimethylarginine level by the NADPH oxidase pathway in endothelial cells. *Vascul Pharmacol.*

Jiang, X.J., M.F. O'Rourke, W.Q. Jin, L.S. Liu, C.W. Li, P.C. Tai, X.C. Zhang, and S.Z. Liu. 2002. Quantification of glyceryl trinitrate effect through analysis of the synthesised ascending aortic pressure waveform. *Heart.* 88:143-8.

John, S., C. Delles, J. Jacobi, M.P. Schlaich, M. Schneider, G. Schmitz, and R.E. Schmieder. 2001. Rapid improvement of nitric oxide bioavailability after lipid-lowering therapy with cerivastatin within two weeks. *J Am Coll Cardiol.* 37:1351-8.

John, S., J. Jacobi, C. Delles, M.P. Schlaich, O. Alter, and R.E. Schmieder. 2000. Plasma soluble adhesion molecules and endothelium-dependent vasodilation in early human atherosclerosis. *Clin Sci (Lond).* 98:521-9.

John, S., M. Schlaich, M. Langenfeld, H. Weihprecht, G. Schmitz, G. Weidinger, and R.E. Schmieder. 1998. Increased bioavailability of nitric oxide after lipid-lowering therapy in hypercholesterolemic patients: a randomized, placebo-controlled, double-blind study. *Circulation.* 98:211-6.

Johnston, C.I., R. Iansek, J.A. Millar, B.P. McGrath, and P.G. Matthews. 1981. Vasoactive peptides and hypertension: role of angiotensin converting enzyme. *Aust N Z J Med.* 11:59-63.

Jugdutt, B.I., and J.W. Warnica. 1988. Intravenous nitroglycerin therapy to limit myocardial infarct size, expansion, and complications. Effect of timing, dosage, and infarct location. *Circulation.* 78:906-19.

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.

Kass, D.A., E.P. Shapiro, M. Kawaguchi, A.R. Capriotti, A. Scuteri, R.C. deGroof, and E.G. Lakatta. 2001. Improved arterial compliance by a novel advanced glycation end-product crosslink breaker. *Circulation.* 104:1464-70.

Kasten, T.P., S.L. Settle, T.P. Misko, D.P. Riley, R.H. Weiss, M.G. Currie, and G.A. Nickols. 1995. Potentiation of nitric oxide-mediated vascular relaxation by SC52608, a superoxide dismutase mimic. *Proc Soc Exp Biol Med.* 208:170-7.

Katsuki, S., W. Arnold, C. Mittal, and F. Murad. 1977. Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J Cyclic Nucleotide Res*. 3:23-35.

Kawano, H., T. Motoyama, N. Hirai, K. Kugiyama, H. Yasue, and H. Ogawa. 2002. Endothelial dysfunction in hypercholesterolemia is improved by L-arginine administration: possible role of oxidative stress. *Atherosclerosis*. 161:375-80.

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.

Kelly, R.P., H.H. Gibbs, M.F. O'Rourke, J.E. Daley, K. Mang, J.J. Morgan, and A.P. Avolio. 1990. Nitroglycerin has more favourable effects on left ventricular afterload than apparent from measurement of pressure in a peripheral artery. *Eur Heart J*. 11:138-44.

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.

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., K. Beck-Oldach, K. McFadden-Lewis, G.A. Murphy, Y.W. Wong, Y. Zhang, and J.D. Horowitz. 2006. Effect of the anti-anginal agent, perhexiline, on neutrophil, valvular and vascular superoxide formation. *Eur J Pharmacol*. 531:13-9.

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., 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.

Kharazmi, A., H. Nielsen, and P.O. Schiotz. 1988. N-acetylcysteine inhibits human neutrophil and monocyte chemotaxis and oxidative metabolism. *Int J Immunopharmacol*. 10:39-46.

Klaus, W., and K. Guttler. 1978. Functional effects of perhexiline maleate on the isolated myocardium. *In* In: Perhexiline maleate. Precoodings of a symposium. Exerpta Medica, Amsterdam. 23-42.

Kleschyov, A.L., M. Oelze, A. Daiber, Y. Huang, H. Mollnau, E. Schulz, K. Sydow, B. Fichtlscherer, A. Mulsch, and T. Munzel. 2003. Does nitric oxide mediate the vasodilator activity of nitroglycerin? *Circ Res*. 93:e104-12.

Klingbeil, A.U., S. John, M.P. Schneider, J. Jacobi, G. Weidinger, and R.E. Schmieder. 2002. AT1-receptor blockade improves augmentation index: a double-blind, randomized, controlled study. *J Hypertens*. 20:2423-8.

Koh, K.K., J.W. Son, J.Y. Ahn, D.K. Jin, H.S. Kim, D.S. Kim, S.H. Han, W.J. Chung, G.S. Park, and E.K. Shin. 2004. Vascular effects of simvastatin combined with ramipril in hypercholesterolemic patients with coronary artery disease, compared with simvastatin alone: a randomized, double-blind, placebo-controlled, crossover study. *Atherosclerosis*. 177:147-53.

Koike, K., and H. Holmsen. 1987. Requirement for thrombin receptor occupancy during platelet secretion under aggregating and non-aggregating conditions. *Thromb Haemost*. 58:1053-9.

Kojda, G., K. Kottenberg, A. Hacker, and E. Noack. 1998. Alterations of the vascular and the myocardial guanylate cyclase/cGMP-system induced by long-term hypertension in rats. *Pharm Acta Helv*. 73:27-35.

Kowalski, J., M. Kosmider, L. Pawlicki, E. Glowacka, M. Banasik, Z. Baj, J. Ciecwierz, and J. Pasnik. 1997. Complement activates neutrophils during PTCA procedure in patients with unstable angina pectoris. *Int J Cardiol*. 58:229-40.

Kratnov, A.E., S.A. Popov, A.A. Kratnov, and O. Dem'iankova Iu. 2005. [Antioxidative defense in patients with acute coronary syndrome and concomitant type 2 diabetes and its modification by lisinopril]. *Kardiologiia*. 45:23-7.

