



Inhibition of human platelet aggregation by perhexiline maleate: mechanisms and therapeutic implications.

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A Thesis submitted to The University of Adelaide as the
requirement for the degree of

Doctor of Philosophy

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May 1999

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SUMMARY

Experiments described in the thesis address the anti-aggregatory effects and mechanism of action of the prophylactic anti-anginal agent perhexiline maleate. In particular, it was sought to examine if perhexiline had an anti-aggregatory effect which may contribute to its proven therapeutic efficacy. Experiments were performed largely utilising an in vitro model of platelet aggregation (whole blood impedance aggregometry). In addition ex vivo studies were performed in blood samples from 1) stable angina pectoris and acute coronary syndrome patients receiving perhexiline as adjunctive therapy and 2) adult female sheep receiving acute or chronic perhexiline therapy.

In vitro studies

Multiple agonist induction of aggregation: Implications regarding platelet reactivity to anti-aggregatory agents

Platelet aggregation has traditionally been investigated in vitro utilising optical aggregometry and single agonists in supraphysiological concentrations. Experiments were designed to develop a more physiologically appropriate method of in vitro induction of aggregation. It was shown that a combination of adenosine diphosphate (ADP), adrenaline, serotonin and thrombin in physiological concentrations enhanced the anti-aggregating effects of such agents as nitroglycerine and prostaglandin E_1 , when compared to ADP alone. These results provide an in vitro experimental method which mimics the in vivo situation.

Perhexiline inhibits platelet aggregation in vitro

Perhexiline is a prophylactic anti-anginal agent utilised in the management of severe stable angina. The interaction between perhexiline and platelet aggregation was investigated utilising both a single agonist (ADP) and the developed multiple agonist technique. Perhexiline proved to be a more effective inhibitor of platelet aggregation when aggregation was induced by the multiple agonist technique. The anti-aggregating effects of perhexiline were found to be 1) disproportionate with its potency as a calcium channel blocker; 2) not accounted for by changes in the cyclic nucleotides cGMP or cAMP; and 3) not mediated via nitric oxide synthase or cyclic nucleotide phosphodiesterase.

Anti-aggregatory effects of perhexiline are not modulated by the inhibition of carnitine palmitoyltransferase-1

Perhexiline inhibits myocardial carnitine palmitoyltransferase-1, which controls the access of long-chain fatty acids to mitochondrial sites of β -oxidation. Experiments compared the platelet carnitine palmitoyltransferase-1 inhibitory and putative anti-aggregatory effects of perhexiline, amiodarone and trimetazidine with those of the specific carnitine palmitoyltransferase-1 inhibitors: etomoxir and hydroxyphenylglyoxylate. It was found that although all agents tested inhibited platelet carnitine palmitoyltransferase-1 activity, to varying degrees, only perhexiline, amiodarone and trimetazidine inhibited platelet aggregation. These findings indicate

that the anti-aggregatory effects of perhexiline are independent of carnitine palmitoyltransferase-1 inhibition.

Ex vivo studies

Effect of perhexiline maleate on ex vivo platelet aggregation

Perhexiline has previously been shown to be effective in reducing symptoms in patients with unstable angina pectoris. As unstable angina is a syndrome associated with platelet hyperaggregability, this observation suggests that perhexiline may affect platelet aggregation in vivo. Patients with ischaemic heart disease demonstrate a poor responsiveness to nitric oxide, so called "nitrate resistance". The ex vivo effects of short-term perhexiline therapy on platelet aggregation and nitric oxide responsiveness were examined in patients with stable angina (n=30) and acute coronary syndromes (n=50), who had refractory angina despite conventional therapy. Baseline nitric oxide responsiveness was compared with normal volunteers (n=24). It was demonstrated that at baseline stable angina and acute coronary syndrome patients had significantly lower anti-aggregatory responses to the nitric oxide donor sodium nitroprusside (SNP). Perhexiline therapy significantly increased platelet responsiveness to SNP. This increase in SNP response was correlated with resolution of symptoms in the unstable angina patients and a decrease in anginal frequency in patients with stable angina. Perhexiline therapy did not significantly affect ADP-induced platelet aggregation. A cohort of unstable angina patients (n=12) not receiving perhexiline did not demonstrate an increase in SNP response after short-term follow-up.

In acute coronary syndrome patients perhexiline therapy did not affect superoxide content, but increased both the guanylate cyclase-independent and -dependent components of nitric oxide effect.

The results suggest that perhexiline in doses which has no incremental anti-aggregatory effect in the presence of background anti-platelet therapy normalises platelet responses to the nitric oxide donor SNP. This normalisation in platelet responsiveness to exogenous nitric donors, does not involve changes in superoxide content. These results suggest that increased nitric oxide responsiveness may contribute to the therapeutic effects of perhexiline.

Perhexiline inhibits sheep platelet aggregation

These studies were performed to examine effect of perhexiline therapy on ADP-induced sheep platelet aggregation and nitric oxide responsiveness. In sheep acutely treated with intravenously infused perhexiline, no anti-aggregatory effect was detected (n=6); similar results were obtained for the specific carnitine palmitoyltransferase-1 inhibitor, etomoxir (n=3). However, when perhexiline was chronically administered orally to sheep (n=2) an anti-aggregatory effect was evident. Sheep platelets were totally unresponsive to the anti-aggregatory nitric oxide donor sodium nitroprusside.

These results suggest the perhexiline has an anti-aggregatory effect independent off its interaction with nitric oxide, which might contribute to its beneficial effects in patients.

DECLARATION

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

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopy.

Scott R Willoughby

(May 1999)

ACKNOWLEDGMENTS

I wish to use this opportunity to express my sincere thanks to those people who have been involved in my studies towards this degree over the last four years.

I am most grateful to my supervisors Professor John Horowitz and Doctor Yuliy Chirkov for their guidance, encouragement and friendship throughout the course of my PhD.

I would like to acknowledge the invaluable support, enthusiasm and friendship of Dr Simon Stewart, who was my “partner in crime” during the perhexiline ex vivo studies (Chapter 6). The work of Ms Gerladine Murphy and Dr Jennifer Kennedy in the carnitine palmitoyltransferase-1 assay (Chapter 5) was invaluable to this thesis. I would also like to acknowledge the assistance of Dr Steve Unger in the sheep experiments (Chapter 7). A special thanks is extended to Mr Andrew Holmes (fellow PhD candidate) who has helped me out when it has been most needed.

Thank you to all the Staff of The Queen Elizabeth Hospital Cardiology Unit for their co-operation, support and patience during the various stages of this thesis.

During my studies I have been a recipient a University of Adelaide Postgraduate Research Scholarship and a North Western Adelaide Health Service (NWAHS) Supplementary Postgraduate Research Scholarship.

Thank you to my family for providing support and enthusiasm during the course of my studies. Finally, I wish to sincerely thank my wife Larissa for her love, inspiration, understanding and encouragement during the past few years.

PUBLICATIONS / PRESENTATIONS

Peer reviewed articles relating to this thesis

Willoughby SR, Chirkova LP, Horowitz JD, Chirkov YY. Multiple agonist induction of aggregation: An approach to examine anti-aggregating effects in vitro. *Platelets*, 1996;7:329-333.

Willoughby SR, Chirkov YY, Kennedy JA, Murphy GA, Horowitz JD. Inhibition of long-chain fatty acid metabolism does not affect platelet aggregation responses. *European Journal of Pharmacology*, 1998;356:207-213.

Accepted presentations at international meetings

Willoughby SR, Chirkov YY, Chirkova LP, Horowitz JD. Perhexiline and amiodarone inhibit platelet aggregation in patients with angina. 69th meeting of the American Heart Association, New Orleans, Louisiana (November 1996).

Chirkov YY, Willoughby SR, Chirkova LP, Horowitz JD. Perhexiline inhibits in vitro platelet aggregation of human platelets in whole blood. International Congress of Cardiovascular Pharmacology, Sydney, Australia (February 1996).

Willoughby SR, Chirkov YY, Kennedy JA, Murphy GA, Horowitz JD. The anti-platelet effects of perhexiline, amiodarone and trimetazidine are not mediated by the inhibition

of carnitine palmitoyltransferase-1 (CPT-1). Cardiac Society of Australia and New Zealand. Hobart, Australia (August 1997).

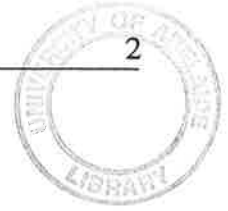
Willoughby SR, Chirkov YY, Chirkova LP, Horowitz JD. Perhexiline in therapeutic concentrations inhibits platelet aggregation in vitro. Cardiac Society of Australia and New Zealand, Brisbane, Australia (August 1996).

Willoughby SR, Chirkov YY, Kennedy JA, Murphy GA, Horowitz JD. Inhibition of long-chain fatty acid metabolism does not affect aggregation responses. XXth Congress of the European Society of Cardiology, Vienna, Austria (August 1998).

Willoughby SR, Stewart S, Horowitz JD, Chirkov YY. Perhexiline maleate normalises platelet responsiveness to the nitric oxide donor sodium nitroprusside (SNP) in patients with stable angina. Cardiac Society of Australia and New Zealand, Perth, Australia (August 1996).

Chapter 1

INTRODUCTION



1 Chapter 1: Introduction

1.1 Platelet physiology

The main physiological task of platelets within the circulation is to arrest the loss of blood when a blood vessel is damaged (Kinlough-Rathbone, et al. 1983; Frishman, et al. 1995). This process involves the rapid adhesion of platelets to the exposed subendothelium followed by platelet to platelet adherence (aggregation) which culminates in the formation of a "platelet plug" which temporally seals off the damaged vessel wall. In the pathological condition of thrombosis "platelet plugs" are formed within blood vessels, potentially arresting the blood supply to nearby tissues, thus causing local ischaemia (Holmsen. 1989; Schrader and Berk. 1990; Holmsen. 1991).

1.1.1 Normal platelet function

Resting platelets circulate as discoid anuclear cells (Siess. 1989), originating from megakaryocytes in the bone marrow. Platelets contain a plasma membrane, internal membranes (open canalicular and dense tubular systems), a cytoskeleton (microtubules and microfilaments), mitochondria, glycogen granules, storage granules (α -granules and dense bodies), lysosomes and peroxisomes (Lind. 1994).

The platelet is surrounded by a plasma membrane that extends through the multiple channels of the surface connected canalicular system, greatly increasing the surface area

of the platelet. The plasma membrane is composed of phospholipids; the negatively charged phosphatidylserine and phosphatidylinositol residues are primarily confined to the cytoplasmic side, where they may serve as substrates for phospholipases. Through this phospholipid bilayer, intrinsic glycoproteins such as glycoprotein (GP) IaIIa, GP Ib, GP IIb/IIIa and GP IV are extruding, serving as platelet receptors for activating and inhibiting agents (Fox and Phillips. 1982).

The platelet cytoskeleton is composed primarily of actin filaments. Upon activation myosin associates with actin filaments, thus generating the tension required for centralisation of the granules. In addition to the cytoplasmic actin filaments, platelets contain membrane skeleton, which stabilises the lipid bilayer and regulates the shape of the plasma membrane. Apart from actin and actin binding proteins, the platelet cytoskeleton is composed of a microtubular coil, just beneath the plasma membrane. This microtubular coil, composed of tubulin, is involved in maintaining the discoid shape of the unstimulated platelet (White. 1971).

The dense tubular system is the equivalent of the smooth endoplasmic reticulum in other cells; it is the site where the majority of calcium is sequestered and where enzymes involved in prostaglandin synthesis are localised (Blockmans, et al. 1995; Kamat and Kleiman. 1995). It lies in close contact with the channels of the open canalicular system, forming a membrane complex.

There are numerous organelles dispersed in the cytoplasm, including mitochondria, glycogen particles, lysosomes and peroxisomes. α -Granules and dense granules are platelet specific storage granules (Holmsen. 1989). α -Granules contain mainly proteins such as platelet factor 4, β -thromboglobulin, platelet derived growth factor, fibrinogen, fibrinonection, thrombospondin, plasminogen activator inhibitor I and von Willebrand factor. Dense bodies are rich in serotonin, ADP and calcium (see Figure 1.1).

Upon platelet activation, platelets lose their discoid shape, become spherical in form and extend long, spiky pseudopods (White. 1972). The organelles are contracted towards the platelet centre and are enclosed by a tight-fitting ring of reassembled microtubules and microfilaments. Finally, the contents of secretory organelles are expelled. During secretion, granule membranes fuse with those of the surface connected canalicular system, with the diffusion of internal granular membrane proteins such as P-selectin into the plasma membrane. Whereas dense body contents are easily secreted, α -granule release requires higher agonist concentrations, while lysosomal granule secretion only occurs with powerful activating agents. In addition to the contents of the three secretory granules, platelets produce and secrete pharmacologically active substances such as thromboxane A_2 and platelet activating factor during their activation and aggregation, establishing a positive feedback system (Holmsen. 1989).

1.1.2 Mechanisms of platelet activation

Platelets are activated by several physiological (thrombin, collagen, ADP, adrenaline, vasopressin, serotonin) and non-physiological (divalent cationophores, cyclic endoperoxide analogues) substances. Although activation is produced by substances that vary markedly in chemical structure, platelets respond with the same series of distinguishable responses: a) shape change; b) aggregation; c) release of materials from secretory granules; and d) liberation of arachidonic acid, which is rapidly converted to prostaglandins and lipoxygenase products (Kinlough-Rathbone, et al. 1983 ;Holmsen. 1989; Siess. 1989).

The platelet plasma membrane contains a large number of receptors which specifically bind agonists such as ADP, adrenaline, collagen, thrombin, serotonin, platelet activating factor, that stimulate the physiological platelet response. The interaction between a platelet-activating agonist and its receptor causes rapid mobilisation of signal molecules within the platelet, notably calcium, diacylglycerol and inositol 1,4,5-trisphosphate which are sufficient to initiate and complete shape change and aggregation responses (Kroll and Schafer. 1989). Small amounts of these molecules cause some dense body secretion and arachidonic acid liberation. The ADP and prostaglandin endoperoxides released through this dense body secretion, and the prostaglandins and thromboxanes formed through the liberated arachidonic acid are themselves potent platelet agonists and interact with their specific receptor causing release of more signal molecules. These platelet-derived agonists mobilise signal molecules by interaction with the primary

agonist in a synergistic way. This autocrine stimulation (positive feedback mechanism) increases the overall stimulus to such an extent that dense body and α -granule secretion is completed (Holmsen. 1989; Holmsen. 1991). Aggregation causes close cell contact which also operates as a positive feedback mechanism. The final feedback mechanism is via platelet activating factor synthesis. However, the relative importance of this mechanism is unclear (see Figure 1.2 for summary).

Some agonists (thrombin and collagen) in high concentrations can mobilise sufficient signal molecules without positive feedback to cause complete activation of all platelet responses; these agonists are “strong agonists”. Other physiological agonists at maximal receptor occupancy cause only mobilisation of submaximal amounts of platelet signal molecules, these agonists are “weak agonists”. These agonists rely on positive feedback to elicit all platelet responses. Interestingly, strong agonists in low concentrations behave as weak agonists, that is they depend on positive feedback to produce full platelet stimulation.

1.1.3 Platelet Inhibitors

Activation of platelets is negatively controlled by biochemical processes which attenuate or prevent agonist-induced responses. Mechanisms of inhibition which are intrinsic to the platelet, produced by normal agonist-induced signal pathways include inositol 1,4,5-trisphosphate, lipocortin, lipoxigenase metabolites and protein kinase-C. Physiologically two molecules of fundamental importance for inhibition of platelet

responses are cyclic guanosine-3',5'-monophosphate (cGMP) and cyclic adenosine-3',5'-monophosphate (cAMP).

Platelets contain guanylate, which converts GTP to cGMP. Physiological activation of guanylate cyclase may be achieved by either platelet derived nitric oxide or by endothelial derived relaxing factor (EDRF). The exogenous nitric oxide donors nitroglycerine (glyceryl trinitrate, (NTG)) and sodium nitroprusside (SNP) also activate guanylate cyclase. However, this requires the preceding release of nitric oxide (Palmer, et al. 1987). In addition to guanylate cyclase platelets also contain adenylate cyclase. The anti-aggregatory agents prostacyclin and prostaglandin E₁ stimulate platelet adenylate cyclase, with a resultant increase in intraplatelet cAMP content. Although the activation of cGMP is independent of cAMP, both cyclic nucleotide pathways act synergistically to inhibit agonist-induced platelet aggregation (Macdonald, et al. 1988).

1.2 Role of platelets in coronary artery disease.

In haemostasis, platelets circulate in close contact with the endothelial cell lining of the vessel wall without adhering to it (Ruggeri. 1997). However, under pathological conditions platelets respond rapidly to alterations of endothelial cells (for example, fatty streak or plaque rupture) and to exposure of subendothelial structures by attaching firmly to the site of the lesion (Frishman, et al. 1995; Body. 1996). Platelet aggregates (thrombi) subsequently formed and are associated with cardiovascular ischaemic events (Figure 1.3).

Upon interruption of the endothelial lining, the subendothelial protein matrix is exposed to circulating platelets and plasma coagulation factors. The major subendothelial proteins involved in platelet adhesion are von Willebrand factor, collagen, fibrinogen and vitronectin. The platelet surface receptor glycoprotein (GP) Ib is responsible for the adherence of von Willebrand factor to platelets under conditions of high shear rates (Bennett. 1992). This receptor, which is exposed on nonactivated platelets, is the major receptor for adhesion. Platelet adhesion occurs without prior platelet activation and produces the conformational changes in platelet structure described in section 1.2. The first monolayer of platelets is connected with the endothelial lesion, whereas subsequent growth of the thrombus depends primarily on platelet-to-platelet interactions. This is catalysed by specific agonist (thrombin, ADP, collagen, and thromboxane A_2) induced platelet vesicle secretions, this promotes further increases in platelet activation, until eventually platelet aggregation occurs.