Krieglstein, C.F., and D.N. Granger. 2001. Adhesion molecules and their role in vascular disease. *Am J Hypertens*. 14:44S-54S.

Kubes, P., M. Suzuki, and D.N. Granger. 1991. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A.* 88:4651-5.

Kurose, I., R. Wolf, M.B. Grisham, T.Y. Aw, R.D. Specian, and D.N. Granger. 1995. Microvascular responses to inhibition of nitric oxide production. Role of active oxidants. *Circ Res.* 76:30-9.

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. and NO2- formation. *Biochem J.* 292:545-50.

Langenstroer, P., and G.M. Pieper. 1992. Regulation of spontaneous EDRF release in diabetic rat aorta by oxygen free radicals. *Am J Physiol.* 263:H257-65.

Lapenna, D., and F. Cuccurullo. 1993. TBA test and "free" MDA assay in evaluation of lipid peroxidation and oxidative stress in tissue systems. *Am J Physiol.* 265:H1030-2.

Latini, R., G. Tognoni, A.P. Maggioni, C. Baigent, E. Braunwald, Z.M. Chen, R. Collins, M. Flather, M.G. Franzosi, J. Kjekshus, L. Kober, L.S. Liu, R. Peto, M. Pfeffer, F. Pizzetti, E. Santoro, P. Sleight, K. Swedberg, L. Tavazzi, W. Wang, and S. Yusuf. 2000. Clinical effects of early angiotensin-converting enzyme inhibitor treatment for acute myocardial infarction are similar in the presence and absence of aspirin: systematic overview of individual data from 96,712 randomized patients. Angiotensin-converting Enzyme Inhibitor Myocardial Infarction Collaborative Group. *J Am Coll Cardiol.* 35:1801-7.

Latson, T.W., W.C. Hunter, N. Katoh, and K. Sagawa. 1988. Effect of nitroglycerin on aortic impedance, diameter, and pulse-wave velocity. *Circ Res.* 62:884-90.

Lau, D.T., E.K. Chan, and L.Z. Benet. 1992. Glutathione S-transferase-mediated metabolism of glyceryl trinitrate in subcellular fractions of bovine coronary arteries. *Pharm Res.* 9:1460-4.

Laursen, J.B., A. Mulsch, S. Boesgaard, P. Mordvintcev, S. Trautner, N. Gruhn, J.E. Nielsen-Kudsk, R. Busse, and J. Aldershvile. 1996. In vivo nitrate tolerance is not associated with reduced bioconversion of nitroglycerin to nitric oxide. *Circulation.* 94:2241-7.

Laursen, J.B., S. Rajagopalan, Z. Galis, M. Tarpey, B.A. Freeman, and D.G. Harrison. 1997. Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. *Circulation.* 95:588-93.

Lee, C., K. Miura, X. Liu, and J.L. Zweier. 2000. Biphasic regulation of leukocyte superoxide generation by nitric oxide and peroxynitrite. *J Biol Chem.* 275:38965-72.

Lee, G., D.T. Mason, and A.N. De Maria. 1978. Effects of long-term oral administration of isosorbide dinitrate on the antianginal response to nitroglycerin. Absence of nitrate cross-tolerance and self-tolerance shown by exercise testing. *Am J Cardiol.* 41:82-7.

Lee, L., R. Campbell, M. Scheuermann-Freestone, R. Taylor, P. Gunaruwan, L. Williams, H. Ashrafian, J. Horowitz, A.G. Fraser, K. Clarke, and M. Frenneaux. 2005a. Metabolic modulation with perhexiline in chronic heart failure: A randomised controlled trial on short term use of a novel treatment. *Circulation.* in press.

Lee, L., R. Campbell, M. Scheuermann-Freestone, R. Taylor, P. Gunaruwan, L. Williams, H. Ashrafian, J. Horowitz, A.G. Fraser, K. Clarke, and M. Frenneaux. 2005b. Metabolic modulation with perhexiline in chronic heart failure: a randomized, controlled trial of short-term use of a novel treatment. *Circulation.* 112:3280-8.

Leidke, A. 1981. Alterations of carbohydrate and lipid metabolism in the acutely ischaemic. *Prog Cardiovasc Disease.* 23:321-336.

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.

LeWinter, M.M., and B. Sobel. 1996. Chronic Angina:Stable. *In* Cardiovascular Therapeutics. T.W. Swith, editor. Saunders, Philadelphia. 85-111.

Liao, J.K., M.A. Bettmann, T. Sandor, J.I. Tucker, S.M. Coleman, and M.A. Creager. 1991. Differential impairment of vasodilator responsiveness of peripheral resistance and conduit vessels in humans with atherosclerosis. *Circ Res.* 68:1027-34.

Lin, S.G., and J.T. Flaherty. 1985. Crossover from intravenous to transdermal nitroglycerin therapy in unstable angina pectoris. *Am J Cardiol.* 56:742-8.

Lindmark, E., L. Wallentin, and A. Siegbahn. 2001. Blood cell activation, coagulation, and inflammation in men and women with coronary artery disease. *Thromb Res.* 103:249-59.

Liuzzo, G., L.M. Biasucci, J.R. Gallimore, R.L. Grillo, A.G. Rebuzzi, M.B. Pepys, and A. Maseri. 1994. The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina. *N Engl J Med.* 331:417-24.

Ludmer, P., A. Selwyn, T. Shook, R. Wayne, G. Mudge, R. Alexander, and P. Ganz. 1986. Parodoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N Engl J Med.* 315:1046-1051.

Lund, R.P., J. Haggendal, and G. Johnsson. 1968. Withdrawal symptoms in workers exposed to nitroglycerine. *Br J Ind Med.* 25:136-8.

Mackenzie, I.S., K.M. Maki-Petaja, C.M. McEniery, Y.P. Bao, S.M. Wallace, J. Cheriyan, S. Monteith, M.J. Brown, and I.B. Wilkinson. 2005. Aldehyde dehydrogenase 2 plays a role in the bioactivation of nitroglycerin in humans. *Arterioscler Thromb Vasc Biol.* 25:1891-5.

Mak, I.T., A.M. Freedman, B.F. Dickens, and W.B. Weglicki. 1990. Protective effects of sulfhydryl-containing angiotensin converting enzyme inhibitors against free radical injury in endothelial cells. *Biochem Pharmacol.* 40:2169-75.

Masoni, A., A.M. Tomasi, and G.A. Oriani. 1975. Clinical evaluation of perhexiline maleate in the treatment of patients with chronic coronary insufficiency. *Am Heart J.* 90:145-52.

Massa, M., V. Rosti, M. Ferrario, R. Campanelli, I. Ramajoli, R. Rosso, G.M. De Ferrari, M. Ferlini, L. Goffredo, A. Bertoletti, C. Klersy, A. Pecci, R. Moratti, and L. Tavazzi. 2005. Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction. *Blood.* 105:199-206.

McDonald, B.J., and B.M. Bennett. 1990. Cytochrome P-450 mediated biotransformation of organic nitrates. *Can J Physiol Pharmacol.* 68:1552-7.

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.

McGuire, J.J., D.J. Anderson, B.J. McDonald, R. Narayanasami, and B.M. Bennett. 1998. Inhibition of NADPH-cytochrome P450 reductase and glyceryl trinitrate biotransformation by diphenyleneiodonium sulfate. *Biochem Pharmacol.* 56:881-93.

McLenachan, J.M., J. Vita, D.R. Fish, C.B. Treasure, D.A. Cox, P. Ganz, and A.P. Selwyn. 1990. Early evidence of endothelial vasodilator dysfunction at coronary branch points. *Circulation.* 82:1169-73.

McLenachan, J.M., J.K. Williams, R.D. Fish, P. Ganz, and A.P. Selwyn. 1991. Loss of flow-mediated endothelium-dependent dilation occurs early in the development of atherosclerosis. *Circulation.* 84:1273-8.

McVeigh, G., G. Brennan, R. Hayes, and D. Johnston. 1994. Primary nitrate tolerance in diabetes mellitus. *Diabetologia.* 37:115-7.

McVeigh, G.E., G.M. Brennan, G.D. Johnston, B.J. McDermott, L.T. McGrath, W.R. Henry, J.W. Andrews, and J.R. Hayes. 1992. Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia.* 35:771-6.

Mehta, J., J. Dinerman, P. Mehta, T.G. Saldeen, D. Lawson, W.H. Donnelly, and R. Wallin. 1989. Neutrophil function in ischemic heart disease. *Circulation.* 79:549-56.

Merillon, J.P., G. Fontenier, J.F. Lerallut, M.Y. Jaffrin, J. Chastre, P. Assayag, G. Motte, and R. Gourgon. 1984. Aortic input impedance in heart failure: comparison with normal subjects and its changes during vasodilator therapy. *Eur Heart J.* 5:447-55.

Mickelson, J.K., N.M. Lakkis, G. Villarreal-Levy, B.J. Hughes, and C.W. Smith. 1996. Leukocyte activation with platelet adhesion after coronary angioplasty: a mechanism for recurrent disease? *J Am Coll Cardiol.* 28:345-53.

Mikolich, J.R., N.B. Nicoloff, P.H. Robinson, and R.B. Logue. 1980. Relief of refractory angina with continuous intravenous infusion of nitroglycerin. *Chest.* 77:375-9.

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.

Mitchell, G.F., H. Parise, E.J. Benjamin, M.G. Larson, M.J. Keyes, J.A. Vita, R.S. Vasan, and D. Levy. 2004. Changes in arterial stiffness and wave reflection with advancing age in healthy men and women: the Framingham Heart Study. *Hypertension.* 43:1239-45.

Mittermayer, F., G. Schaller, J. Pleiner, A. Vychytil, G. Sunder-Plassmann, W.H. Horl, and M. Wolzt. 2005. Asymmetrical dimethylarginine plasma concentrations are related to basal nitric oxide release but not endothelium-dependent vasodilation of resistance arteries in peritoneal dialysis patients. *J Am Soc Nephrol.* 16:1832-8.

Mohanty, P., W. Homouda, R. Garg, A. Aljada, H. Ghanim, and P. Dandona. 2000. Glucose challenge stimulates Reactive oxygen species (ROS) generation by leucocytes. *J Clin Endocrinol Metab.* 85:2970-73.

Morledge, J. 1973. Proceedings: Effects of perhexiline maleate in angina pectoris: a double-blind clinical evaluation with ECG-treadmill exercise testing. *Postgrad Med J.* 49:Suppl 3:64-7.

Muller, S., I. Konig, W. Meyer, and G. Kojda. 2004. Inhibition of vascular oxidative stress in hypercholesterolemia by eccentric isosorbide mononitrate. *J Am Coll Cardiol.* 44:624-31.

Mulsch, A., J. Bauersachs, A. Schafer, J.P. Stasch, R. Kast, and R. Busse. 1997. Effect of YC-1, an NO-independent, superoxide-sensitive stimulator of soluble guanylyl cyclase, on smooth muscle responsiveness to nitrovasodilators. *Br J Pharmacol.* 120:681-9.

Murad, F., R.M. Rapoport, and R. Fiscus. 1985. Role of cyclic-GMP in relaxations of vascular smooth muscle. *J Cardiovasc Pharmacol.* 7:S111-8.

Nabel, E.G., A.P. Selwyn, and P. Ganz. 1990. Large coronary arteries in humans are responsive to changing blood flow: an endothelium-dependent mechanism that fails in patients with atherosclerosis. *J Am Coll Cardiol.* 16:349-56.