1.3 Endothelial function and platelet aggregation

The endothelium is subject to both acute and chronic injury (Moore. 1985; Packham and Mustard. 1986). However, the precise nature of the damage responsible for the pathogenesis of atherosclerosis is uncertain, although processes of mechanical injury, chemical damage, levels and type of lipids, aging and genetic background are involved (Ross. 1986; Ross and Glomset. 1976).

The presence of atherosclerotic vascular disease is highly correlated with the development and clinical presentation of platelet thrombi. There are two phases of atherosclerotic progression. The first involves the primary progression of growth of intimal lesions from fatty streaks to fibrous plaques. The second phase is the development of the platelet thrombus as a result of plaque rupture or ulceration (Davies, 1994; Davies, 1994).

Fatty streaks are slightly raised, intimal lesions which first appear at an early age and are considered as the precursors of atherosclerotic plaques (Ross and Glomset, 1976; Adams, et al. 1987). Fibrous plaques develop from fatty streaks and appear at points of stress along the coronary artery and are firm protrusions into the lumen of the vessel (Packham and Mustard, 1986; Ross, 1986; Adams, et al. 1987). The plaques usually consist of a cholesterol-filled centre surrounded by a layer of foam cells. Overlying the lipid layer is a fibromuscular cap composed of collagen, smooth muscle and elastin with an intact endothelial wall separating the plaque from the vessel lumen. Degenerating platelet-rich thrombi are also found in the plaque.

Larger plaques impinge into the lumen decreasing luminal diameter and affecting coronary flow (Ross, 1993). However, their appearance does not necessarily cause occlusive disease (Falk, 1987). The acute clinical manifestations of coronary syndromes result from the eventual rupture and ulceration of these large plaques, probably preceded by focal or generated inflammatory change of uncertain origin (Kinlay, et al. 1998; Libby and Aikawa, 1998; Maseri and Sanna, 1998). Plaque rupture occurs when the

fibrous cap of the plaque is disrupted by physical stresses and either tears or is completely removed (Richardson, et al. 1989). Platelets are exposed to the ruptured plaque and undergo activation and subsequent aggregation (see section 1.1.2.) to form a thrombus adjacent to the plaque. These plaques may transiently reduce coronary blood flow, leading to the condition of unstable angina pectoris (Davies and Thomas. 1985; Lee, et al. 1986; Falk. 1987; Kroll and Schafer. 1989; White. 1994), if these plaques grow rapidly and totally occlude an artery patients are predisposed to an acute myocardial infarction (Conti and Mehta. 1987; Ip, et al. 1994). Most arterial thrombi formed in ruptured plaques are continually incorporating and losing thrombotic material. The lost material in the form of large or small emboli impacts on the microcirculation downstream from the plaque. Depending on the site of thrombosis, these emboli may cause transient ischaemia, stroke, myocardial or peripheral vascular disease (White. 1994).

1.3.1.1 Endothelial factors affecting platelet aggregation

Platelet activation and recruitment are tightly regulated by products of the endothelium, including prostacyclin, nitric oxide and endothelin (Radomski, et al. 1987; de Graaf, et al. 1992; Noll and Luscher. 1998). Prostacyclin, a product of arachidonic metabolism, promotes vasodilation and serves as a major inhibitor of platelet activation, adhesion and aggregation (Moncada and Vane. 1978). Nitric oxide (endothelial derived relaxing factor) is synthesised by endothelial cells and platelets and stimulates elevation of cGMP in smooth muscles and platelets (Brockman, et al. 1991) causing vasodilation and

inhibition of aggregation. For example, endothelial derived nitric oxide inhibited platelet adhesion under flow conditions (de Graaf, et al. 1992) and the disaggregation of platelets aggregated with either collagen or U46619 (Radomski, et al. 1987). Endothelin, a potent endothelial vasoconstrictor has no direct effects on platelet function (Noll and Luscher. 1998).

Endothelial dysfunction is associated with cardiovascular disease (Noll and Luscher. 1998) and contributes to enhanced vasoconstriction responses, adhesion of platelets and monocyte proliferation on vascular smooth muscle cells. A possible explanation for these responses is a decreased endothelial release of nitric oxide, either by impaired synthesis or excessive oxidative degradation (Cannon. 1998; Noll and Luscher. 1998).

1.3.1.2 Intra vs extra-platelet influences on aggregability

As mentioned earlier, platelets *in vivo* are influenced by a vast number of pro- (ADP, thrombin, thromboxane A₂) and anti- (nitric oxide, prostacyclin) aggregatory substances. The interaction between these opposing forces dictates the relative state of platelet activation. However, platelets are not solely under the "control" of external influences; several internal systems exist. The "classical" example is the autocrine release of ADP from within the platelet after activation (see Figure 1.2). Platelets contain both constitutive and inducible nitric oxide synthase (Chen and Mehta. 1996) and thus release nitric oxide when aggregated (Freedman, et al. 1997). Nitric oxide released from activated platelets markedly inhibits platelet recruitment (Freedman, et al. 1997).

Freedman et al has recently demonstrated that the release of nitric oxide from platelets is impaired in patients with acute coronary syndromes when compared to patients with stable angina (Freedman, et al. 1998) or normal controls (Freedman, et al. 1997).

1.3.2 Evidence for role of platelets in cardiovascular disease

1.3.2.1 Animal studies

Severe coronary artery stenosis produces turbulent flow and relative stasis. This, in conjunction with endothelial disruption, may activate platelets proximal to coronary narrowing. Several studies in dogs have shown transient platelet adhesion and aggregation at the site of a stenotic coronary artery with resultant transient reductions in coronary blood flow (Uchida, et al. 1975; Folts, et al. 1976). In a study by Folts et al constrictors were placed on the circumflex coronary artery after the endothelium was damaged by compressing the artery. Cyclic reductions in circumflex blood flow and distal coronary perfusion pressure were observed despite maintenance of normal aortic pressure (Folts, et al. 1976). These results, together with imaging of indium-labelled platelet distribution, provide pathological evidence that platelets accumulate at the site of the severe stenosis in coronary arteries at the time of reduction in distal coronary blood flow and pressure. Folts et al also demonstrated that these reductions could be prevented by the administration of aspirin and other platelet-aggregation-inhibiting agents (Folts, et al. 1976).

Bolli et al performed similar experiments and found that the reductions in distal coronary blood flow were not affected after administration of heparin (1000U/kg) (Bolli, et al. 1984). In contrast, intravenous aspirin (30 mg/kg) reversed flow reductions. The authors concluded that platelet aggregation rather than fibrin deposition is the cause for the observed cyclic reductions in coronary flow.

Platelets may be implicated in the formation of a thrombus in a coronary artery by the following methods: 1) flow abnormalities caused by atherosclerotic lesions seem to operate on the red cells; these indirectly activate the platelets. This mechanism could be mediated by ADP coming from a small proportion of haemolysed erythrocytes and 2) platelets could be activated directly by the haemodynamic forces, which would lead to distortion of their membrane, leading to the formation of the endogenous platelet activator thromboxane A_2 (Born and Kratzer. 1981).

1.3.2.2 Role of platelets in unstable angina pectoris

Patients with acute coronary syndromes such as unstable angina pectoris, demonstrate hypersensitive platelets, circulating platelet aggregates or elevated levels of circulating platelet secretory products (Smitherman, et al. 1981; Fitzgerald, et al. 1986; Gray, et al. 1993; Becker, et al. 1994; Langford, et al. 1996). For example, Langford et al demonstrated that patients with unstable angina and acute myocardial infarction exhibit systemic platelet activation when blood samples were assessed using flow cytometry (Langford, et al. 1996). Available data are consistent with not only localised

intracoronary (Vaitkus, et al. 1995) but also generalised (Hamm, et al. 1987; Dorn, et al. 1990) activation of platelets in unstable angina pectoris.

Another large body of evidence for the importance of platelets in acute coronary syndromes comes from the clinical efficacy of anti-platelet therapies. For example; aspirin is effective for the secondary prevention of myocardial infarction post unstable angina (Cairns, et al. 1985; Frishman and Miller. 1986; ISIS-2. 1988; RISC. 1990), while glycoprotein IIb/IIIa antagonists have been demonstrated to be effective in reducing acute ischaemic complications of percutaneous coronary intervention (EPIC. 1994; EPILOG. 1997; CAPTURE. 1997; IMPACT. 1997; RESTORE. 1997) and in improving clinical outcomes among patients with acute coronary syndromes without persistent ST-segment elevation (Ferguson. 1997).

1.3.2.3 Role of platelets in stable angina pectoris

In a study by Meade et al in patients with angina, or history of myocardial infarction, or electrocardiographic evidence of ischaemia demonstrated increased (although not significant) responses towards ADP compared to those without (Meade, et al. 1985). Further to this observation Elwood et al demonstrated a significant relationship between ischaemic heart disease and platelet aggregation in patients with past myocardial infarction and electrocardiographic evidence of ischaemia. However, no correlation was detected for ADP-induced platelet aggregation and angina (Elwood, et al. 1991). Thaulow et al in a large prospective study demonstrated that platelet concentration and

ADP-induced aggregation were significantly correlated with only long-term fatal coronary heart disease. No association was evident between platelet concentration or aggregation and development of angina pectoris or positive exercise electrocardiographic response (Thaulow, et al. 1991).

Previously, Chirkov et al demonstrated that patients with stable angina exhibited significantly greater extents of aggregation than normal subjects, when assessed in platelet-rich plasma using ADP as the inductor of aggregation (Chirkov, et al. 1993). More recently, utilising whole blood aggregometry Chirkov et al demonstrated that stable angina patients are more aggregable than normal subjects (Chirkov, et al. 1999).

1.4 Conventional pharmacological therapy in the management of acute and chronic myocardial ischaemia

Current therapeutic options for the treatment of acute and chronic coronary syndromes are extensive; the standard agents utilised in the treatment of acute coronary syndromes are mentioned below. Details of any associated anti-aggregatory effects are discussed in a section 1.6.

1.4.1 Nitrates

Organic nitrates, such as NTG, isosorbide dinitrate and isosorbide mononitrate are the most commonly used anti-anginal agents. These organic nitrates act as exogenous nitric oxide donors. Therefore, the clinical benefits (coronary and peripheral arterial and

venous vasodilatation and anti-aggregatory effects) of these agents are related to the effect of nitric oxide on the vasculature and platelets (Horowitz and Henry. 1987; Chirkov, et al. 1993).

In the management of stable angina, oral or transdermal preparations are utilised for the prevention of myocardial ischaemia and are usually used in conjunction with other anti-anginal medications (Flaherty. 1989). Sublingual nitrate preparations rapidly relieve myocardial ischaemia while intravenous NTG is utilised for the treatment of unstable angina pectoris with the primary objective of reducing frequency of angina (Curfman, et al. 1983). Intravenous NTG also improves cardiac haemodynamics (Jugdutt. 1991; Stone, et al. 1983) and decreases infarct size (Jugdutt and Warnica. 1988; Yusuf, et al. 1988).

The major clinical problem related to nitrate therapy is the potential development of nitrate tolerance (Abrams. 1986). Nitrate tolerance is essentially the diminution of clinical effects of the drug and is at least in part caused by reduced rate of nitric oxide generation, after continuous use (Horowitz and Henry. 1987; Meredith, et al. 1993; Boesgaard, et al. 1994). Tolerance development is correlated with frequent dosage, large doses and continuous treatment without adequate drug-free periods (Henry, et al. 1989; Abrams. 1991). Furthermore, it is becoming apparent that nitrate therapy may be limited by de novo “nitrate resistance”, which is defined as a poor responsiveness to nitrates (in vivo or in vitro) in the absence of prior nitrate therapy. The large literature on mechanisms of nitrate tolerance has not resolved the various component causes (for

review see: Abrams. 1993; Abrams. 1995); this is outside the scope of the current work. However, a discussion of previous studies in “nitrate resistance” is provided in section 1.7.3 and Chapter 6.

1.4.2 L-type calcium channel antagonists

These agents, such as verapamil, diltiazem and nifedipine, are selective inhibitors of the L-type calcium channel and interfere with the entry of calcium into 1) myocytes, producing negative inotropy (Schwartz, et al. 1985) and 2) coronary and peripheral vascular smooth muscle, producing vasodilation (Fleckenstein, et al. 1975; Schwartz, et al. 1985).

Nifedipine binds to a specific (dihydropyridine) receptor subclass, distinct from those associated with verapamil and diltiazem (Opie. 1990). Nifedipine affects mainly the peripheral and coronary vasculature and has only minor negative inotropic effects at clinical doses. The vasodilation induced by nifedipine can result in a reflex tachycardia, thereby limiting its clinical use as monotherapy in the management of myocardial ischaemia. It has recently been argued that many dihydropyridine's may have deleterious effects on outcomes in patients with potential or actual myocardial ischaemia (Yusuf, et al. 1991; Furberg and Psaty. 1996).

Verapamil and diltiazem have more marked negative chronotropic and inotropic effects in addition to their coronary and peripheral vasodilator effects (Opie. 1990). Verapamil

and diltiazem are effective at reducing myocardial ischaemia in stable and unstable angina pectoris (Khurmi and Raftery. 1987; Theroux, et al. 1985).

1.4.3 β -Adrenoceptor blockers

These agents competitively inhibit binding of catecholamines to β -adrenoceptors. They are extremely useful in the management of exertional angina via reducing myocardial oxygen demand by decreasing heart rate, blood pressure and contractility during times of increased sympathetic activity despite vasoconstrictor effects (Rutherford and Braunwald. 1992). However, the utility in unstable angina is less than clear-cut (HINT. 1986).

1.4.4 Anti-aggregatory and anti-coagulant agents

With the emerging role of platelets in coronary syndromes, there have been extensive developments as regards the therapeutic utility of agents interfering with either platelet aggregation or thrombus formation.

1.4.4.1 Aspirin

The most commonly used anti-aggregatory agent is aspirin and is used for both the prevention and treatment of coronary syndromes. Aspirin irreversibly inhibits the cyclooxygenase pathway, which is essential for the production of both thromboxane A_2 and prostacyclin from arachidonic acid. However, there is an apparent selective

inhibition of thromboxane A_2 synthesis. The mechanism for this is unclear but may be due to rapid recovery of cyclooxygenase in vascular endothelial cells or presystemic inhibition of portal arterial system, where the concentration of aspirin may be higher than the systemic circulation (Pedersen and FitzGerald. 1984). Irrespective of the mechanism, the clinical benefit of aspirin is due to inhibition of aggregation via irreversible inhibition of thromboxane A_2 . While aspirin appears to exert only minor effects on ischaemia within the first 48 hours (Theroux, et al. 1988); its longer-term efficacy is well-established (Lewis. 1983; Theroux, et al. 1988). Further discussion on this topic is in section 1.6.1.

1.4.4.2 ADP receptor antagonists

These agents (ticlopidine and clopidogrel) selectively and specifically interfere with ADP-mediated platelet activation and cause aspirin-like irreversible, non-competitive inhibition of platelet aggregation (Schorr. 1995). Ticlopidine is more effective than aspirin in stroke prevention and in treatment of peripheral arterial disease. Similarly, the CAPRIE trial data suggest that clopidogrel is marginally more effective than aspirin (CAPRIE. 1996). The full anti-platelet action of ticlopidine requires 3 to 5 days of oral administration and persist up to 10 days after cessation of therapy (McTavish, et al. 1990). The current clinical usage of ADP antagonists is largely in patients having intracoronary stent insertion (Kereiakes, et al. 1997; EPISTENT. 1998).

1.4.4.3 GPIIb/IIIa antagonists

The binding of fibrinogen to activated platelets is the final step in platelet aggregation and is mediated by the GPIIb/IIIa receptor. GPIIb/IIIa is unique to the platelet and is the most abundant platelet surface glycoprotein. GPIIb/IIIa antagonists have been widely investigated in patients with acute coronary syndromes recently. There is good evidence that abciximab (7e3) reduced ischaemic complications in patient populations with high angioplasty/stent intervention rates. Data for other GPIIb/IIIa antagonists (tirofiban, lamifiban) are less clear-cut, although they are likely to offer some additive anti-ischaemic effects when combined with aspirin and heparin (PRISM-PLUS. 1998).

1.4.4.4 Anti-thrombin Anticoagulants

Heparin is in widespread use in the management of acute coronary syndromes. Intravenous heparin infusion has been shown to decrease risk of myocardial infarction in unstable angina pectoris and has been found to be superior to aspirin. No synergism has been documented on combination of the two drugs (Theroux, et al. 1985) but aspirin limits “rebound” ischaemia on cessation of heparin (Waters, et al. 1991). For the management of acute myocardial infarction, heparin prevents early re-occlusion of the reperfused infarct related artery (Paskernak, et al. 1992) and is commonly used either simultaneously with or directly after thrombolytic therapy

There are many new anticoagulant preparations (for example, direct anti-thrombins, low molecular weight heparins) currently used or in development for the management of

acute ischaemic syndromes. Of these, only the low molecular weight heparin, enoxaparine, has demonstrated superiority over unfractionated heparin (Cohen, et al. 1997).

Warfarin, an oral anticoagulant, acts as a competitive inhibitor of Vitamin K which is essential for the hepatic production of coagulant factor II, VI, IX and X, and protein C. Warfarin is not used in the management of acute coronary syndromes, but rather during follow-up in selected patients.

1.4.4.5 Thrombolytic therapy

Intravenous thrombolysis is available for the treatment of acute myocardial infarction. These agents include streptokinase, urokinase, anisoylated plasminogen streptokinase activator complex and recombinant tissue-type plasminogen activator (rTPA). There is strong evidence in favour of intravenous thrombolysis as a mortality-reducing strategy in myocardial infarction (GISSI. 1986; ISIS-2. 1988).

1.5 Superoxide in platelet activation

Platelet activation is an energy-requiring, oxidative process that requires oxygen consumption by the platelet and results inter alia, in release of the oxygen free radicals superoxide anion, hydroxyl radical and other reactive oxygen species such as hydrogen peroxide. Baseline superoxide is measurable in resting platelets (Marcus, et al. 1977)

while platelet activation is associated with an increase in superoxide production by platelets (Iuliano, et al. 1997).