Napoli, C., V. Sica, F. de Nigris, O. Pignalosa, M. Condorelli, L.J. Ignarro, and A. Liguori. 2004. Sulfhydryl angiotensin-converting enzyme inhibition induces sustained reduction of systemic oxidative stress and improves the nitric oxide pathway in patients with essential hypertension. *Am Heart J.* 148:e5.

Naruko, T., M. Ueda, K. Haze, A.C. van der Wal, C.M. van der Loos, A. Itoh, R. Komatsu, Y. Ikura, M. Ogami, Y. Shimada, S. Ehara, M. Yoshiyama, K. Takeuchi, J. Yoshikawa, and A.E. Becker. 2002. Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation.* 106:2894-900.

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.

Nigam, R., T. Whiting, and B.M. Bennett. 1993. Effect of inhibitors of glutathione S-transferase on glyceryl trinitrate activity in isolated rat aorta. *Can J Physiol Pharmacol.* 71:179-84.

Numaguchi, Y., T. Sone, K. Okumura, M. Ishii, Y. Morita, R. Kubota, K. Yokouchi, H. Imai, M. Harada, H. Osanai, T. Kondo, and T. Murohara. 2006. The impact of the capability of circulating progenitor cell to differentiate on myocardial salvage in patients with primary acute myocardial infarction. *Circulation.* 114:I114-9.

Okouchi, M., N. Okayama, H. Omi, K. Imaeda, T. Fukutomi, A. Nakamura, and M. Itoh. 2004. Protective actions of gliclazide on high insulin-enhanced neutrophil-endothelial cell interactions through inhibition of mitogen activated protein kinase and protein kinase C pathways. *Microvasc Res.* 67:1-8.

O'Rourke, M. 1990. Arterial stiffness, systolic blood pressure, and logical treatment of arterial hypertension. *Hypertension.* 15:339-47.

O'Rourke, M.F., and R.P. Kelly. 1993. Wave reflection in the systemic circulation and its implications in ventricular function. *J Hypertens.* 11:327-37.

Ozuyaman, B., P. Ebner, U. Niesler, J. Ziemann, P. Kleinbongard, T. Jax, A. Godecke, M. Kelm, and C. Kalka. 2005. Nitric oxide differentially regulates proliferation and mobilization of endothelial progenitor cells but not of hematopoietic stem cells. *Thromb Haemost.* 94:770-2.

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.

Panza, J.A., A.A. Quyyumi, J.E. Brush, Jr., and S.E. Epstein. 1990. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med.* 323:22-7.

Paravicini, T.M., L.M. Gulluyan, G.J. Dusting, and G.R. Drummond. 2002. Increased NADPH oxidase activity, gp91phox expression, and endothelium-dependent vasorelaxation during neointima formation in rabbits. *Circ Res.* 91:54-61.

Parker, J.D., and J.O. Parker. 1998. Nitrate therapy for stable angina pectoris. *N Engl J Med.* 338:520-31.

Parker, J.O., B. Farrell, K.A. Lahey, and G. Moe. 1987. Effect of intervals between doses on the development of tolerance to isosorbide dinitrate. *N Engl J Med.* 316:1440-4.

Parker, J.O., K.A. Vankoughnett, and B. Farrell. 1985. Comparison of buccal nitroglycerin and oral isosorbide dinitrate for nitrate tolerance in stable angina pectoris. *Am J Cardiol.* 56:724-8.

Pauca, A.L., N.D. Kon, and M.F. O'Rourke. 2005. Benefit of glyceryl trinitrate on arterial stiffness is directly due to effects on peripheral arteries. *Heart.* 91:1428-32.

Pepine, C.J., L.M. Lopez, D.M. Bell, E.M. Handberg-Thurmond, R.G. Marks, and S. McGorray. 1997. Effects of intermittent transdermal nitroglycerin on occurrence of ischemia after patch removal: results of the second transdermal intermittent dosing evaluation study (TIDES-II). *J Am Coll Cardiol.* 30:955-61.

Pepine, C.J., W.W. Nichols, R.C. Curry, Jr., and C.R. Conti. 1979. Aortic input impedance during nitroprusside infusion. A reconsideration of afterload reduction and beneficial action. *J Clin Invest.* 64:643-54.

Pepne, C.J., S.J. Schang, and C.R. Bemiller. 1974. Effects of perhexiline on symptomatic and hemodynamic responses to exercise in patients with angina pectoris. *Am J Cardiol.* 33:806-12.

Perticone, F., A. Sciacqua, R. Maio, M. Perticone, R. Maas, R.H. Boger, G. Tripepi, G. Sesti, and C. Zoccali. 2005. Asymmetric dimethylarginine, L-arginine, and endothelial dysfunction in essential hypertension. *J Am Coll Cardiol.* 46:518-23.

Pfeffer, M.A., M. Domanski, J. Verter, M. Dunlap, G.C. Flaker, B. Gersh, J. Hsia, A.D. Goldberg, M.C. Limacher, A.P. Maggioni, Y. Rosenberg, J.L. Rouleau, J.W. Warnica, A.G. Wasserman, and E. Braunwald. 2001. The continuation of the Prevention of Events With Angiotensin-Converting Enzyme Inhibition (PEACE) Trial. *Am Heart J.* 142:375-7.

Pfeifer, A., P. Klatt, S. Massberg, L. Ny, M. Sausbier, C. Hirneiss, G.X. Wang, M. Korth, A. Aszodi, K.E. Andersson, F. Krombach, A. Mayerhofer, P. Ruth, R. Fassler, and F. Hofmann. 1998. Defective smooth muscle regulation in cGMP kinase I-deficient mice. *Embo J.* 17:3045-51.

Philpott, A., S. Chandy, R. Morris, and J.D. Horowitz. 2004. Development of a regimen for rapid initiation of perhexiline therapy in acute coronary syndromes. *Intern Med J.* 34:361-3.

Pruefer, D., R. Scalia, and A.M. Lefer. 1999. Homocysteine provokes leukocyte-endothelium interaction by downregulation of nitric oxide. *Gen Pharmacol.* 33:487-98.

Quyyumi, A.A. 1998. Does acute improvement of endothelial dysfunction in coronary artery disease improve myocardial ischemia? A double-blind comparison of parenteral D- and L-arginine. *J Am Coll Cardiol.* 32:904-11.

Radomski, M.W., R.M. Palmer, and S. Moncada. 1987. The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br J Pharmacol.* 92:639-46.

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.

Rapoport, R.M., M. Ashraf, and F. Murad. 1989. Effects of melittin on endothelium-dependent relaxation and cyclic GMP levels in rat aorta. *Circ Res.* 64:463-73.

Rapoport, R.M., M.B. Draznin, and F. Murad. 1983. Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation. *Nature.* 306:174-6.

Ratz, J.D., J.J. McGuire, D.J. Anderson, and B.M. Bennett. 2000. Effects of the flavoprotein inhibitor, diphenyleneiodonium sulfate, on ex vivo organic nitrate tolerance in the rat. *J Pharmacol Exp Ther*. 293:569-77.

Reinhard, M., K. Giehl, K. Abel, C. Haffner, T. Jarchau, V. Hoppe, B.M. Jockusch, and U. Walter. 1995. The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. *Embo J.* 14:1583-9.

Rocic, P., and P.A. Lucchesi. 2005. NAD(P)H oxidases and TGF-beta-induced cardiac fibroblast differentiation: Nox-4 gets Smad. *Circ Res*. 97:850-2.

Roth, A., D. Kulick, L. Freidenberger, R. Hong, S.H. Rahimtoola, and U. Elkayam. 1987. Early tolerance to hemodynamic effects of high dose transdermal nitroglycerin in responders with severe chronic heart failure. *J Am Coll Cardiol*. 9:858-64.

Ruth, P., G.X. Wang, I. Boekhoff, B. May, A. Pfeifer, R. Penner, M. Korth, H. Breer, and F. Hofmann. 1993. Transfected cGMP-dependent protein kinase suppresses calcium transients by inhibition of inositol 1,4,5-trisphosphate production. *Proc Natl Acad Sci U S A*. 90:2623-7.

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, J.L. Rouleau, and et al. 1994. Effects of captopril on ischemic events after myocardial infarction. Results of the Survival and Ventricular Enlargement trial. SAVE Investigators. *Circulation*. 90:1731-8.

Sadowska, A.M., Y.K.B. Manuel, T. Vertongen, G. Schippers, D. Radomska-Lesniewska, E. Heytens, and W.A. De Backer. 2006. Effect of N-acetylcysteine on neutrophil activation markers in healthy volunteers: In vivo and in vitro study. *Pharmacol Res*. 53:216-25.

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.

Sato, K., M. Kinoshita, M. Kojima, K. Miyagawa, H. Takase, S. Suzuki, and Y. Dohi. 2000. Failure of L-arginine to induce hypotension in patients with a history of accelerated-malignant hypertension. *J Hum Hypertens*. 14:485-8.

Schachinger, V., M.B. Britten, M. Elsner, D.H. Walter, I. Scharrer, and A.M. Zeiher. 1999. A positive family history of premature coronary artery disease is associated with impaired endothelium-dependent coronary blood flow regulation. *Circulation*. 100:1502-8.

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.

Schafer, A., M. Burkhardt, T. Vollkommer, J. Bauersachs, T. Munzel, U. Walter, and A. Smolenski. 2003. Endothelium-dependent and -independent relaxation and VASP serines 157/239 phosphorylation by cyclic nucleotide-elevating vasodilators in rat aorta. *Biochem Pharmacol*. 65:397-405.

Schafer, A., J. Widder, M. Eigenthaler, G. Ertl, and J. Bauersachs. 2004a. Reduced basal nitric oxide bioavailability and platelet activation in young spontaneously hypertensive rats. *Biochem Pharmacol*. 67:2273-9.

Schafer, A., F. Wiesmann, S. Neubauer, M. Eigenthaler, J. Bauersachs, and K.M. Channon. 2004b. Rapid regulation of platelet activation in vivo by nitric oxide. *Circulation*. 109:1819-22.

Schlaifer, J.D., G.B. Mancini, B.J. O'Neill, B. Pitt, H.E. Haber, and C.J. Pepine. 1999. Influence of smoking status on angiotensin-converting enzyme inhibition-related improvement in coronary endothelial function. TREND Investigators. Trial on Reversing Endothelial Dysfunction. *Cardiovasc Drugs Ther*. 13:201-9.

Schmidt, K., E.R. Werner, B. Mayer, H. Wachter, and W.R. Kukovetz. 1992. Tetrahydrobiopterin-dependent formation of endothelium-derived relaxing factor (nitric oxide) in aortic endothelial cells. *Biochem J*. 281:297-300.

Schroder, H. 1992. Cytochrome P-450 mediates bioactivation of organic nitrates. *J Pharmacol Exp Ther*. 262:298-302.

Secco, D.D., J.A. Paron, S.H. de Oliveira, S.H. Ferreira, J.S. Silva, and Q. Cunha Fde. 2003. Neutrophil migration in inflammation: nitric oxide inhibits rolling, adhesion and induces apoptosis. *Nitric Oxide*. 9:153-64.

Segers, P., A. Qasem, T. De Backer, S. Carlier, P. Verdonck, and A. Avolio. 2001. Peripheral "oscillatory" compliance is associated with aortic augmentation index. *Hypertension*. 37:1434-9.

Serrano, C.V., Jr., R. Rocha Giraldez, J. de Lara Fernandes, J.C. Nicolau, J.L. Zweier, and J.A. Ramires. 2005. Platelet and leukocyte adhesion and activation in unstable angina and post-PTCA. *Int J Cardiol.* 99:423-8.

Servent, D., M. Delaforge, C. Ducrocq, D. Mansuy, and M. Lenfant. 1989. Nitric oxide formation during microsomal hepatic denitration of glyceryl trinitrate: involvement of cytochrome P-450. *Biochem Biophys Res Commun.* 163:1210-6.