There is evidence that at least some of the oxygen-derived free radicals formed during aggregation may modulate the aggregation process (Leo, et al. 1997). During aggregation other free radicals are also released from platelets. Among these is nitric oxide (Freedman, et al. 1997; Freedman, et al. 1998) which exerts anti-aggregatory effects (see section 1.7) as well as deactivating superoxide (Munzel, et al. 1995).

1.5.1 Mechanisms of oxygen free radical effects in platelet activation

Platelets are activated upon exposure to oxygen free radical generating systems, including those derived from white and red blood cells. Platelets are in close contact with these generating systems; this has been demonstrated in the clinical settings of unstable angina and myocardial infarction (Davies and Thomas. 1985; Kroll and Schafer. 1989). The presence of oxygen species in close proximity to the fissuring plaque provides important stimuli for platelet aggregation and thus contributes to thrombus formation.

Oxygen free radicals activate tyrosine kinases associated with the plasma membrane, this induces tyrosine phosphorylation which in turn phosphorylates mitogen activated protein kinase. This kinase subsequently phosphorylates and activates cytosolic phospholipase A_2 and produces an increase in arachidonic acid which through

prostaglandin endoperoxide synthase activates the platelet activator thromboxane A_2 . Oxygen free radicals also stimulate arachidonic acid metabolism by increasing the activity of cyclooxygenase enzyme. Hydrogen peroxide has been shown to stimulate this enzyme, thus inducing an increased production of thromboxane A_2 (Taylor, et al. 1983). Consistent with this, aspirin inhibits aggregation of platelets stimulated with hydrogen peroxide (Pratico, et al. 1991). However, this is not the only pathway for to activate platelets as oxygen free radicals also stimulate cyclooxygenase-independent pathways. This is observed as the aggregation (utilising high agonist concentrations) of aspirin treated platelets, are inhibited by oxygen free radical scavenger, such as vitamin E (Halliwell, et al. 1988).

1.6 Assessment of platelet aggregation and function

A vast number of in vitro, ex vivo and in vivo tests exist to assess platelet aggregability and the effects of drugs on platelets. The clinical relevance of these methodologies is debatable as they do not account for biochemical and circulatory factors that influence platelet activity in vivo. Nevertheless, in vitro and ex vivo methodologies have been utilised to determine the effectiveness of drugs and to classify patients in large clinical trials.

1.6.1 History

Since the identification of platelets in 1881 (Bizzozero. 1881) and the increased understanding of the involvement of platelets in cardiovascular disease various methods

for measuring platelet aggregation have been employed. Platelet aggregation has predominantly been measured by the turbidometric technique first described by Born in 1962 (Born, 1962). This technique utilises the separation of platelets from other blood cells by centrifugation (platelet-rich plasma). In 1977 Feinman et al extended the work of Born by simultaneously measuring platelet aggregation and dense granule secretion (Feinman, et al. 1977). This was achieved by adding buffered firefly luciferin-luciferase to the platelet-rich plasma, which subsequently becomes luminescent in the presence of secreted ATP. Soon after this in 1980 Cardinal and Flower described the impedance method for measuring platelet aggregation in whole blood (Cardinal and Flower, 1980).

The above techniques (or slight modifications of) have formed the backbone of physiological platelet research. However, more recently a newer in vitro technique (flow cytometric analysis of platelet markers) has been introduced to measure platelet activation. The various techniques are reviewed in more detail below.

1.6.2 Current platelet aggregation / activation techniques

1.6.2.1 Physiological

1.6.2.1.1 Optical (turbidometric) platelet aggregometry

This is the most commonly used assay of platelet function and involves the optical detection of platelet aggregation in platelet-rich plasma. For this assay, citrated blood is centrifuged to prepare platelet-rich plasma. After removing the platelet-rich plasma,

platelet-poor plasma is prepared from the blood to serve as a control. A cuvette of platelet-rich plasma is then continually stirred and incubated at 37°C. An infrared light beam passing through the cuvette is scattered by the platelets, with the methodology calibrated such that the amount of light transmitted through the platelet-poor plasma is defined as 100% aggregation, whereas the light transmitted through platelet-rich plasma is defined as 0% aggregation. The aggregation reaction is initiated by addition of an agonist (for example, ADP, adrenaline or collagen) and the light transmitted through the platelet-rich plasma is monitored.

A variant of this technique, designed to examine aggregability in an environment minimally dependent on circulating physiological activators / inhibitors of aggregation, utilises identical methodology in washed platelets (Judge, et al. 1995; Rodriguez-Linares and Cano. 1995). This technique, while theoretically “pure”, is ultimately extremely unphysiological and thus has limited applications.

The advantages of turbidometric platelet aggregation include being able to distinguish between the primary and secondary phases of aggregation and the preparation of platelet-rich plasma focuses results on platelet function as opposed to that of other blood constituents. However, the advantages of this method are outweighed by its many disadvantages. These include the fact that platelet function in vitro does not necessarily reflect platelet function in vivo. Furthermore, sample aging occurs as a result of the time required to prepare platelet-rich plasma and the presence of substances such as lipids in the platelet-rich plasma or platelet-poor plasma can alter absorbance at the chosen

wavelength. However, the major problem with turbidometric aggregometry is that centrifugation modulates platelet behaviour; as platelets are heterogeneous in size, density and metabolic activities, it is likely that subpopulations of platelets are lost during the preparation of platelet-rich plasma (Hjemdahl. 1995).

1.6.2.1.2 Impedance platelet aggregometry

This technique can be performed in either platelet-rich plasma or whole blood. When platelet-rich plasma is utilised, essentially the same disadvantages as the turbidometric method apply. When whole blood is used, it is diluted 1:1 with physiological saline, placed in a cuvette and warmed at 37°C. An electrode, comprised of two closely spaced platinum wires, is inserted in the cuvette and the electrical resistance between the wires is measured. After the addition of a platelet agonist, platelet aggregates accumulate between the wires and the resistance increases.

The advantages of this technique are that whole blood is a closer reflection of the *in vivo* situation; no modification of the blood sample is involved. Impedance aggregometry only requires small blood quantities and can be performed quickly and conveniently. The only disadvantage of whole blood aggregometry is the greater degree of inter-assay variability for replicate samples in comparison with platelet-rich plasma.

Taking into account the advantages and disadvantages of this and the other techniques mentioned, whole blood impedance aggregometry was chosen as the preferred technique for this thesis.

1.6.2.1.3 Flow cytometry

Flow cytometry enables rapid measurement of specific characteristics of a large number of individual platelets. Platelets are prepared by fluorescent labelling with a conjugated monoclonal antibody. The suspended solution of platelets is placed in the flow chamber of a flow cytometer and passed through the light beam of a laser. Exposure of the platelets to light results in emitted fluorescence, which is then detected and processed along with the forward and side light-scattering properties of the platelet (Givan. 1992). To assess the activation state of platelets, activation-dependent monoclonal antibodies are added to the platelets. These bind strongly to activated platelets and weakly to resting or unstimulated platelets. Other antibodies are available that bind to the ligand occupied by P-selectin and GPIIb/IIIa. The latter is used to evaluate the proportion of receptors occupied by GPIIb/IIIa antagonists.

Advantages of this technique are essentially the same as for whole blood impedance aggregometry. In addition flow cytometry can also detect the activation state and the reactivity of circulating platelets. The major disadvantage of flow cytometry is the expensive equipment involved and the high cost of maintenance. Blood sample also needs to be processed rapidly (> 45 min) to avoid ex vivo platelet activation.

1.6.2.1.4 Indium labelled platelets

Another available technique is indium labelled [^{111}In] platelets, which are utilised to determine the extent of platelet aggregation in vivo (May, et al. 1991). However, their use is restricted to animal models. Washed platelet are prepared from whole blood (by washing with a calcium-free Tyrode's solution containing prostacyclin) and incubated with [^{111}In]oxine (25-50 μCi). Any unbound isotope is removed and the labelled platelets are reinjected into the animal. Platelet aggregation is induced by intravenous administration of ADP (or other agonist) and is measured as an increase in radioactivity (γ -counter) in the pulmonary circulation.

1.6.2.2 Biochemically orientated measures of platelet activation / aggregation

1.6.2.2.1 Calcium concentrations

The activation of platelets is accompanied by an increase in intracellular cytoplasmic calcium. This calcium flux is measured by various techniques; for example Fura 2 and aequorin. Fura 2 is incubated with platelet-rich plasma and aliquots of the platelet suspension are studied in a spectrofluorimeter. Samples are excited every 340 to 380nm, and emission is recorded every 500ms at 510nm. Fura 2 signals are then expressed as the ratio of fluorescence intensities (Grynkiewicz, et al. 1985). On the other hand, the fluorescent photoprotein aequorin is loaded into washed platelets using dimethylsulfoxide (see Chapter 2 for detailed methodology). Total agonist induced

calcium mobilisation is measured as well as the release of internal calcium (by removal of cytoplasmic calcium with EGTA). Calcium concentrations are then calculated from a calibration curve as fractional aequorin luminescence to calcium in the presence 1 mM Mg^{2+} . Platelet aggregation is also able to be simultaneously measured with calcium mobilisation (Yamaguchi, et al. 1986).

1.6.2.2.2 Platelet cGMP/cAMP content

To determine the intraplatelet concentration of either cGMP platelet-rich plasma is prepared from whole blood either after drug administration (ex vivo experiments) or after incubation with a nitric oxide donor (in vitro experiment). The platelet-rich plasma is then filtered through glass filters to harvest the platelets. The filters are then rinsed with physiological saline and placed in EDTA for further extraction of cGMP in a boiling water bath for 5 min (Chirkov, et al. 1991). After centrifugation of samples at 3000g for 10 min, cGMP concentration in supernatant was assayed by a radioimmunoassay system.

A similar protocol is employed for determination of intraplatelet cAMP content.

1.6.2.2.3 Thromboxane A_2 formation

Platelet aggregation is mediated, in part by the intracellular synthesis and release of thromboxane A_2 (Arita, et al. 1989). Thromboxane A_2 formation is measured by the concentration of thromboxane B_2 , its stable hydrolysis product, is determined after 3 min

incubation with an agonist. Thromboxane B₂ was analysed using and Elisa kit after purification of the samples on C18 Sep-Pak light columns.

1.6.3 Activators of aggregation: re in vitro methodology

In vitro aggregation can be induced by a variety of chemical and mechanical methods that work through several distinct intracellular pathways. Historically, turbidometric platelet aggregation (utilising platelet-rich plasma) has been measured in response to agonists such as ADP, adrenaline, collagen, thrombin and arachidonic acid. As well as choice of agonist, another consideration is the concentration of the agonist utilised, as some agonists in high concentrations will directly stimulate release of platelet granule contents, which include ADP, ATP, serotonin, calcium, platelet factor 4, Factor V, fibrinogen and other molecules that participate in thrombosis.

Impedance aggregometry in whole blood exhibits the same considerations for the choice of agonist as turbidometric aggregation. ADP has been extensively utilised for whole blood impedance aggregometry, both for in vitro and ex vivo experiments, and produces a dose-dependent increase in aggregation at physiological concentrations (Jabs, et al. 1978). In addition, ADP does not produce granule release at the above concentrations. For these reasons ADP was chosen as the agonist of choice for this thesis.

1.7 Anti-aggregatory effects of prophylactic anti-anginal agents and other cardioactive drugs

1.7.1 Is aspirin enough?

The inhibition of platelet aggregation has obvious beneficial possibilities in the management of atherosclerotic-induced ischaemic heart disease, especially in acute ischaemic syndromes. Aspirin's effect on platelet aggregation is described in section 1.3.4.1. Aspirin has been investigated for both primary and secondary prevention of myocardial ischaemia. Currently, low dose aspirin is the antiplatelet treatment of choice for long-term prevention of myocardial ischaemia and certain forms of ischaemic stroke (Matchar, et al. 1994; Patrono. 1994; Roth and Calverley. 1994; Barnett, et al. 1995).

As a primary preventative measure, aspirin has been shown to reduce the occurrence of cardiovascular complications in patients who never had myocardial infarction (Fuster, et al. 1993; Antiplatelet Trialists' Collaboration. 1994; Patrono. 1994). However, a number of studies have demonstrated increased frequency of haemorrhagic stroke with aspirin therapy (Antiplatelet Trialists' Collaboration. 1994; Patrono. 1994).

The administration of aspirin to patients with previous myocardial infarction has consistently decreased the rate of nonfatal myocardial infarction, overall mortality or both (Antiplatelet Trialists' Collaboration. 1994). Aspirin has also been shown to prevent myocardial infarction in the long-term management of unstable angina pectoris (Cairns, et al. 1985; Theroux, et al. 1988) and death after acute myocardial infarction

(ISIS-2. 1988). Aspirin's benefit on prognosis post myocardial infarction is additive to that obtained from treatment with streptokinase (ISIS-2. 1988).

However, several problems exist with aspirin therapy. The major problem associated with aspirin therapy is adverse gastrointestinal effects (Schrör. 1995). These have been reported to occur in up to 35% of patients taking the drug (Fuster, et al. 1993; Patrono. 1994). The high frequency of adverse effects suggests that compliance to the aspirin regimen might be poor. Another problem with aspirin therapy is the increased incidence of cerebral haemorrhage, which occurs when aspirin is used of both primary and secondary prevention (Physician's Health Study Research Group. 1989). However, the major concern with aspirin is that it is a relatively weak anti-aggregating agent (Schrör. 1995). Consistent with this in spite of the beneficial effects of aspirin, either alone or in combination with anticoagulants such as heparin, between 6% and 14% of patients with unstable angina progress to recurrent myocardial ischaemia or death within the first month in most studies (RISC. 1990; GUSTO-IIb. 1996). Clearly this value is unacceptable; therefore the opportunity exists for anti-platelet agents to lower this incidence. Newer anti-platelet agents may need to be developed or recognition of existing anti-platelet effects in currently used anti-anginal agents.

In response to the limitation of aspirin's efficacy, a number of more potent anti-aggregatory agents have been developed. These include ADP antagonists such as ticlopidine and clopidogrel, which have some advantage over aspirin in practice (CAPRIE. 1996). Furthermore, GPIIb/IIIa antagonists represent a theoretically

attractive group of drugs for management of acute ischaemia; results with some but not all members of this class of drugs are promising (PRISM-PLUS. 1998, PURSUIT. 1998).

1.7.2 Examples of antiplatelet effects of anti-anginal agents

It is also important to appreciate that several agents, initially developed for treatment of exertional angina, may exert important anti-aggregatory effects.

1.7.2.1 Nitrates

Organic nitrates, such as nitroglycerine, which act as donors of nitric oxide, were once thought only to be vasodilators, also affect platelet aggregation. Nitric oxide effects platelet cell signalling via many mechanisms; for an overview see Figure 1.4. Early in vitro work (Synek, et al. 1970; Schafer, et al. 1980) demonstrated that NTG inhibits platelet aggregation. However, the concentrations utilised were above those observed therapeutically. Nevertheless, more recent studies have provided convincing evidence that nitrates have antiplatelet effects at clinically relevant doses in humans. For example, Diodati and Waters showed that in patients with unstable angina pectoris or acute myocardial infarction, NTG infusion, at a clinically relevant infusion rate (although relatively high) of 1.2 µg/kg/min, inhibited ex vivo platelet aggregation induced by ADP or thrombin (Diodati, et al. 1990). Chirkov et al in stable angina patients have also shown that sublingual nitroglycerine (300 µg) significantly decreases platelet aggregation (Chirkov, et al. 1993;). These results have also been observed in an

animal model of periodic platelet formation in coronary arteries (Folts. 1991). These authors showed that intravenous nitroglycerine (10-15 µg/kg/min) inhibited the cyclic platelet thrombus formation, and that prolonged exposure (20-40 min) was required for its antiplatelet effects to become manifest.

The clinical relevance of the anti-aggregatory effects of NTG and other organic nitrates has been investigated to only a limited extent. However, the following represents a summary of currently widely held opinion:-

- a) The effects of nitrates on platelet aggregation are potentiated by sulphydryl donors such as N-acetylcysteine (Loscalzo. 1985; Stamler, et al. 1988; Chirkov, et al. 1993) reflecting in part formation of S-nitroso-N-acetylcysteine.
- b) The process of inhibition of platelet aggregation by nitrates is likely to be relevant in acute coronary syndromes (characterised by increased platelet aggregation).
- c) In patients with stable angina, onset of tolerance (attenuation of effect) to organic nitrates may limit long-term anti-aggregatory activity (Chirkov, et al. 1997).

1.7.2.2 Verapamil / Diltiazem

The L-type calcium antagonist, verapamil has previously been shown to inhibit in vitro platelet aggregation (Ono and Kimura. 1981; Jaffrezou, et al. 1992). In addition verapamil has also been reported to inhibit platelet aggregation and intracoronary

thrombus formation. Wallen et al demonstrated that in patients with stable angina, verapamil (40 to 80 mg/day) attenuated platelet aggregation (Wallen, et al. 1995). Similar results were obtained by Winther et al who demonstrated that verapamil (120-240 mg/day) decreased platelet secretory products in blood samples from healthy volunteers (Winther, et al. 1990). Verapamil decreased cyclic flow variations in an animal model of stenosed coronary arteries (Beaughard, et al. 1995). However, Benedict and Sheng demonstrated no effect of verapamil in an animal model of thrombus formation (Benedict and Sheng. 1988); similar results were obtained by Bonebrake et al (Bonebrake, et al. 1986).

Like verapamil, diltiazem (another calcium antagonist) has been shown to inhibit platelet aggregation in some studies but not in others. Colica et al demonstrated that in healthy subjects diltiazem (240 mg/day) inhibited platelet aggregation (Colica, et al. 1990). Altman et al showed that diltiazem, added in vitro to blood samples from healthy subjects treated with aspirin, enhanced the anti-aggregatory effect of aspirin alone (Altman, et al. 1988). These results are opposed by those of Rostagno et al who did not demonstrate any inhibitory effect of diltiazem in a double blind randomised trial of healthy subjects (Rostagno, et al. 1990). Similar results were obtained in an animal model of stenosed coronary arteries where diltiazem (0.1 mg/kg) did not affect cyclic flow variations (Morishima, et al. 1995).