Seth, P., R. Kumari, M. Dikshit, and R.C. Srimal. 1994. Modulation of rat peripheral polymorphonuclear leukocyte response by nitric oxide and arginine. *Blood.* 84:2741-8.

Shah, R.R., N.S. Oates, J.R. Idle, R.L. Smith, and J.D. Lockhart. 1982. Impaired oxidation of debrisoquine in patients with perhexiline neuropathy. *Br Med J (Clin Res Ed).* 284:295-9.

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.

Shurtz-Swirski, R., S. Sela, A.T. Herskovits, S.M. Shasha, G. Shapiro, L. Nasser, and B. Kristal. 2001. Involvement of peripheral polymorphonuclear leukocytes in oxidative stress and inflammation in type 2 diabetic patients. *Diabetes Care.* 24:104-10.

Sihm, I., A.P. Schroeder, C. Aalkjaer, M. Holm, B. Morn, M. Mulvany, K. Thygesen, and O. Lederballe. 1995. Regression of media-to-lumen ratio of human subcutaneous arteries and left ventricular hypertrophy during treatment with an angiotensin-converting enzyme inhibitor-based regimen in hypertensive patients. *Am J Cardiol.* 76:38E-40E.

Skowasch, D., A. Viktor, M. Schneider-Schmitt, B. Luderitz, G. Nickenig, and G. Bauriedel. 2006. Differential antiplatelet effects of angiotensin converting enzyme inhibitors : Comparison of ex vivo platelet aggregation in cardiovascular patients with ramipril, captopril and enalapril. *Clin Res Cardiol.* 95:212-6.

Soejima, H., H. Ogawa, H. Yasue, K. Kaikita, K. Takazoe, K. Nishiyama, K. Misumi, S. Miyamoto, M. Yoshimura, K. Kugiyama, S. Nakamura, and I. Tsuji. 1999. Angiotensin-converting enzyme inhibition reduces monocyte chemoattractant protein-1 and tissue factor levels in patients with myocardial infarction. *J Am Coll Cardiol.* 34:983-8.

Sorensen, K.E., D.S. Celermajer, D. Georgakopoulos, G. Hatcher, D.J. Betteridge, and J.E. Deanfield. 1994. Impairment of endothelium-dependent dilation is an early event in children with familial hypercholesterolemia and is related to the lipoprotein(a) level. *J Clin Invest.* 93:50-5.

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, A.D. 1973. [Perhexiline maleate in angina pectoris. Study of tolerance to treadmill exercise]. *Arq Bras Cardiol.* 26 Suppl 1:83-8.

Steen, V.M., and H. Holmsen. 1985. Synergism between thrombin and epinephrine in human platelets: different dose-response relationships for aggregation and dense granule secretion. *Thromb Haemost.* 54:680-3.

Stephens, N.G., A. Parsons, P.M. Schofield, F. Kelly, K. Cheeseman, and M.J. Mitchinson. 1996. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet.* 347:781-6.

Stepien, J.M., R.M. Prideaux, S.R. Willoughby, Y.Y. Chirkov, and J.D. Horowitz. 2003. Pilot study examining the effect of cholesterol lowering on platelet nitric oxide responsiveness and arterial stiffness in subjects with isolated mild hypercholesterolaemia. *Clin Exp Pharmacol Physiol.* 30:507-12.

Stokes, K.Y., L. Calahan, J.M. Russell, S. Gurwara, and D.N. Granger. 2006. Role of platelets in hypercholesterolemia-induced leukocyte recruitment and arteriolar dysfunction. *Microcirculation.* 13:377-88.

Sturrock, A., B. Cahill, K. Norman, T.P. Huecksteadt, K. Hill, K. Sanders, S.V. Karwande, J.C. Stringham, D.A. Bull, M. Gleich, T.P. Kennedy, and J.R. Hoidal. 2006. Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol.* 290:L661-L673.

Sugano, R., H. Matsuoka, N. Haramaki, H. Umei, E. Murase, K. Fukami, S. Iida, H. Ikeda, and T. Imaizumi. 2005. Polymorphonuclear leukocytes may impair endothelial function: results of crossover randomized study of lipid-lowering therapies. *Arterioscler Thromb Vasc Biol.* 25:1262-7.

Swedberg, K., and J. Kjekshus. 1988. Effects of enalapril on mortality in severe congestive heart failure: results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). *Am J Cardiol.* 62:60A-66A.

Sydow, K., and T. Munzel. 2003. ADMA and oxidative stress. *Atheroscler Suppl.* 4:41-51.

Takase, B., A. Hamabe, K. Satomura, T. Akima, A. Uehata, T. Matsui, F. Ohsuzu, M. Ishihara, and A. Kurita. 2006. Comparable prognostic value of vasodilator response to acetylcholine in brachial and coronary arteries for predicting long-term cardiovascular events in suspected coronary artery disease. *Circ J.* 70:49-56.

Taylor, A.L., S. Ziesche, C. Yancy, P. Carson, R. D'Agostino, Jr., K. Ferdinand, M. Taylor, K. Adams, M. Sabolinski, M. Worcel, and J.N. Cohn. 2004. Combination of isosorbide dinitrate and hydralazine in blacks with heart failure. *N Engl J Med.* 351:2049-57.

Teo, K.K., J.G. Kelly, J.F. Darby, J.T. Ennis, and J.H. Horgan. 1983. Perhexiline during exercise training in coronary heart disease. *Clin Pharmacol Ther.* 34:744-8.

Teragawa, H., K. Ueda, K. Matsuda, M. Kimura, Y. Higashi, T. Oshima, M. Yoshizumi, and K. Chayama. 2005. Relationship between endothelial function in the coronary and brachial arteries. *Clin Cardiol.* 28:460-6.

Thadani, U. 1997. Nitrate tolerance, rebound, and their clinical relevance in stable angina pectoris, unstable angina, and heart failure. *Cardiovasc Drugs Ther.* 10:735-42.