The clinical investigations corresponding to findings of anti-aggregatory activity for verapamil include demonstrations of stabilisation in patients with unstable angina

pectoris (Opie. 1996) and reduction in reinfarction after Non-Q-wave infarction (Hansen. 1991). Similar data are available for diltiazem (Diltiazem Postinfarction Trial research Group. 1988).

However, uncertainty as to the anti-aggregatory effects of diltiazem demonstrates that all characteristics of one agent within a certain class of drug cannot be routinely assigned to other agents within the class.

1.7.2.3 Statins

The 3 hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) greatly reduce cardiovascular-related mortality and morbidity in patients with and without cardiovascular disease (Scandinavian Simvastatin Survival Study. 1994; Shepherd, et al. 1995). These drugs inhibit the rate-limiting enzyme in cholesterol synthesis in the liver, thereby decreasing hepatic production of low-density lipoproteins (LDL).

Hypercholesterolaemia is associated with hypercoagulability as well as enhanced platelet reactivity at sites of acute vascular disease (Badimon, et al. 1991). In addition to lowering circulating LDL's in this group of patients statins also decrease platelet aggregation and platelet activation markers. Lovastatin therapy has been shown to decreased serum fibrinogen levels and ADP-induced platelet aggregation (Mayer, et al. 1992), while after 2-3 months of pravastatin therapy, platelet aggregation was decreased

at both low and high shear rates assessed via an ex vivo system (Lacoste, et al. 1995). In addition, simvastatin therapy in dyslipidaemia patients decreases ex vivo platelet aggregation and the production of thromboxanes B₂ and A₂ (Notarbartolo, et al. 1995).

1.8 Current issues re nitric oxide donors and platelet function and myocardial ischaemia

1.8.1 Mechanistic

1.8.1.1 Pathways for organic nitrates, especially in platelets

Organic nitrates are indirect donors of nitric oxide (Schorr, et al. 1991). However, the precise mechanism by which nitric oxide is produced are not fully understood. It is likely that there are several mechanisms involved for each agent, due to the differing molecular structures and redox states of nitrogen within each molecule (Harrison and Bates. 1993).

Nitric oxide is released from NTG by a thiol-dependent metabolic step. There is strong evidence that this sulphydryl-dependent step is at least in part enzyme mediated (Seth and Fung. 1993; Wheatly, et al. 1994). However, the nature of the enzyme involved remains uncertain. A correlation exists between the rate of NTG metabolism and the enzyme activity of glutathione-S-transferase. This enzyme catalyses the attachment of glutathione to the nitrate group of NTG, when two glutathione molecules bind to NTG the reaction yields glycerol dinitrate, nitric oxide and oxidised glutathione (Harrison and Bates. 1993). Sulphydryls are also necessary to facilitate formation of S-nitrothiols from

nitric oxide; this process occurs in equilibrium with that of nitric oxide (Horowitz and Henry, 1987).

The nitric oxide released from organic nitrates has multiple targets within the platelet (see Figure 1.4). Nitric oxide diffuses readily across cell membranes, and binds to guanylate cyclase. This leads to the activation of the enzyme and elevation of cGMP, which in turn activates cGMP-dependent protein kinases and subsequently inhibition of platelet activation/aggregation.

Nitric oxide also activates cellular processes which are not mediated by binding to guanylate cyclase. Trepakova et al have recently demonstrated that the nitric oxide mediated inhibition of thrombin-induced increase in free cytoplasmic calcium concentrations is attenuated but not prevented by inhibition of guanylate cyclase with H-(1,2,4)-oxadiazolo(4,3-a)quinoxalin-1-one (ODQ), but when an inhibitor of the sarcoplasmic/endoplasmic reticulum calcium-ATPase (SERCA) was utilised, nitric oxide mediated inhibition was abolished (Trepakova, et al. 1999). These results suggest the nitric oxide inhibition is mediated via mechanism(s) other than guanylate cyclase. Furthermore, Tsikas et al have shown that ODQ did not inhibit collagen or arachidonic acid-induced platelet aggregation by S-nitroso-cysteine, although ODQ inhibited S-nitroso-cysteine induced formation of cGMP (Tsikas, et al. 1999). Therefore, S-nitroso-cysteine inhibits aggregation by cGMP-independent mechanisms.

1.8.1.2 Do nitric oxide donors produce oxidative stress plus sulphhydryl depletion

Organic nitrates have been shown to increase the formation of reactive oxygen species. Munzel et al demonstrated that the metabolism of NTG was associated with enhanced superoxide production both in vitro and in vivo during tolerance (Munzel, et al. 1995). It was postulated that the increased flux of superoxide acted as the final mediator of nitrate tolerance by inactivating nitric oxide release from either the administered drug or the endothelium (Munzel and Bassenge. 1996; Rajagopalan, et al. 1996). Increased superoxide formation has also been shown to occur after acute NTG administration in cultured vascular and endothelial cells, washed ex vivo platelets and whole blood (Dikalov, et al. 1997). Reactive oxygen species interact with platelets to increase the extent of aggregation. Bassenge et al demonstrated that 3 day nonintermittent NTG administration to healthy subjects, and therefore the development of tolerance, coincided with the progressive upregulation in activity of washed ex vivo platelets. This progressive upregulation was reversed by the antioxidant ascorbate (vitamin C; 1000 mg, three times a day for three days); nitrate tolerance was also reversed (Bassenge, et al. 1998).

In addition to reactive oxygen species limiting nitric oxide release from organic nitrates, sulphhydryl depletion may also be involved in the development of nitrate tolerance (Horowitz, et al. 1983). Sulphydryl donors (for example N-acetylcysteine (NAC)) have been shown to potentiate the effects of NTG (Horowitz, et al. 1983; May, et al. 1987; Horowitz. 1991; Boesgaard, et al. 1994). Horowitz et al demonstrated the NTG intravenous infusion rate required to induced a 10% fall in mean arterial blood pressure

and a 30% fall in mean pulmonary capillary wedge pressure was significantly less when NAC was infused just prior to NTG infusions as compared to NTG alone infusions (Horowitz, et al. 1983). The antiplatelet effects of organic nitrates are also potentiated by NAC. For example, inhibition of aggregation by NTG in vitro is potentiated by NAC (Loscalzo. 1985; Chirkov, et al. 1993), while ex vivo platelet responsiveness to NTG is increased by the addition of NAC (Stamler, et al. 1988).

1.8.2 Tolerance in platelets

Nitrate tolerance represents a major limitation to the vasodilator efficacy of nitrate therapy (Abrams. 1995). In addition to tolerance developing at the vascular level, platelets from patients treated with nitrates also display nitrate tolerance. Chirkov et al recently demonstrated that nitrate tolerance developed in platelets from stable angina patients both after a single sublingual dose (300µg) and after 24 hour low rate (5µg/min) intravenous infusion of NTG. Importantly, the observed NTG tolerance was not associated with cross tolerance to the nitric oxide donor sodium nitroprusside (SNP) or to down-regulation of platelet guanylate cyclase.

1.8.3 Nitric oxide resistance

Nitrate tolerance is not the sole reason for decreased responsiveness to nitrate therapy. Recent data suggest that nitrate therapy is limited by a de novo resistance to the vasodilatory and anti-aggregatory effects of nitrates; this poor responsiveness was termed "nitrate resistance". This has been documented haemodynamically in patients

with congestive heart failure (Armstrong, et al. 1980; Elkayam, et al. 1985; Kulick, et al. 1988; Abrams. 1991) and in platelets obtained from patients with stable angina (Chirkov, et al. 1993; Chirkov, et al. 1996). Recently Chirkov et al have shown that nitrate resistance may be attributable to either inactivation of released nitric oxide by the superoxide anion radical and/or reduced guanylate cyclase sensitivity to nitric oxide associated with a defect in nitric oxide/ cGMP pathway (Chirkov, et al. 1999).

1.9 Perhexiline Maleate

Perhexiline maleate has been in clinical use as a prophylactic anti-anginal agent for over 25 years. Despite demonstrating remarkable clinical efficacy in the treatment of stable angina pectoris, particularly in those patients resistant to other anti-anginal medications (Mir and Kafetzakis. 1978; White and Lowe. 1983), perhexiline was virtually withdrawn from use around 1990 due to the emergence of serious side-effects related to its potential toxicity and the availability of apparently safer and more effective anti-anginal agents. Recently, improved understanding of perhexiline's mechanism of action and the ability to correlate its potential clinical efficacy and toxic profile with concentrations in the plasma, perhexiline has once again established itself as effective clinical management in the treatment of individuals with severe stable angina pectoris particularly in those where conventional therapy including surgery/angioplasty has failed or is contra-indicated (Cole, et al. 1990). In addition to this clinical use recent developments in respect to the unique properties of perhexiline and the ability to predict its therapeutic impact, based on plasma concentration has raised the possibility for the utilisation of perhexiline in the

management of individuals with acute coronary syndromes (such as unstable angina pectoris) refractory to conventional maximal therapy.

1.9.1 Clinical efficacy

During the early development and clinical use of perhexiline a large number of controlled studies were conducted. The vast majority of these involved patients with stable angina pectoris and demonstrated that perhexiline reduces anginal frequency and increases exercise tolerance (Morledge. 1973; Souza. 1973; Alcocer, et al. 1974; Audier, et al. 1974; Pepne, et al. 1974; Masoni, et al. 1975; Dernier and Colen. 1977; Hitchcock, et al. 1977; Teo, et al. 1983). These studies utilised perhexiline as either monotherapy or in combination with a limited number of prophylactic agents (usually β -adrenoceptor blockers) and made no attempt to take into account inter-individual variability in the pharmacokinetics (which was unknown at that time) of the drug. However, at this time perhexiline was typically administered according to symptomatic response with doses ranging from 200-400 mg/day.

More recently, perhexiline has been investigated in patients unresponsive to other anti-anginal agents, with evidence initially obtained for incremental effects in patients who remain symptomatic while being treated with β -adrenoceptor blockers (Mir and Kafetzakis. 1978; White and Lowe. 1983; Rees. 1983) and combinations of other anti-anginal agents (Horowitz and Mashford. 1979; Cole, et al. 1990). For example, Horowitz et al, examined the long-term clinical efficacy of perhexiline among patients

with severe angina pectoris refractory to maximal therapy of nitrates, β -adrenoceptor blockers and calcium channel antagonists (Horowitz and Mashford. 1979). In this study 21 of the 26 patients enrolled reported a significant reduction in the frequency of anginal attacks with 15 patients reporting anginal frequency less than one third of previous levels. Results of this study were extended by Cole et al who examined the clinical efficacy of perhexiline in a double-blind controlled crossover study of 17 patients with angina refractory to maximal anti-anginal therapy including nitrates, a β -adrenoceptor blocker and a calcium channel antagonist. Anginal frequency and severity was reduced in 11 patients whilst receiving perhexiline compared to no patients when receiving placebo. The reduction in anginal frequency was accompanied by a marked clinical improvement in exercise tolerance as measured by formal exercise test (Cole, et al. 1990).

Until recently, there was very little information available concerning the efficacy of perhexiline in unstable angina pectoris. This may relate in part to the inherent difficulty of rapidly initiating therapy without an intravenous preparation of the drug (Horowitz, et al. 1986), and the large number and combination of drugs used in the treatment of unstable angina pectoris. However, in 1996 Stewart et al investigated the concentration effect / relationship for perhexiline in 40 patients treated for unstable angina (Stewart, et al. 1996). The majority of patients (31 of 40) had remained symptomatic despite combined use of aspirin, heparin, intravenously infused nitroglycerine and verapamil. In 16 of these patients N-acetylcysteine had already been co-infused with nitroglycerine (Horowitz, et al. 1988; Horowitz, et al. 1988) without elimination of symptoms. After

72 hours of perhexiline therapy anginal symptoms had resolved in 28 of the 40 patients. The results suggested ($p=0.055$) an association between resolution of anginal symptoms and plasma perhexiline concentration. The authors also established a relationship between the development of toxic symptoms (nausea/dizziness) and elevated plasma perhexiline concentration ($p=0.002$).

Preliminary data suggest that perhexiline is also beneficial in the treatment of severe aortic stenosis. Unger et al investigated the effect of long-term (3 month's) perhexiline therapy in 15 elderly patients with symptomatic aortic stenosis, who were unsuitable for aortic valve replacement (Unger, et al. 1997). Symptomatic status improved in 13 of the 15 patients and 5 became asymptomatic. Perhexiline was well tolerated with no withdrawals due to toxicity or deteriorating clinical status. The improvement in symptomatic status in this group of patients was tentatively attributed to the oxygen sparing effect of perhexiline caused by inhibition of carnitine palmitoyltransferase-1. A larger blinded investigation in this area is about to commence.

1.9.2 Perhexiline toxicity: relationship to plasma perhexiline concentrations

The adverse effects of perhexiline can essentially be divided into two categories, the first associated with short-term (less than 3 months) therapy and the second with long-term (greater than 3 months) therapy.

Short-term adverse effects of perhexiline include nausea and dizziness, approximately 10% of patients initially treated with perhexiline will develop these effects (Cole, et al. 1990). There is a close relationship between appearance of toxic symptoms and the elevation of plasma perhexiline concentrations beyond the therapeutic range (upper therapeutic level 0.6 mg/L) (Stewart, et al. 1996). Any nausea and dizziness are usually transient and does not require cessation of the drug but rather a lowering of the prescribed dosage (Horowitz, et al. 1986; Horowitz, et al. 1995). For example, Cole et al reported that after rapid perhexiline loading 4 of the 17 patients studied developed nausea and/or dizziness associated with plasma perhexiline concentrations > 0.6 mg/L which resolved within two weeks of reduced dosage; this paralleled reductions in plasma concentrations (Cole, et al. 1990). The other potential short-term adverse effect is symptomatic hypoglycaemia in diabetics (Roger, et al. 1975; Schlienger, et al. 1978; Luccioni, et al. 1978); this can be prevented by appropriate adjustment of other hypoglycaemic medications.

The adverse effects (hepatitis and peripheral neuropathy) of perhexiline during long-term therapy reflect the extensive deposition of various phospholipids in hepatocytes and Schwann cells (Pollet, et al. 1977; Hauw, et al. 1978; Hauw, et al. 1978; Hoenig and Werner. 1979; Hoenig and Werner. 1979 Pollet, et al. 1979; Hauw, et al. 1980; Hauw, et al. 1981; Albert and Lullmann-Rauch. 1983). Similar phospholipid deposition is associated with many adverse effects of amiodarone (Goldman, et al. 1985; Pirovino, et al. 1988; Reasor and Kacew. 1996). It is likely that these long-term adverse effects are due to excessive inhibition of carnitine palmitoyltransferase-1 activity leading to

deposition of non-metabolised lipids (Fromenty and Pessayre. 1995; Kennedy, et al. 1996; Berson, et al. 1998).

The potential long-term toxic effects of hepatitis and peripheral neuropathy have been extensively reported (Beaugrand, et al. 1977; Beaugrand, et al. 1978; Bertrand, et al. 1978; Lenoir and Blanchon. 1978; Myers and Ronthal. 1978; Nick, et al. 1978; Said. 1978; J; Daniell, et al. 1979; Fardeau, et al. 1979; Forbes, et al. 1979; Myers. 1979; Mikol. 1979; Pessayre, et al. 1979; Caruzzo, et al. 1980; Laplane and Bousser. 1981; Paliard, et al. 1981; Satz, et al. 1991; Vic, et al. 1981). In 1978 a relationship between these toxic effects and higher than normal concentrations of perhexiline was established by Singlas et al who observed that plasma perhexiline concentrations were greater in patients developing peripheral neuropathy and hepatitis than in unaffected patients (Singlas, et al. 1978). A possible explanation for the above effect was proposed by Shah et al who suggested that the phenomenon was confined to “slow” hydroxylators (Shah, et al. 1982). However, the number of patients reportedly developing hepatitis and peripheral neuropathy was far greater than the known proportion of “slow hydroxylators” (Horowitz and Mashford. 1979).

Horowitz et al subsequently reported that clinically detectable adverse effects of perhexiline were largely confined to those patients with plasma perhexiline concentration $> 0.7 \mu\text{g/L}$ (Horowitz, et al. 1986). A prospective study utilising a therapeutic range for perhexiline of 0.15-0.6 mg/L demonstrated that the toxic effects of peripheral neuropathy and hepatitis could be avoided by adjusting the perhexiline dosage

to maintain this range (Horowitz, et al. 1986). The same therapeutic range was utilised by Cole et al, with titration of dosage on the basis of plasma perhexiline concentrations; no patient developed hepatitis or peripheral neuropathy (Cole, et al. 1990).

1.9.3 Recommended treatment regimen

Perhexiline may be utilised for short-term management of both stable angina and unstable angina. In many patients, especially those with unstable angina, perhexiline is usually introduced with a loading dose (200 mg twice daily for three days) followed by a maintenance dose of 100 mg twice daily (Horowitz, et al. 1986). Close blood glucose monitoring is essential for diabetic patients because of the possibility of hypoglycaemia at day 3. Plasma perhexiline concentration may be checked if clinical response is suboptimal. However, there is little risk of severe toxicity.

In patients who require long-term perhexiline therapy, an initial treatment regimen of 100 mg twice daily should be followed by determination of plasma perhexiline concentration after 1-2 weeks of therapy. Dosage should then be adjusted to maintain plasma concentrations of between 0.15-0.6 mg/L (Horowitz, et al. 1995). Eventually maintenance doses may vary between 50 mg/week to 400 mg/day depending on hydroxylation status.

1.9.4 Pharmacokinetics

The pharmacokinetic profile of perhexiline is complex and therefore relatively unpredictable. Perhexiline demonstrates two unusual pharmacokinetic features;- 1) marked pharmacogenetic variability with the Caucasian population and 2) saturable (Michaelis-Menten) hepatic metabolism within the normal dosing range.