Theroux, P., J.T. Willerson, and P.W. Armstrong. 2000. Progress in the treatment of acute coronary syndromes: a 50-year perspective (1950-2000). *Circulation.* 102:IV2-IV13.

Tiefenbacher, C.P. 2001. Tetrahydrobiopterin: a critical cofactor for eNOS and a strategy in the treatment of endothelial dysfunction? *Am J Physiol Heart Circ Physiol.* 280:H2484-8.

Tiefenbacher, C.P., T. Bleeke, C. Vahl, K. Amann, A. Vogt, and W. Kubler. 2000. Endothelial dysfunction of coronary resistance arteries is improved by tetrahydrobiopterin in atherosclerosis. *Circulation.* 102:2172-9.

Tiefenbacher, C.P., S. Friedrich, T. Bleeke, C. Vahl, X. Chen, and F. Niroomand. 2004. ACE inhibitors and statins acutely improve endothelial dysfunction of human coronary arterioles. *Am J Physiol Heart Circ Physiol.* 286:H1425-32.

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.

Touyz, R.M., X. Chen, F. Tabet, G. Yao, G. He, M.T. Quinn, P.J. Pagano, and E.L. Schiffrin. 2002. Expression of a functionally active gp91phox-containing neutrophil-type NAD(P)H oxidase in smooth muscle cells from human resistance arteries: regulation by angiotensin II. *Circ Res.* 90:1205-13.

Touyz, R.M., G. Yao, M.T. Quinn, P.J. Pagano, and E.L. Schiffrin. 2005. p47phox associates with the cytoskeleton through cortactin in human vascular smooth muscle cells: role in NAD(P)H oxidase regulation by angiotensin II. *Arterioscler Thromb Vasc Biol.* 25:512-8.

Towell, J.F., 3rd, J.J. Barboriak, W.F. Townsend, J.H. Kalbfleisch, and R.I. Wang. 1986. Erythrocyte aldehyde dehydrogenase: assay of a potential biochemical marker of alcohol abuse. *Clin Chem.* 32:734-8.

Tritto, I., P. Wang, P. Kuppusamy, R. Giraldez, J.L. Zweier, and G. Ambrosio. 2005. The anti-anginal drug trimetazidine reduces neutrophil-mediated cardiac reperfusion injury. *J Cardiovasc Pharmacol.* 46:89-98.

Unger, S.A., J.A. Kennedy, K. McFadden-Lewis, K. Minerds, G.A. Murphy, and J.D. Horowitz. 2005. Dissociation between metabolic and efficiency effects of perhexiline in normoxic rat myocardium. *J Cardiovasc Pharmacol.* 46:849-55.

van de Wal, R.M., H.W. Plokker, D.J. Lok, F. Boomsma, F.A. van der Horst, D.J. van Veldhuisen, W.H. van Gilst, and A.A. Voors. 2006. Determinants of increased angiotensin II levels in severe chronic heart failure patients despite ACE inhibition. *Int J Cardiol.* 106:367-72.

van den Berg, E.K., J.M. Schmitz, C.R. Benedict, C.R. Malloy, J.T. Willerson, and G.J. Dehmer. 1989. Transcardiac serotonin concentration is increased in selected patients with limiting angina and complex coronary lesion morphology. *Circulation*. 79:116-24.

van der Wal, A.C., A.E. Becker, and P.K. Das. 1993. Medial thinning and atherosclerosis--evidence for involvement of a local inflammatory effect. *Atherosclerosis*. 103:55-64.

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.

Varin, R., P. Mulder, F. Tamion, V. Richard, J.P. Henry, F. Lallemand, G. Lerebours, and C. Thuillez. 2000. Improvement of endothelial function by chronic angiotensin-converting enzyme inhibition in heart failure : role of nitric oxide, prostanoids, oxidant stress, and bradykinin. *Circulation*. 102:351-6.

Vaughan Williams, E.M. 1980. In: (3rd Edn ed.),Anti-Arrhythmic Action and the Puzzle of Perhexiline,. Academic Press, London.

Verbeuren, T.J., F.H. Jordaens, L.L. Zonnekeyn, C.E. Van Hove, M.C. Coene, and A.G. Herman. 1986. Effect of hypercholesterolemia on vascular reactivity in the rabbit. I. Endothelium-dependent and endothelium-independent contractions and relaxations in isolated arteries of control and hypercholesterolemic rabbits. *Circ Res*. 58:552-64.

Vlachopoulos, C., K. Hirata, and M.F. O'Rourke. 2001. Pressure-altering agents affect central aortic pressures more than is apparent from upper limb measurements in hypertensive patients: the role of arterial wave reflections. *Hypertension*. 38:1456-60.

Wainwright, R.J., J.P. Foran, S.F. Padaria, F. Akhras, G. Jackson, and A.R. Clark. 1993. The long-term safety and tolerability of transdermal glyceryl trinitrate, when used with a patch-free interval in patients with stable angina. *Br J Clin Pract*. 47:178-82.

Wang, H.D., P.J. Pagano, Y. Du, A.J. Cayatte, M.T. Quinn, P. Brecher, and R.A. Cohen. 1998. Superoxide anion from the adventitia of the rat thoracic aorta inactivates nitric oxide. *Circ Res*. 82:810-8.

Wang, J.P., L.C. Chang, S.L. Raung, M.F. Hsu, L.J. Huang, and S.C. Kuo. 2002. Inhibition of superoxide anion generation by YC-1 in rat neutrophils through cyclic GMP-dependent and -independent mechanisms. *Biochem Pharmacol*. 63:577-85.

Wanikiat, P., D.F. Woodward, and R.A. Armstrong. 1997. Investigation of the role of nitric oxide and cyclic GMP in both the activation and inhibition of human neutrophils. *Br J Pharmacol*. 122:1135-45.