All pharmacokinetic studies of perhexiline in human subjects have utilised orally administered drug. There is no commercially available intravenous form of perhexiline at present. Perhexiline is well absorbed from the gastro-intestinal tract and is cleared via extensive hepatic metabolism to mono- and di-hydroxy-perhexiline's (Wright, et al. 1973). The extent of biological activity of these metabolites is largely unknown, although the major monohydroxy-perhexiline is an even weaker carnitine palmitoyltransferase-1 antagonist than the parent drug (Kennedy, et al. 1996). Perhexiline's concentration is higher within the tissues than the plasma; this has been attributed to perhexiline's lipophilic nature (Cooper, et al. 1987; Amoah, et al. 1984; Amoah, et al. 1986). There is further intra-mitochondrial concentrating of perhexiline relative to cytoplasmic concentrations (Deschamps, et al. 1994).

1.9.5 Pharmacogenetic variability

Perhexiline's hydroxylation status is controlled by cytochrome P₄₅₀ 2D6 (Shah, et al. 1982). Approximately 6-10% of the Caucasian population, but only 1% of Chinese, have a defect in this cytochrome system and are referred to as "poor" hydroxylators. Upon therapy these individuals clear perhexiline at approximately 10% of the rate of the

majority of patients (“normal” metabolisers), and therefore require markedly reduced dosages of the drug (Shah, et al. 1982). A very small proportion of patients are “ultra-fast” hydroxylators, and require increased steady-state doses of perhexiline.

1.9.6 Saturable hepatic metabolism

Within the population of “normal” hydroxylators the clearance of perhexiline is readily saturable (Michaelis-Menten pharmacokinetics) within the clinical dosing range (Horowitz, et al. 1981). This results in disproportionate changes in steady-state plasma perhexiline concentrations per unit change in daily dosage of perhexiline. For example, increasing daily dosage of perhexiline from 150 to 300 mg in a patients whose steady-state plasma perhexiline concentration is 100 µg/L is likely to result in a new steady-state drug concentration far in excess of the 200 µg/L which would be expected from linear pharmacokinetics (Horowitz, et al. 1995).

1.9.7 Mechanism of Action

1.9.7.1 Historical

Perhexiline was initially developed in the late 1960’s as a derivative of the coronary vasodilator hexadine (Hudak, et al. 1970). At the time of development it was considered that any drug which increased coronary blood flow was likely to be useful in the treatment of angina pectoris. Subsequently it has been demonstrated that non-specific small vessel dilators usually have clinically detrimental effects in the context of acute

myocardial ischaemia due primarily to the phenomenon of “coronary steal” (Gaya, et al. 1993; Guideri, et al. 1994; Harrison and Bates. 1993; Seiler, et al. 1997). Although perhexiline exerts coronary vasodilator effects in some animal models (Rowe, et al. 1970), there has been no observed major coronary vasomotor effect in either normal subjects or patients with ischaemic heart disease; it is also notable that perhexiline does not induce hypotension, suggesting little systemic vasodilator effect.

1.9.7.2 Calcium antagonism

In 1978 Fleckenstein-Grun et al demonstrated in vitro that perhexiline inhibited L-type calcium channels, but only in relatively high (above therapeutic) concentrations, from these observations the authors suggested that the therapeutic efficacy of perhexiline might be due to L-type calcium channel blockade (Fleckenstein-Grun and al. 1978). However, Barry et al later demonstrated that whilst perhexiline was indeed a calcium channel antagonist in myocardial cells, it was far less potent than the established calcium channel antagonists verapamil, nifedipine and diltiazem (Barry, et al. 1985). The notion that the therapeutic efficacy of perhexiline occurs via calcium channel antagonism is also difficult to comprehend given that perhexiline lacks marked hypotensive, vasodilator or negative inotropic effects at doses used clinically (Vaughan Williams. 1980). This view is further supported by the clinical reports suggesting that perhexiline is more efficacious than other calcium antagonists (Horowitz and Mashford. 1979; Cole, et al. 1990).

1.9.7.3 Carnitine palmitoyltransferase -1 inhibition

In 1980 Vaughan Williams demonstrated that perhexiline induced increased tissue phospholipid concentrations and postulated that perhexiline produces a metabolic shift by promoting glucose utilisation whilst simultaneously limiting lipid catabolism in the myocardium (Vaughan Williams. 1980). From these results Vaughan Williams hypothesised that perhexiline may act by causing a shift from fatty acid to glucose metabolism, thereby exerting an oxygen-sparing effect during periods of cardiac ischaemia. This potential mechanism has until recently received little attention, although it is consistent with the known histological toxicity of the drug.

1.9.7.3.1 Role of fatty acids and carbohydrates

As with most living tissue, myocardial tissue is able to produce energy from a wide range of substrates, including fatty acids and carbohydrates, but also from various molecules produced by metabolism, such as lactate, pyruvate and ketone bodies. The cardiac muscle shifts continuously from fatty acids to carbohydrates to fuel production of adenosine triphosphate (ATP), according to the supply availability in the blood elicited by food, exercise, or pathophysiologic state (the most important being presence/absence of coronary artery disease) (Grynberg and Demaison. 1996). While acute changes in cardiac function are buffered by acute changes in substrate utilisation, chronic changes in cardiac function result from changes in gene expression.

Glucose metabolism is divided into two main components, glycolysis and glucose oxidation. In glucose oxidation pyruvate derived from glycolysis is taken up into the mitochondria and decarboxylated by pyruvate dehydrogenase complex (Figure 1.5). The product of this is acetyl coenzyme-A (CoA), which is further metabolised by the mitochondria eventually leading to ATP production. Fatty acids are the major oxidative fuel for the heart consuming 60-100% of the utilised oxygen at any one time, compared to 0-20% each for lactate and glucose (Grynberg and Demaison. 1996) they require more oxygen than glucose metabolism to produce an equivalent amount of ATP. For example, one glucose molecule requires 12 oxygen atoms to produce 38 ATP molecules compared to one molecule of palmitate which requires 46 oxygen atoms to produce 130 ATP molecules. Thus, in terms of oxygen, fatty acids are not as efficient as glucose as a source of energy (Grynberg and Demaison. 1996).

Under normal physiological conditions the supply of oxygen to the myocardium is not limited and the advantage of fatty acids as regards efficiency of ATP production is one that explains it being the predominant metabolic substrate in the heart. However, under pathological conditions a preferential shift to carbohydrate utilisation will theoretically result in a 15-20% increased efficiency in the use of oxygen.

Previous experiments by Jeffery et al have provided evidence for a decrease in fatty acid utilisation in the isolated perfused working rat heart following perhexiline infusion at therapeutic concentrations (Jeffrey, et al. 1995). The metabolism of palmitate was reduced while simultaneously lactate utilisation was increased. This study was

important in that the demonstrated changes in substrate utilisation corresponded to an increase in cardiac output while no significant change in oxygen consumption was observed, that is cardiac efficiency increased following perhexiline perfusion. Further evidence for perhexiline's metabolic shift is provided by Deschamp et al who demonstrated impaired β -oxidation of long-chain fatty acids in hepatocytes exposed to perhexiline (Deschamps, et al. 1994). Recently Kennedy et al have demonstrated that perhexiline has a direct effect on fatty acid oxidation in rat heart by inhibiting a key enzyme regulating fatty acid utilisation, mitochondrial carnitine palmitoyltransferase-1 (Kennedy, et al. 1996).

The myocardial tissue exhibits a tight coupling between fatty acid and carbohydrate metabolism. Long chain fatty acids once in the cytoplasm, are activated and converted to long chain acyl-CoA. Long chain acyl-CoA are then transported to the mitochondrial matrix by carnitine palmitoyltransferase-1, located in the inner surface of the outer mitochondrial membrane (see Figure 1.6) (McGarry, et al. 1978; Kashfi, et al. 1994; Kennedy, et al. 1996). Carnitine palmitoyltransferase-1, the rate limiting enzyme in fatty acid metabolism, converts long chain acyl-CoA to long chain acyl carnitine (LCAC). LCAC is then reconverted to long chain acyl-CoA via carnitine palmitoyltransferase-2 (McGarry, et al. 1978). Long chain acyl-CoA then undergoes β -oxidation to produce hydrogen and acetyl-CoA. Acetyl-CoA is further converted to hydrogen in the Krebs cycle. There is reciprocal interplay between fatty acid and glucose metabolism, where inhibition of one automatically triggers up-regulation of the other. Acetyl-CoA causes negative feedback effect on the pyruvate dehydrogenase

system and thus acts as a regulatory mechanism for glucose utilisation. Acetyl- CoA is transported to the cytoplasm where it is converted to malonyl-CoA. Malonyl-CoA has been shown to inhibit carnitine palmitoyltransferase-1 (McGarry and Foster. 1980; Kashfi, et al. 1994; Kennedy, et al. 1996).

Carnitine palmitoyltransferase-1 is the main regulatory enzyme in the process of fatty acid oxidation with its activity being affected by the concentration of malonyl-CoA within the cell (see Figure 1.6). Thus, this is the rate limiting step in fatty acid oxidation. Kennedy et al demonstrated that perhexiline produced a dose-dependent inhibition of carnitine palmitoyltransferase-1 in rat heart and liver (Kennedy, et al. 1996). Although perhexiline was less potent than the physiological carnitine palmitoyltransferase-1 inhibitor, malonyl-CoA or the specific inhibitor 4-hydroxyphenylglyoxylate, perhexiline was shown to be partially specific as regards inhibition of the cardiac enzyme isoform of carnitine palmitoyltransferase-1 as opposed to the hepatic isoform (Kennedy, et al. 1996). This may be relevant to perhexiline's proven efficacy in the management of myocardial ischaemia.

The inhibition of carnitine palmitoyltransferase-1 is likely to have two consequences: increased myocardial efficiency and decreased potential for impairment of myocardial function during ischaemia. Protection against myocardial dysfunction during ischaemia may additionally reflect prevention of accumulation of LCAC's which have been implicated in the pathogenesis of left ventricular dysfunction and arrhythmogenesis during ischaemia (Yamada, et al. 1994; Clarke, et al. 1996).

A number of other agents have been shown to inhibit carnitine palmitoyltransferase-1. These include etomoxir, POCA (2(5[4-chlorophenyl]pentyl)oxyrane-2-carboxylate), 2-tetradecylglycidic acid and oxfenicine. Etomoxir and POCA, have been shown to induced metabolic changes in animal models in order to identify useful effect in the treatment of diabetes (Rosen and Reinauer. 1984; Wolf and Engel. 1985), cardiac ischaemia (Lopaschuk, et al. 1988; Reinauer, et al. 1990), or arrhythmia (Yamada, et al. 1994). Oxfenicine, which is converted to its active carnitine palmitoyltransferase-1 inhibitor hydroxyphenylglyoxylate, has been investigated as a potential anti-anginal agent (Bergman, et al. 1980). However, few studies to date have linked carnitine palmitoyltransferase-1 inhibition with clinically proven anti-anginal efficacy. The exceptions are 2 investigations by Kennedy et al which demonstrate carnitine palmitoyltransferase-1 inhibition not only by perhexiline, but to a lesser extent by amiodarone and trimetazidine, both of which have prophylactic anti-anginal effects (Kennedy, et al. 1996; Kennedy and Horowitz. 1998).

1.9.7.4 Hypoglycaemic effects

Perhexiline has been shown to lower blood sugar levels, especially among diabetic patients receiving either insulin or sulphonylureas. This decrease in blood sugar levels increases the patients risk of developing hypoglycaemia (Roger, et al. 1975; Dally, et al. 1977; Fournier, et al. 1978; Schlienger, et al. 1978; Luccioni, et al. 1978; Erhart, et al. 1981). Feldman et al found increased tissue responsiveness to insulin among perhexiline

patients (Feldman. 1974), whilst Luccioni et al observed that insulin secretion was increased in the presence of perhexiline (Luccioni, et al. 1978). The exact mechanism for the above effect is not fully understood. However, there is a possibility that this effect could occur through carnitine palmitoyltransferase-1 inhibition. In a study by Kashiwagi et al in diabetic patients, it was demonstrated that fasting hyperglycaemia was correlated with accelerated hepatic glucose production secondary to glucogenesis induced by elevated free fatty acids (Kashiwagi. 1995). As the inhibition of carnitine palmitoyltransferase-1 results in both reduced free fatty acids and increased glucose oxidation (Lopaschuk, et al. 1989), the use of agents which inhibit this enzyme may be potentially useful in the management of diabetes (Kashiwagi. 1995). Evidence for this effect comes from the carnitine palmitoyltransferase-1 inhibitor etomoxir, which has been demonstrated in rats to significantly reduce fasting glucose concentrations (Barnett, et al. 1992). Similarly, a study by Iida et al demonstrated that in diabetic rats carnitine palmitoyltransferase-1 activity was increased when compared with non-diabetic rats. As a consequence of this fatty acid oxidation was also enhanced. When insulin was administered to the diabetic rats responses mirrored that of non-diabetic rats. The authors concluded that fatty acid oxidation was regulated by carnitine palmitoyltransferase-1 activity and insulin (Iida, et al. 1993). Therefore inhibition of carnitine palmitoyltransferase-1 is likely to result in decreased fatty acid oxidation. These hypoglycaemic effects of perhexiline (and other carnitine palmitoyltransferase-1 inhibitors) are also observed with the thiazolidinediones (for example troglitazone), which produce metabolic shifts similar to those seen with carnitine palmitoyltransferase-1 inhibition (Horton, et al. 1998).

1.9.7.5 Inhibition of platelet aggregation

Perhexiline has been shown to inhibit platelet aggregation in only one study previously. Ono and Kimura undertook a study to examine the anti-aggregatory efficacy of a number of calcium antagonists: diltiazem, nifedipine, verapamil and perhexiline (Ono and Kimura. 1981). Unexpectedly, perhexiline, the weakest calcium antagonist of the agents used when classified on vasodilator activity, exhibited the greatest anti-aggregatory effect. This observation was left without any further exploration possibly because of inconsistencies between anti-aggregatory and calcium-blocking efficacy of the agents tested. Nevertheless, the documented phenomenon has important clinical implication not only given the recent suggestion by Stewart et al that perhexiline decreases symptoms in patients with unstable angina pectoris (Stewart, et al. 1996). As discussed earlier unstable angina pectoris is a syndrome associated with increased platelet activation, therefore a reduction in symptoms among this group of patients may imply an interaction between perhexiline and platelet aggregation. However, the mechanism of inhibition of aggregation by perhexiline remains unknown to date, despite the evidence that this may contribute to its clinical efficacy.

1.9.7.6 Other possible mechanisms

1.9.7.6.1 Effects on potassium channel activity

Perhexiline has previously been shown to block rapidly activating delayed rectifier potassium channels. Rampe et al demonstrated by utilising inside-out macropatches that perhexiline (IC_{50} 1.5 μ M) inhibited rectifier potassium channels (cloned from human heart) currents in a time- and voltage-dependent manner (Rampe, et al. 1995). Perhexiline also reduced potassium channel tail current amplitude and slowed its delay relative to control. These data are consistent with the blockade of open channels. In addition perhexiline also blocked an ultra-rapid delayed rectifier potassium channel in human atrial myocytes (Rampe, et al. 1995). In addition, perhexiline also inhibits the ether-a-go-go-related (HERG) potassium current. Walker et al demonstrated that perhexiline ($\bullet$ 10 μ M) increased the rate of HERG inactivation, but had not effect on recovery from inactivation (Walker, et al. 1999). The results of the above studies suggest that the blockade of one or more types of voltage-dependent potassium channels by perhexiline may explain some of the electrophysiological effects (QT interval prolongation) observed with its use in humans (Antman, et al. 1980).

1.10 Could perhexiline represent an important anti-aggregatory agent?

1.10.1 Platelet metabolism: normal and in ischaemia

As discussed in section 1.8.7.3.1 under normal physiological conditions the preferential substrate for ATP production within the heart is long-chain fatty acids. However, under

physiological conditions when the supply of oxygen is limited a shift towards carbohydrate utilisation occurs, resulting in a 15-20 % increase in oxygen efficiency. In contrast to the heart, platelets utilise carbohydrates as their main source of energy.

Carnitine palmitoyltransferase-1 inhibitors have previously been investigated for useful effects in cardiac ischaemia (Lopaschuk, et al. 1988; Reinauer, et al. 1990) and arrhythmia's (Yamada, et al. 1994). In addition, data suggest that in vivo some inhibitors of carnitine palmitoyltransferase-1 inhibit platelet aggregation (Ishikura, et al. 1992), thus raising the possibility that perhexiline may inhibit platelet aggregation

1.10.2 Clinical considerations

1.10.2.1 Lack of studies

Perhexiline has been shown to decrease the symptoms in patients with unstable angina pectoris (Stewart, et al. 1996). As unstable angina is a disease state associated with increased platelet aggregability, this result suggests that perhexiline may have an anti-aggregatory component associated with its clinical effectiveness. However, that study by Stewart et al (Stewart, et al. 1996) is the only one of its type as the vast majority of studies utilising perhexiline have involved patients with stable angina. Although increased platelet aggregability has been identified in this group of patients no study has investigated a potential interaction between perhexiline and platelet aggregation.

1.10.2.2 Polypharmacy

A potential problem for the investigation of an anti-aggregatory effect of perhexiline in both stable and unstable angina is the amount of existing anti-platelet therapy (see section 1.6). Unless perhexiline exerts a large degree of inhibition of aggregation (for example to the same extent as GPIIb/IIIa inhibitors) an anti-aggregatory effect may be to be masked by the concurrent anti-platelet therapy.

1.10.3 Potential interaction with nitric oxide / free radicals

Recently, perhexiline, amiodarone and diethylaminoethoxyhexestrol (DEAEH), all carnitine palmitoyltransferase-1 inhibitors, have been shown to increase the formation of reactive oxygen species from intact rat liver mitochondria (Berson, et al. 1998). As reactive oxygen species, or more specifically superoxide, inactivate nitric oxide it would be anticipated that if the above observation in isolated mitochondria is applicable to the whole organism, then nitric oxide content would be expected to decrease. However, the concentration of perhexiline examined in the study by Berson et al were approximately 200 fold higher than its therapeutic concentration (Berson, et al. 1998); hence the clinical relevance of this observation is questionable.

Carnitine palmitoyltransferase-1 exists in two isoforms (liver and skeletal muscle). These two isoforms have differing degrees of sensitivity to various agents:- for example the skeletal muscle isoform is 100 fold more sensitive to malonyl-CoA (the endogenous carnitine palmitoyltransferase-1 inhibitor) than the liver isoform (McGarry, et al. 1983).

The heart and platelets contain predominantly the skeletal muscle isoform of carnitine palmitoyltransferase-1 and thus the increase in reactive oxygen species produced by perhexiline in isolated intact hepatic mitochondria, may not necessarily be applicable to the heart or platelets.

1.11 Scope of current study

Despite being in clinical use for over 30 years little or nothing is known between the interaction of perhexiline and platelet aggregation. This thesis was designed to explore this interaction (both in vitro and ex vivo) and to study the mechanism(s) by which perhexiline inhibits platelet aggregation.

Aims of this thesis include:-

- 1) To develop an in vitro model of aggregation more closely reflecting the in vivo situation.
- 2) To compare this model with existing platelet methodology by examining the platelet responsiveness of the anti-aggregatory agents nitroglycerine, prostaglandin E₁ and verapamil.
- 3) To examine the putative anti-aggregatory effects of perhexiline in vitro in whole blood from patients with stable angina.
- 4) To study the possible roles of the cyclic nucleotides, cGMP and cAMP in the anti-aggregatory mechanism of perhexiline.

- 5) To examine if carnitine palmitoyltransferase-1 inhibition modulates the anti-aggregatory effects of perhexiline.
- 6) To examine the effect of perhexiline therapy on ex vivo platelet aggregation and sodium nitroprusside responsiveness in blood samples from patients with stable angina or acute coronary syndromes.
- 7) To examine the effect of acute and chronic perhexiline administration on ADP-induced sheep platelet aggregation and platelet nitric oxide responsiveness.

In order to achieve the above aims, experiments were performed utilising an in vitro model of platelet aggregation (Chapter 3-5). Additional studies were performed in blood samples from 1) stable angina and acute coronary syndrome patients treated with perhexiline, and 2) sheep, either acutely or chronically treated with perhexiline.

Chapter 3 describes the work of developing a multiple agonist method for inducing in vitro platelet aggregation. Anti-aggregatory effects of nitroglycerine, prostaglandin E₁ and verapamil were compared using the developed technique and the existing single (ADP) agonist method.

Chapter 4 described the work of assessing in vitro anti-aggregatory effects of perhexiline, utilising both single and multiple agonist methodology. In addition, the possible role of cGMP and cAMP in the anti-aggregatory effect of perhexiline was investigated.

Chapter 5 compares the association between the in vitro effects of perhexiline, amiodarone and trimetazidine on carnitine palmitoyltransferase-1 activity and aggregation in human platelets. A comparison was made with the specific carnitine palmitoyltransferase-1 inhibitors, etomoxir and hydroxyphenylglyoxylate.

Chapter 6 studies the effects of perhexiline therapy, in both stable angina and acute coronary syndromes, on ex vivo platelet aggregation and sodium nitroprusside responsiveness. Platelet responsiveness was compared with that of normal volunteers and acute coronary syndrome patients not receiving perhexiline. In addition, the possible interaction between perhexiline, superoxide and guanylate cyclase was tested.

Chapter 7 briefly studies the effect of in vivo perhexiline administration (acutely and chronically) to normal sheep on ADP-induced platelet aggregation and nitric oxide responsiveness.

Chapter 8 summarises the conclusions to be drawn from the various experiments and comments on possible directions for future studies.

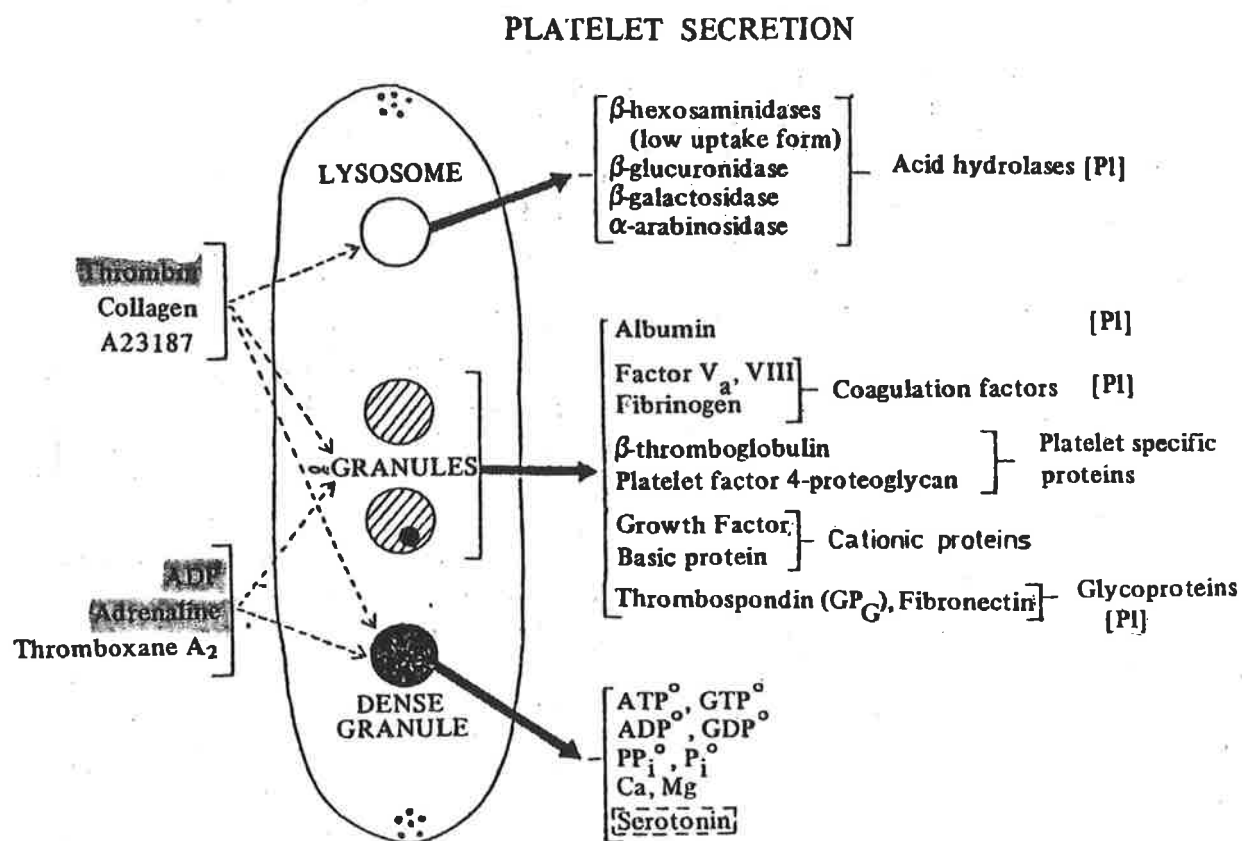


Figure 1.1.

Platelet secretion (Holmsen. 1989).

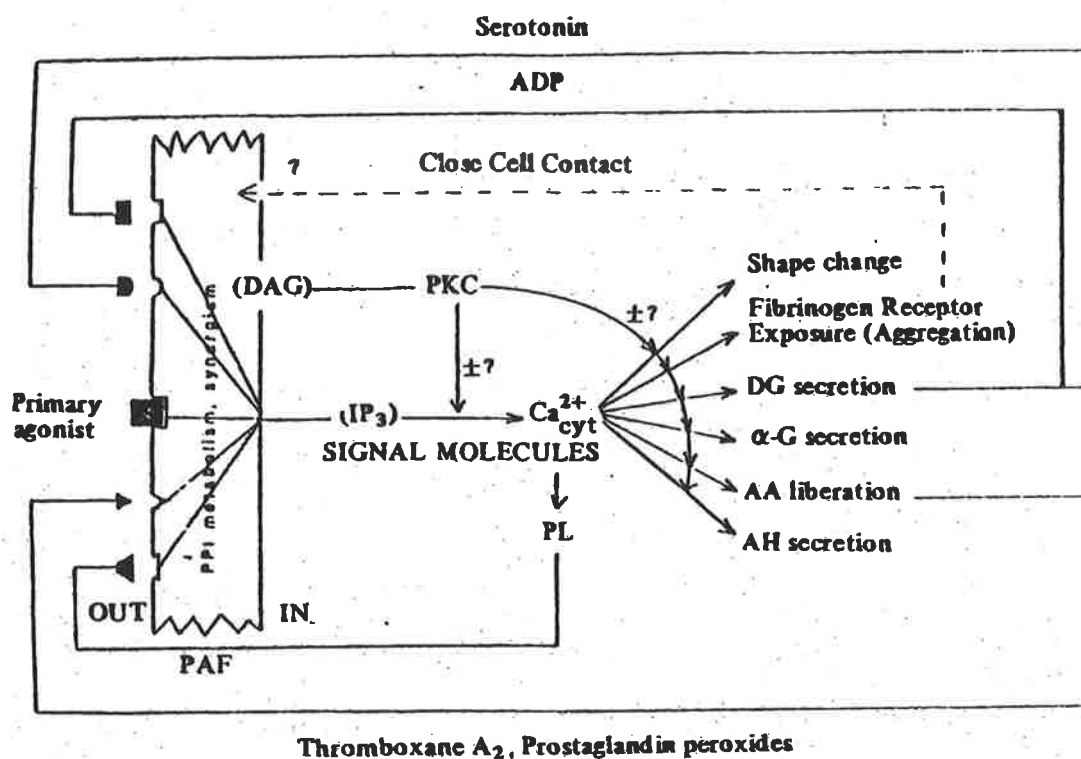


Figure 1.2.

Schematic representation of platelet activation and response (Holmsen. 1991).

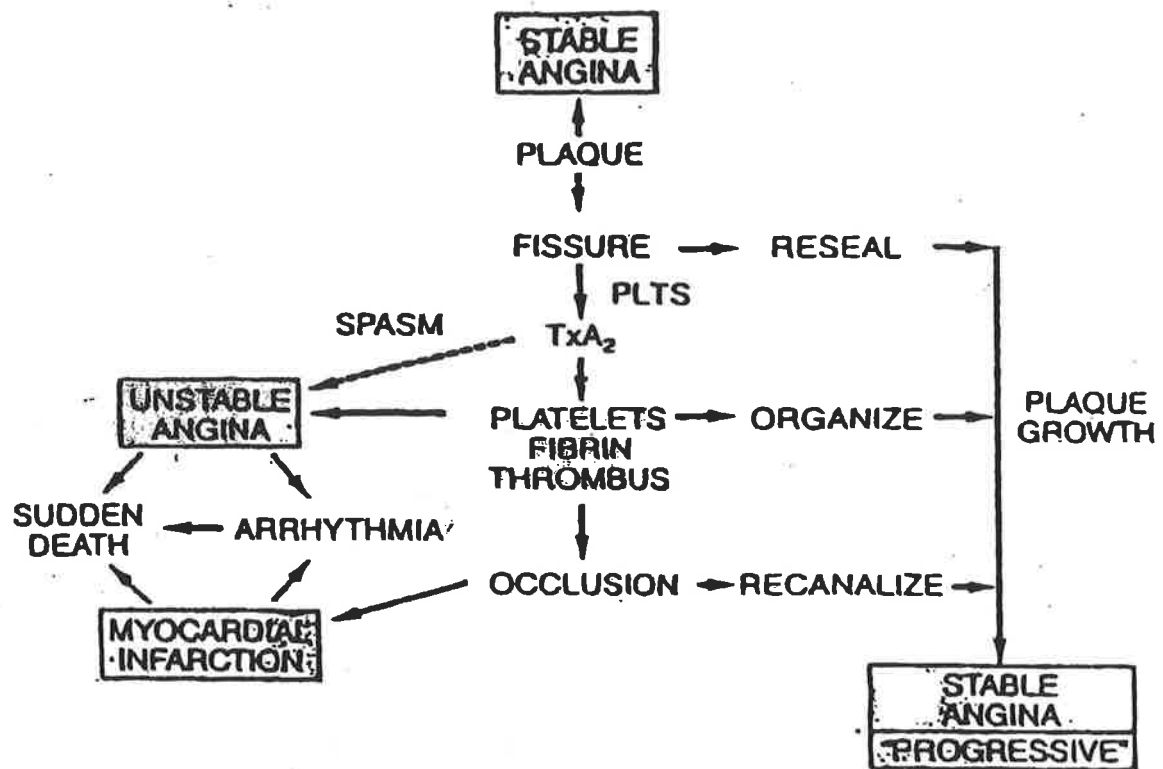


Figure 1.3.

Schematic representation of angina (stable/unstable) and the involvement of platelets (Bashour, et al. 1988).

TxA₂: Thromboxane A₂

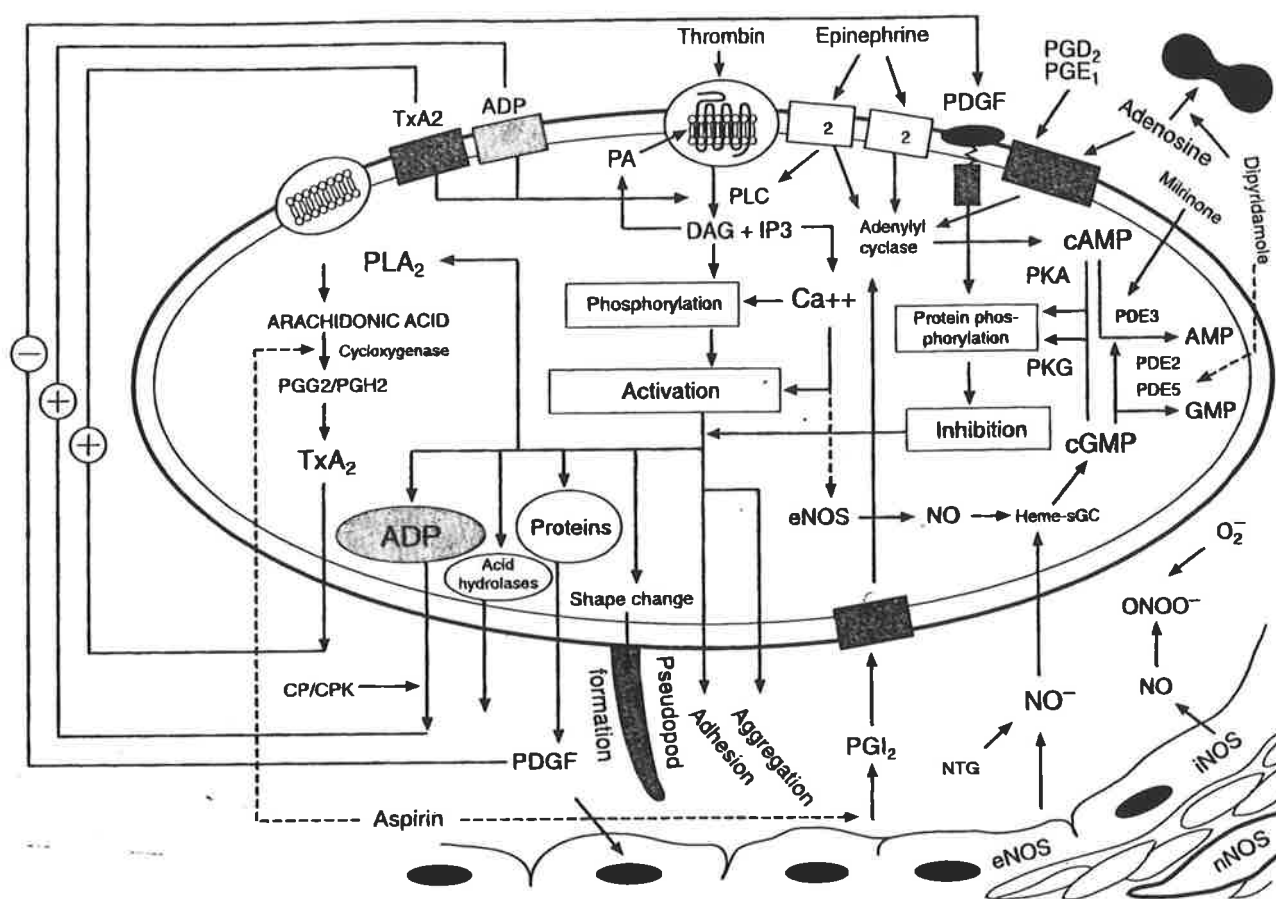


Figure 1.4.

Effects of nitric oxide on cell signalling pathways in human platelets. Effects of autocrine stimulation of platelet activation are illustrated by (+) for potentiation and (-) for inhibition (Jensen. 1996).

Abbreviations: NOS; nitric oxide synthase, NO; nitric oxide, sGC; soluble guanylate cyclase, cGMP; guanosine 3',5'-cyclic monophosphate, cAMP; adenosine 3',5'-cyclic monophosphate, PKG; cGMP-dependent protein kinase, CP/CPK; creatine-phosphate/creatine phosphokinase, PDE; phosphodiesterase.