Waring, W.S., H.M. Sinclair, and D.J. Webb. 2006. Effects of salbutamol and glyceryl trinitrate on large arterial stiffness are similar between patients with hypertension and adults with normal blood pressure. *Br J Clin Pharmacol*.

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. 1998. 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.

Watts, G.F., S.F. O'Brien, W. Silvester, and J.A. Millar. 1996. Impaired endothelium-dependent and independent dilatation of forearm resistance arteries in men with diet-treated non-insulin-dependent diabetes: role of dyslipidaemia. *Clin Sci (Lond)*. 91:567-73.

We, G.C., M.G. Siroi, R. Qu, P. Liu, and J.L. Roulea. 2002. Effects of quinapril on myocardial function, ventricular remodeling and cardiac cytokine expression in congestive heart failure in the rat. *Cardiovasc Drugs Ther*. 16:29-36.

Westerbacka, J., M. Tamminen, J. Cockcroft, and H. Yki-Jarvinen. 2004. Comparison of in vivo effects of nitroglycerin and insulin on the aortic pressure waveform. *Eur J Clin Invest*. 34:1-8.

Wiemer, G., W. Linz, S. Hatrik, B.A. Scholkens, and T. Malinski. 1997. Angiotensin-converting enzyme inhibition alters nitric oxide and superoxide release in normotensive and hypertensive rats. *Hypertension*. 30:1183-90.

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. 2002a. 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, J.R. Cockcroft, and D.J. Webb. 2002b. Inhibition of basal nitric oxide synthesis increases aortic augmentation index and pulse wave velocity in vivo. *Br J Clin Pharmacol.* 53:189-92.

Wilkinson, I.B., H. MacCallum, P.C. Hupperetz, C.J. van Thoor, J.R. Cockcroft, and D.J. Webb. 2001. Changes in the derived central pressure waveform and pulse pressure in response to angiotensin II and noradrenaline in man. *J Physiol.* 530:541-50.

Williams, F.M., K. Tanda, M. Kus, and T.J. Williams. 1993. Trimetazidine inhibits neutrophil accumulation after myocardial ischaemia and reperfusion in rabbits. *J Cardiovasc Pharmacol.* 22:828-33.

Williams, S.B., J.A. Cusco, M.A. Roddy, M.T. Johnstone, and M.A. Creager. 1996. Impaired nitric oxide-mediated vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Am Coll Cardiol.* 27:567-74.

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. *Eur Heart J.* 23:1946-54.

Willoughby, S.R., S. Stewart, A.S. Holmes, Y.Y. Chirkov, and J.D. Horowitz. 2005. Platelet nitric oxide responsiveness: a novel prognostic marker in acute coronary syndromes. *Arterioscler Thromb Vasc Biol.* 25:2661-6.

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.

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.

Wirth, K., G. Breipohl, J. Stechl, J. Knolle, S. Henke, and B. Scholkens. 1991. DesArg9-D-Arg[Hyp3,Thi5,D-Tic7,Oic8]bradykinin (desArg10-[Hoe140]) is a potent bradykinin B1 receptor antagonist. *Eur J Pharmacol.* 205:217-8.

Wolin, M.S., P.D. Cherry, J.M. Rodenburg, E.J. Messina, and G. Kaley. 1990. Methylene blue inhibits vasodilation of skeletal muscle arterioles to acetylcholine and nitric oxide via the extracellular generation of superoxide anion. *J Pharmacol Exp Ther.* 254:872-6.

Woo, K.S., P. Chook, L.L. Chan, A.S. Cheung, W.H. Fung, M. Qiao, Y.I. Lolin, G.N. Thomas, J.E. Sanderson, C. Metreweli, and D.S. Celermajer. 2002. Long-term improvement in homocysteine levels and arterial endothelial function after 1-year folic acid supplementation. *Am J Med.* 112:535-9.

Woo, K.S., P. Chook, Y.I. Lolin, J.E. Sanderson, C. Metreweli, and D.S. Celermajer. 1999. Folic acid improves arterial endothelial function in adults with hyperhomocystinemia. *J Am Coll Cardiol.* 34:2002-6.

Yaginuma, T., A. Avolio, M. O'Rourke, W. Nichols, J.J. Morgan, P. Roy, D. Baron, J. Branson, and M. Feneley. 1986. Effect of glyceryl trinitrate on peripheral arteries alters left ventricular hydraulic load in man. *Cardiovasc Res.* 20:153-60.

Yeates, R.A., M. Schmid, and M. Leitold. 1989. Antagonism of glycerol trinitrate activity by an inhibitor of glutathione S-transferase. *Biochem Pharmacol.* 38:1749-53.

Youn, T.J., H.S. Kim, and B.H. Oh. 1999. Ventricular remodeling and transforming growth factor-beta 1 mRNA expression after nontransmural myocardial infarction in rats: effects of angiotensin converting enzyme inhibition and angiotensin II type 1 receptor blockade. *Basic Res Cardiol.* 94:246-53.

Yusuf, S., G. Dagenais, J. Pogue, J. Bosch, and P. Sleight. 2000a. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med.* 342:154-60.

Yusuf, S., P. Sleight, J. Pogue, J. Bosch, R. Davies, and G. Dagenais. 2000b. 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.

Zeiher, A.M., H. Drexler, B. Saurbier, and H. Just. 1993. Endothelium-mediated coronary blood flow modulation in humans. Effects of age, atherosclerosis, hypercholesterolemia, and hypertension. *J Clin Invest.* 92:652-62.

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, X., S.P. Zhao, X.P. Li, M. Gao, and Q.C. Zhou. 2000. Endothelium-dependent and -independent functions are impaired in patients with coronary heart disease. *Atherosclerosis.* 149:19-24.

Zhou, X.B., P. Ruth, J. Schlossmann, F. Hofmann, and M. Korth. 1996. Protein phosphatase 2A is essential for the activation of Ca2+-activated K+ currents by cGMP-dependent protein kinase in tracheal smooth muscle and Chinese hamster ovary cells. *J Biol Chem.* 271:19760-7.