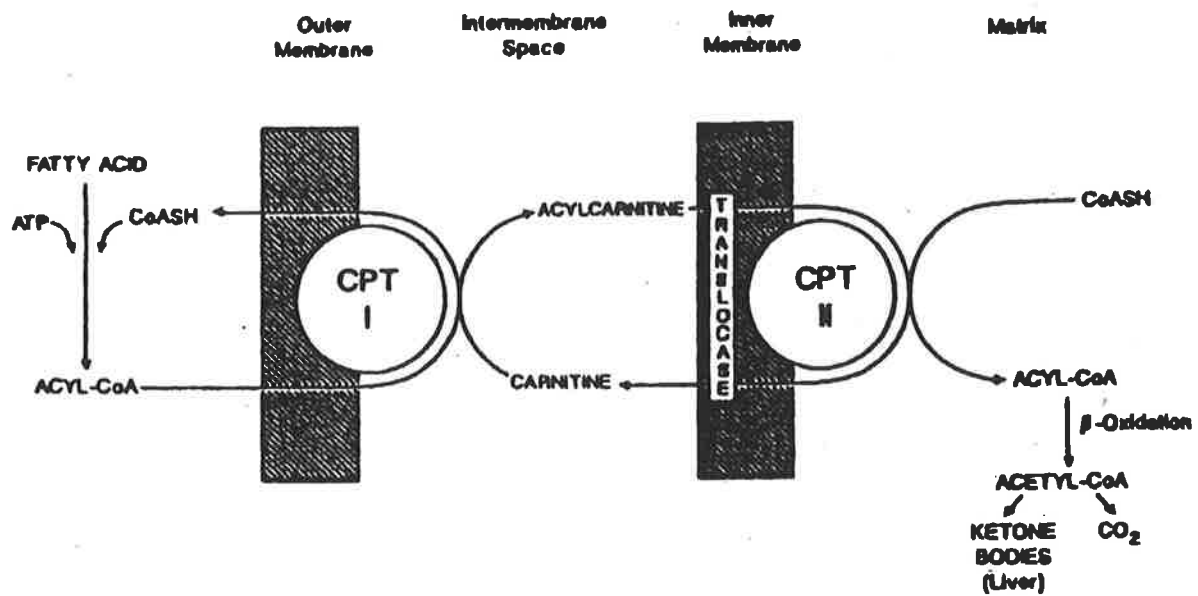


Figure 1.5.

Roles of carnitine palmitoyltransferase-1 and II (CPT-1 and II) in the mitochondrial transport of fatty acids (McGarry, et al. 1991).

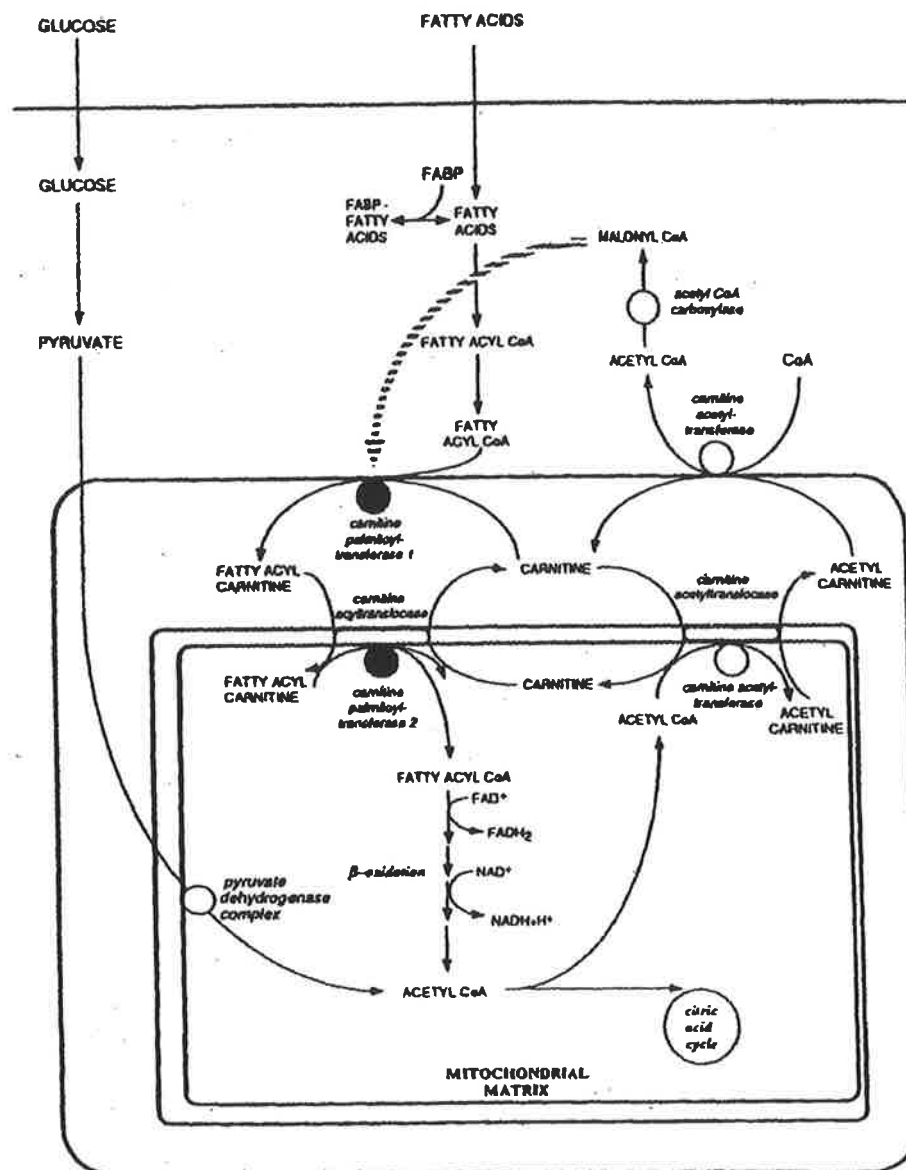


Figure 1.6.

Schematic diagram of fatty acid oxidation in the heart (Lopaschuk, et al. 1994).

Chapter 2

MATERIALS AND METHODS

2 Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Subjects studied

All experimental procedures involving patients or normal volunteers were approved by the Ethics of Research Committee of the Queen Elizabeth Hospital and informed consent was obtained prior to subject entry in all cases.

2.1.2 Blood sampling

Blood samples from normal volunteers were drawn from a venesection of an antecubital vein, while blood samples from patients were drawn from either the antecubital vein or through a femoral arterial sheath during cardiac catheterisation. In all cases, blood was withdrawn into a plastic syringe utilising minimal suction, as to prevent activation of the platelets during collection. Blood was transferred slowly to plastic screw top tubes containing 1:10 volume of citric acid-sodium anticoagulant (two parts of 0.1 mM citric acid to three parts 0.1 mM trisodium citrate, pH 5); acidified citrate was utilised in order to minimise the deterioration of platelet function during experiments (Kinlough-Rathbone, et al. 1983). The time interval between collection of blood samples and platelet aggregation studies was 10-15 min.

2.1.3 Preparation of Platelet-rich plasma and washed platelets

2.1.3.1 *Platelet-rich plasma for cGMP/cAMP assay*

Platelet-rich plasma was prepared by centrifugation of blood at 500 g for 8 min at room temperature. The supernatant was retained and a platelet count was performed utilising a STKS Coulter counter (Coulter Electronics Inc. Hialeah, Fl, USA). The volume of platelet-rich plasma required for the cGMP/cAMP assays were calculated in order to obtain the optimal concentration of cGMP/cAMP for the radioimmunoassay, according to the following formula:

$$\text{Volume platelet-rich plasma (ml)} = \frac{150 \times 10^6}{\text{Platelet count}}$$

2.1.3.2 *Washed platelets for intraplatelet calcium and aggregation*

Platelet-rich plasma was prepared as above by centrifugation of the anticoagulated blood (500g, 8 min) and collected into a 50 ml plastic tube. The remaining red blood cell and white blood cell suspension was mixed with 2 mls of a solution containing 140 mM NaCl, 2.7 mM KCl, 0.1% albumin, 0.1% glucose, 3.8 mM HEPES, 5 mM EGTA, 1 μ M PGE₁, pH=7.6 (buffer A) and spun at 500 g for 5 min. The supernatant was removed and added to the platelet-rich plasma. 5-10 ml of buffer A was added to the tube of platelet-rich plasma and then spun at 800g for 15 min to pellet the platelets. The supernatant was removed and the platelet pellet resuspended in 5-10 ml of buffer A and spun at 800 g for 10 min. The supernatant was removed and the platelets were resuspended in 90 μ l of

buffer A. The washed platelet suspension was used for intraplatelet calcium assay and washed platelet aggregation as described in sections ?.

2.1.3.3 *Washed platelets for CPT-1 assay*

Washed platelets were prepared essentially as described by Iida et al (Iida, et al. 1991). Platelet-rich plasma was centrifuged at 500 g for 8 min at room temperature and platelets were washed twice (centrifugation at 800 g, 15 min, room temperature then resuspension) with a solution containing 36 mM citric acid, 5 mM glucose, 5 mM KCl, 90 mM NaCl, 1 μ M prostaglandin E₁, pH 6.5. The platelet pellet was then resuspended in modified Tyrode's solution consisting of 11.9 mM NaHCO₃, 0.55 mM glucose, 2.68 mM KCl, 137 mM NaCl, 0.416 mM NaH₂PO₄, 1 mM MgCl₂, 5 mM Hepes, 1 μ M PGE₁, pH 7.35 at a final concentration of 10⁹ platelets/ml.

2.1.4 Chemicals

Materials and chemical substances used were of the highest possible purity and quality. Frequently used chemical are mentioned below, other chemicals are mentioned in relevant sections.

Adenosine diphosphate (ADP)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solution were made in 0.9% saline and stored at -20°C. Dilution's of ADP were made in 0.9% saline prior to use.

L-adrenaline [(-) epinephrine (+) bitartrate salt crystalline] (Adr)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were made in 0.01 M HCl and stored at -20 °C. Dilution's of adrenaline were made in 0.01 M HCl prior to use. Control experiments with matched concentrations of HCl showed no effect on platelet aggregation.

Serotonin [5-hydroxytryptamine - creatinine sulfate complex] (5HT)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were made in 0.9 % saline and stored at -20 °C. Dilution's of serotonin were made in 0.9 % saline prior to use.

Thrombin [from human plasma] (Thr)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were made in 0.9 % saline and stored at -20 °C. Dilution's of thrombin were made in 0.9 % saline prior to use.

Perhexiline - hydrochloride

Perhexiline was a gift from Sigma Pharmaceuticals Australia, Melbourne, Victoria.

Stock solutions of perhexiline were prepared in ethanol and dilution's were made in 0.9 % saline on the day of experiment. Control experiments with matched concentrations of ethanol showed no effect on platelet aggregation.

Verapamil - hydrochloride (Ver)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were prepared in 0.9 % saline. Dilution's of verapamil were made in 0.9 % saline prior to use.

Prostaglandin - E₁ (PGE₁)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were prepared in ethanol. Lower concentrations were diluted in 0.9 % saline. Control experiments with matched concentrations of ethanol showed no effect on platelet aggregation.

Glycerol trinitrate (NTG; nitroglycerine)

Purchased from Fison Pty Ltd, Sydney, Australia. Dilution's were made prior to use in 0.9 % saline from a 22 mM stock in ethanol. Control experiments with matched concentration of ethanol showed no effect on platelet aggregation.

Amiodarone - hydrochloride (Ami)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were prepared in methanol. Lower concentrations were prepared using 0.9 % saline. Control experiments with matched concentrations of methanol showed no effect on platelet aggregation.

Etomoxir (Eto)

Purchased from Research Biochemicals International. Stock solutions of etomoxir were made in 0.9 % saline and stored at -20 °C. Dilution's of etomoxir were made in 0.9 % saline prior to use.

Oxfenicine [L-(+)-Hydroxyphenylglycine] (Oxf)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were made in distilled water and stored at -20 °C. Dilution's of oxfenicine were made in distilled water prior to use.

Hydroxyphenylglyoxylate (HPG)

The active metabolite of oxfenicine. Was a gift from Hoffman-La Roche, Nutley, New Jersey, USA. Stock solutions of HPG were made in 0.9 % saline and stored at -20 °C. Dilution's of HPG were made in 0.9 % saline prior to use.

Trimetazidine - hydrochloride (TMZ)

Was a gift from Servier, France. Stock solutions were made on 0.9 % saline and stored at -20 °C. Dilution's were made in 0.9 % saline prior to use.

Sodium nitroprusside (SNP)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were made in 0.9 % saline at least 30 minutes prior to use. Subsequent dilution's were made in 0.9 % saline.

2.1.5 Other Chemicals

Aequorin

Purchased from Dr J Blinks, Friday Harbor Photoproteins, Friday Harbor, WA, USA.

The photoprotein aequorin is harvested from the jellyfish *Aequora aequora* and emits a blue light on binding calcium. Aequorin is lyophilised from a solution of 1 mg aequorin/ml in 150 mM KCl, 5 mM HEPES buffer. The lyophilised aequorin is dissolved in 333 μ L of Chelex resin filtered water containing 7 mM EGTA adjusted to pH 7.4.

Analytical grade Chelex 100 resin was purchased from Bio-Rad Laboratories, Hercules, CA, USA. Chelex resin is utilised to remove trace amounts of calcium from distilled water. Chelex resin filtered water was prepared by adding 5 mg of resin to every 100 ml of distilled water. The sample was then stirred for 1 hour and the resin was removed by centrifugation at 1500 g for 20 min and removing the supernatant.

The stock solution of aequorin was then divided into 10 μ L aliquots each containing 3 mg/ml aequorin and stored at -80°C until use.

ethylene glycol-bis(β -aminoethyl ester) N,N,N',N'-tetraacetic acid (EGTA)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions (10 mM) were made in Chelex resin filtered water and adjusted to pH 7.4 utilising NaOH and HCl.

Oxyhaemoglobin (HBO₂)

Was prepared by dissolving 1 mM haemoglobin (bovine lyophilised powder, Sigma Chemical Co; St Louis Mo, USA) in 4 ml of distilled water and adding it to 1 ml of 2 mM sodium dithionite (Sigma Chemical Co; St Louis Mo, USA), prepared immediately before use, to form approximately 200 µM of oxyhaemoglobin. The oxyhaemoglobin was then dialysed in distilled water in the dark at 4 °C for 1 hour. Aliquots were stored at -70 °C until use.

N ω -nitro-L-arginine methyl ester, hydrochloride (L-NAME)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were made in 0.9 % saline and stored at -20 °C.

1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)

Purchased from Tocris Cookson, Bristol, United Kingdom. Stock solutions were made in dimethylsulfoxide (DMSO [Pierce, Rockford, Illinois, USA]) and stored at -20 °C until use. Dilution's from the stock solution were made in 0.9 % saline.

3-isobutyl-1-methyl-xanthine (IBMX)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were made in 0.9 % saline and stored at -20 °C.

Superoxide dismutase [from bovine erythrocytes] (SOD)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were made in 0.9 % saline and stored at -20 °C.

Catalase [from bovine liver]

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were made in 0.9 % saline and stored at -20 °C.

Bis-N-methylacridinium nitrate (Lucigenin)

Purchased from Sigma Chemical Co; St Louis Mo, USA. Stock solutions were made in 0.9 % saline and prepared fresh each experimental day.

[3H] l-carnitine (specific activity 77 Ci/mM)

Purchased from Amersham Int plc, UK.

cGMP [¹²⁵I] radioimmunoassay Kit and cAMP [¹²⁵I] radioimmunoassay Kit

Purchased from Amersham Int plc, UK.

2.2 Methods

2.2.1 Instrumentation

Platelet aggregation in whole blood and aequorin luminescence in washed platelets were examined utilising a dual channel whole blood impedance Lumi-Aggregometer (Model

560CA, Chrono-Log, Havertown, P.A, USA). Data was collected via a Chrono-log Model 810CA Aggro/Link computer interface connected to an IBM (486DX) compatible computer utilising Windows version 3.1. Continuous measurements of the aggregation response enables automatic calculation of the maximal amplitude of the aggregation curve. An amplifier/integrator (Model 810CA, Chrono-Log, Havertown, P.A, USA) was utilised to record and determine the intracellular ionised calcium concentration.

2.2.2 Whole Blood Aggregometry

The principle underlying whole blood impedance aggregometry is that a very small electrical current is passed between two electrodes immersed in the blood sample. During initial contact with the blood the electrodes become coated with a monolayer of platelets. When an agonist is added, platelets aggregate on the monolayer, increasing the impedance. This increase in impedance is recorded over time and is directly proportional to the mass of the platelet aggregate. This method allows assessment of platelet function is without the necessity for mechanical processing of the blood sample (Cardinal and Flower. 1980; Ingberman-Wojenski, et al. 1983; Ingberman-Wojenski and Silver. 1984) thus providing a sensitive system with a more physiological environment than other in vitro systems (Boyd and Davis. 1988).

Blood was placed in plastic cuvettes, diluted 2 fold with physiological saline (final volume 1 ml), and warmed in a heating block at 37 °C for 7 min. During this time any incubation with inhibitors of aggregation or vehicle controls were performed.

Siliconised stir bars were used to stir the blood at a rate of 1000 rpm. To initiate aggregation experiments, inductors of aggregation (eg. ADP or thrombin) were added at the end of the pre-incubation period. Solutions of inductors of aggregation were added in volumes of 1-5 μ L. Aggregation experiments were monitored continually for 7 min and maximal responses were recorded (Rikadenki chart recorder, Rikadenki Kogyo Co. Ltd. Tokyo, Japan or Model 810-CA Aggro-link computer interface, Chrono-Log, Haverstown, P.A. USA) for electrical impedance, in Ohms (see Figure 2.1 for representative aggregation traces). Each test was performed in triplicate and from these average values were calculated.

Stability of platelets' responsiveness to ADP (1 μ M) was tested every 30 min over periods up to 2 hours after blood collection, with coefficient of variation <10% in all cases.

2.2.3 Platelet cGMP assay

This procedure was performed as described by Chirkov et al (Chirkov, et al. 1993). Essentially, platelet-rich plasma (0.5 ml) was incubated for either 2.5 min with SNP or 5 min with NTG at 37°C. In control experiments 0.9% saline was utilised. After incubation the plasma was filtered to harvest platelets. To achieve this plastic filter holders Swinnex-25 (Millipore, USA) containing GF/C Glass Microfibre Filters (Whatman, UK) were attached to 5 ml plastic syringes and plasma was injected across the filter. Filters with absorbed platelets were rinsed with 0.5 ml of 0.9% saline, and

placed into glass tubes containing 0.5 ml (cGMP assay) of 4 mM EDTA. EDTA was utilised to prevent cGMP decay.

Tubes were then placed in a boiling water bath for 5 min. After this time filters were removed and samples centrifuged at 3000 g for 10 min, cGMP concentrations in the supernatant were assayed using cGMP [^{125} I] radioimmunoassay Kit (Amersham, UK).

2.2.4 Platelet cAMP Assay

The procedure for preparing platelet for cAMP assay was performed as described by Chirkov et al (Chirkov, et al. 1995) and is essentially the same as for cGMP above. cAMP concentration in samples after extraction were assayed using cAMP [^{125} I] radioimmunoassay Kit (Amersham, UK).

2.2.4.1 cGMP and cAMP radioimmunoassay

The cGMP radioimmunoassay is based on the competition between unlabelled cGMP and a fixed quantity of ^{125} I-labelled cGMP for a limited number of binding sites on a cGMP-specific antibody. With fixed amounts of antibody and ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand. Measurement of the radioactivity in the pellet enables the amount of labelled cGMP to be calculated. The concentration of unlabelled cGMP in the sample is then determined by interpolation from a standard curve (Figure 2.2).

The assay system utilised a high specific activity [^{125}I] 2'-O-succinyl-cGMP tyrosine methyl ester tracer, together with a highly specific and sensitive antiserum. Separation of the antibody bound from free fraction is achieved with a second antibody Amerlex-M preparation allowing for a simple magnetic separation.

cGMP may be measured in the range of 2-128 fmol/tube (0.7-44 pg/tube).

The cAMP assay is essentially the same as the cGMP assay described above. The cAMP assay system utilised a high specific activity [^{125}I] 2'-O-succinyl-cAMP tyrosine methyl ester tracer.

cAMP may be measured in the range of 2-128 fmol/tube (0.7-42 pg/tube).

Intraplatelet cGMP/cAMP content results are expressed as pmol of cGMP/cAMP per 10^9 platelets and were calculated using the following formula:

$$\text{cGMP/cAMP [pmol/}10^9\text{ platelets]} = \frac{A \times 10 \times V_1 \text{ (volume of EDTA, ml)}}{\text{PRP count} \times V_2 \text{ (volume of PRP, ml)}}$$

A = cGMP/cAMP content per tube

PRP= platelet-rich plasma

2.2.5 Intraplatelet Calcium

2.2.5.1 *Aequorin loading*

Aequorin was loaded into platelets (preparation of washed platelets described in section 2.1.3.2) using dimethylsulfoxide (DMSO [Pierce, Rockford, Illinois, USA]) as described by Yamaguchi et al (Yamaguchi, et al. 1986). Briefly, at room temperature (20-22 °C), DMSO was stepwisely added in 1 µl portions over 7.5 min to obtain a concentration of 6% in the suspension, time delay between successive steps was 1.5 min. The suspension was incubated for 2 min afterwards, and diluted with 10 times volume of buffer A. Two minutes later, platelets were sedimented (10000g for 90 sec), the supernatant was removed and platelets were washed with 1 ml of buffer A and transferred to a clean tube (to remove any excess buffer containing DMSO) and resedimented (10000g for 60 sec). The supernatant was removed and platelets were resuspended in 1 ml buffer A and resedimented (10000 g for 60 sec). Supernatant was removed and platelets were resuspended in buffer B, which is essentially the same as buffer A except EGTA and PGE_1 were omitted and 1 mM MgCl_2 and 1 mM CaCl_2 was added. Platelet counts were performed utilising a STKS Coulter counter (Coulter Electronics Inc. Hialeah, Fl, USA) and the platelet suspension was diluted utilising buffer 2 to a final concentration of 200-300 x 10³ platelets / µl.

Before commencement of experiment platelets were allowed to equilibrate for 15 min.

2.2.6 Intraplatelet calcium assay protocol

1 ml of the platelet suspension was incubated in a cuvette at 37°C for 3-5 min. The total amount of calcium in the platelets can be measured ($L_{\max}$) by adding 10 μ L of 10 % Triton X-100 solution to the platelet suspension.

To measure the total flux of calcium (L) the platelet suspension is incubated as above. When placed in the test well 10 μ L of thrombin (0.1 U/ml) is injected into the system, and the subsequent luminescence signal measured.

Total internal calcium (L_{egta}) is measured essentially the same as for total flux of calcium except 10 μ L of EGTA is added just before the addition of thrombin. EGTA binds external calcium therefore any measured luminescence signal is entirely due to the release of intraplatelet calcium.

Total and internal calcium concentration was calculated according to the method described below.

2.2.7 Calculation of total and internal calcium concentration

Intracellular calcium ion concentration ($[Ca^{2+}]_i$) was calculated from the fractional luminescence ($L/L_{\max}$) of the aequorin-loaded platelet suspension and the maximal luminescence ($L_{\max}$) recorded for appropriate aliquots of aequorin-loaded platelet lysed with Triton X-100 (final concentration 0.1 %) [Pierce, Rockford, Illinois, USA]. Log_{10}

$L/L_{\max}$ was then converted to $[Ca^{2+}]_i$ by reference to a calibration curve (Figure 2.3) which relates fractional aequorin luminescence to $[Ca^{2+}]$ in the presence of 1 mM Mg^{2+} .

2.2.8 Washed platelet aggregation

Washed platelet aggregation was measured essentially the same as for whole blood aggregation as described in section 2.2.2. The only differences were 1) aggregation was induced by thrombin 0.1U/mL and 2) aggregation was measured simultaneously with measurement of calcium release.

2.2.9 Platelet carnitine palmitoyltransferase-1 assay

Carnitine palmitoyltransferase-1 activity was estimated in saponin-permeabilized platelets as the formation of palmitoyl- $[^3H]$ carnitine from palmitoyl-CoA and $[^3H]$ l-carnitine, essentially as described by McGarry et al (McGarry, et al. 1978). Platelets were permeabilized by incubating with 60 μ g/ml saponin for 5 min at 37 °C. The disruption of the plasma membrane by saponin, without significant effect on the mitochondrial membrane, was verified by the release of the cytoplasmic enzyme, lactate dehydrogenase (EC 1.1.1.27), into the medium without any appreciable release of the mitochondrial matrix enzyme, glutamate dehydrogenase (EC 1.4.1.3). Lactate dehydrogenase release was 61 % and glutamate dehydrogenase 1 % of total releasable enzyme (estimated by incubation with Triton X-100, 0.1 % w/v). 2.5×10^8 platelets were added to the incubation medium (final volume 1 ml) containing 50 mM mannitol, 25 mM HEPES, 0.2 mM EGTA, 75 mM KCl, pH 7.0, 5 mM dithiothreitol,

2 mM KCN, 0.25 mM NaH_2PO_4 , 2.6 mg fatty acid-free bovine serum albumin, 60 μM palmitoyl-CoA and 0.4 mM l-carnitine. The platelets were preincubated for 15 min at 37 °C with inhibitors, or control vehicle, with the exception of malonyl-CoA (or its control) which was preincubated with the cells for 5 min only. The reaction was started by the addition of saponin and continued for 5 min, after which the reaction was stopped by the addition of 0.1 ml of concentrated HCl and tubes placed on ice. Blanks were identical except that concentrated HCl was added at the start of incubation. Preliminary experiments indicated that the reaction was linear up to 1) platelet concentrations of 5×10^8 platelets/ml, 2) a palmitoyl-CoA concentration of 100 μM , and 3) 15 min incubation. At the end of incubation, samples were diluted from 1 ml to 4 ml with distilled water and the product, [^3H] palmitoylcarnitine, was extracted with 2 ml n-butanol as described by Kiorpes et al (Kiorpes, et al. 1984).

2.2.10 Chemiluminescence assay of Superoxide (O_2^-)

Detection of O_2^- in whole blood was performed utilising a chemiluminescence technique (Gyllenhammar. 1987), with lucigenin as a probe for O_2^- . Blood samples were diluted two-fold with 0.9% saline (final volume 1 ml) and prewarmed for 5 min at 37°C before the addition of lucigenin (final concentration 125 μM). Chemiluminescence was monitored using a photoluminometer component of the dual channel lumi-aggregometer (Model 560CA, Chrono-Log). Intensity of lucigenin chemiluminescence was expressed in millivolts (mV). Specificity of the O_2^- detection was verified with superoxide dismutase; addition of superoxide dismutase (300 U/ml)

instantly cancelled the lucigenin signal. Coefficient of variation for lucigenin chemiluminescence was <15%.

2.3 Data Analysis

Data were processed using Microsoft Excel 5.0 and Prism v2.01 software. In experiments where the influence of anti-aggregatory agents were examined, inhibition of aggregation was evaluated as a percentage of maximal aggregation achieved in the absence of the inhibitor.

Data were expressed throughout as mean $\pm$ SEM (unless otherwise indicated). P values of less than 0.05 are considered statistically significant. Details of data analysis are described in each Chapter.

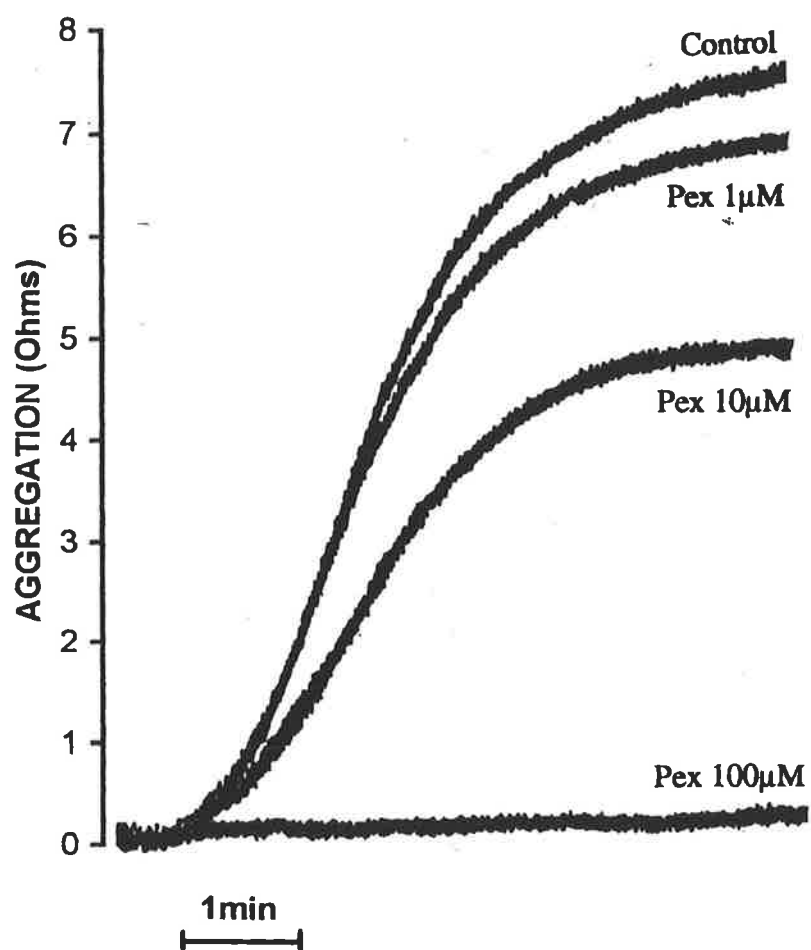


Figure 2.1.

A representative aggregogram demonstrating inhibition of aggregation.

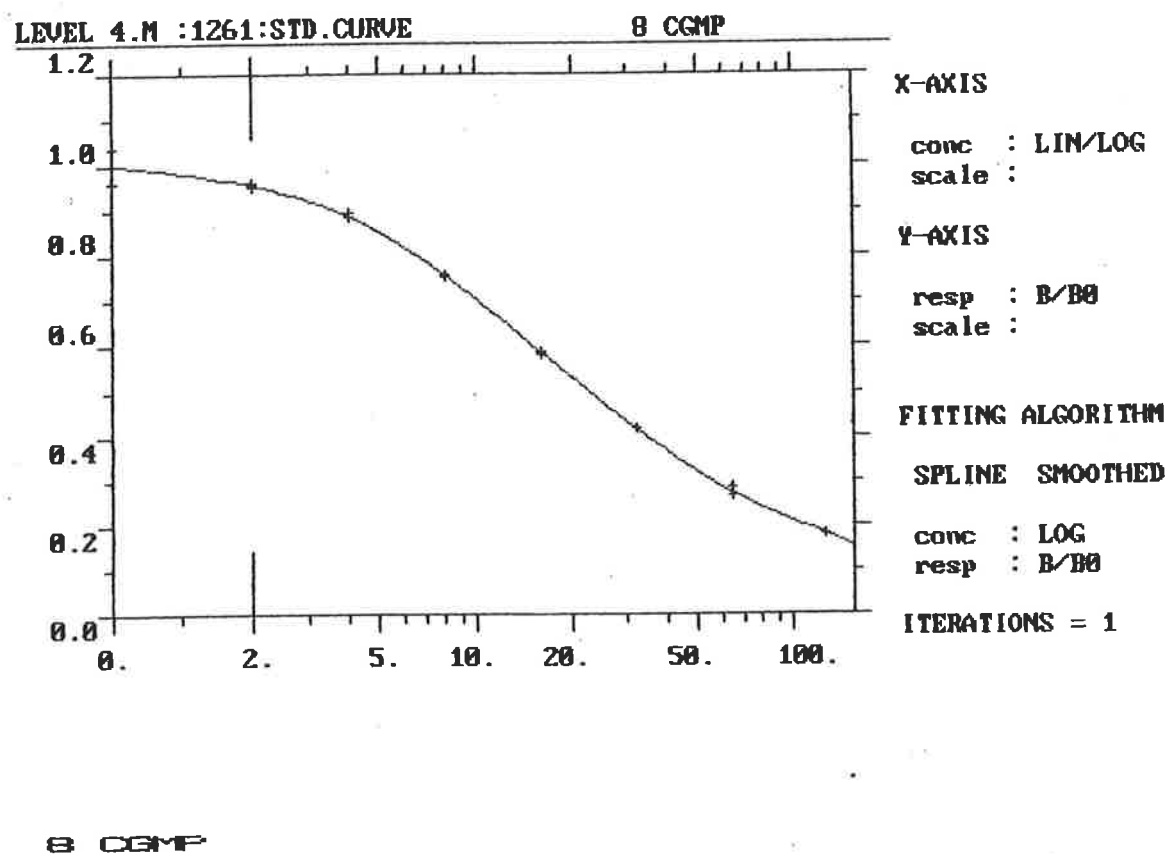


Figure 2.2.

A representative standard curve for cGMP assay.

STANDARD CURVE

(LOG) L/L_{max}	[Ca++] Std. Curve	Anti-Log	$\mu\text{M Ca}$	(LOG) L/L_{max}	[Ca++] Std. Curve	Anti-Log	$\mu\text{M Ca}$
-0.50	-4.35	44.6 x 10 ⁻⁶ M	44.67	-2.40	-5.40	3.16 x 10 ⁻⁶ M	3.98
-0.55	-4.40		39.81	-2.45	-5.45		3.54
-0.60	-4.45		35.48	-2.50	-5.45		3.54
-0.65	-4.50		31.62	-2.55	-5.50		3.26
-0.70	-4.50		31.62	-2.60	-5.50		3.16
-0.75	-4.55		28.18	-2.65	-5.50		3.16
-0.80	-4.60		25.12	-2.70	-5.55		2.82
-0.85	-4.65		22.39	-2.75	-5.55		2.82
-0.90	-4.65		22.39	-2.80	-5.60		2.51
-0.95	-4.70		19.95	-2.90	-5.65		2.24
-1.0	-4.70		19.95	-3.00	-5.70		2.00
-1.05	-4.75		17.78	-3.05	-5.70		2.00
-1.10	-4.75		17.78	-3.10	-5.70		2.00
-1.15	-4.80		15.84	-3.15	-5.70		2.00
-1.20	-4.85		14.13	-3.20	-5.75		1.78
-1.25	-4.85	8.91 x 10 ⁻⁶ M	14.13	-3.25	-5.75		1.78
-1.30	-4.90		12.59	-3.30	-5.75		1.78
-1.35	-4.95		11.22	-3.35	-5.80		1.58
-1.40	-4.95		11.22	-3.40	-5.80		1.58
-1.45	-5.00		10.00	-3.45	-5.85		1.41
-1.50	-5.05		8.91	-3.50	-5.85		1.41
-1.55	-5.05		8.91	-3.55	-5.85		1.41
-1.60	-5.10		7.94	-3.60	-5.90		1.26
-1.65	-5.10		7.94	-3.65	-5.90		1.26
-1.70	-5.10		7.94	-3.70	-5.90		1.26
-1.75	-5.15		7.08	-3.75	-5.95		1.12
-1.80	-5.15		7.08	-3.80	-5.95		1.12
-1.85	-5.20		6.31	-3.85	-6.00		1.00
-1.90	-5.20		6.31	-3.90	-6.00		1.00
-1.95	-5.70		6.31	-3.95	-6.05		0.89
-2.00	-5.25		5.62	-4.00	-6.05		0.89
-2.05	-5.25		5.62				
-2.10	-5.30		5.01				
-2.15	-5.30		5.01				
-2.20	-5.35		4.47				
-2.25	-5.35		4.47				
-2.30	-5.35		4.47				
-2.35	-5.40		3.98				

Figure 2.3.

Intraplatelet Calcium standard curve.

Chapter 3

MULTIPLE AGONIST INDUCTION OF AGGREGATION: IMPLICATIONS REGARDING PLATELET REACTIVITY TO ANTI- AGGREGATORY AGENTS.

3 Chapter 3: Multiple Agonist Induction of Aggregation: Implications Regarding Platelet Reactivity to Anti-Aggregatory Agents.

3.1 Summary

Platelet aggregation has traditionally been investigated in vitro utilising optical aggregometry and single agonists in supraphysiological concentration. However, in vivo platelets are exposed to more than one agonist at any one time. Hence the primary objective of this study was to develop a more physiologically appropriate method of in vitro induction of aggregation. This was achieved in whole blood samples from patients by utilising ADP in combination with subthreshold, physiologically relevant concentrations of adrenaline, serotonin and thrombin (multiple agonist approach). When aggregation was induced by multiple agonists [ADP (0.1-0.5 μ M), adrenaline (1 nM), serotonin (1 nM) and thrombin (0.005 U/ml)] an increase in extent of aggregation was observed relative to ADP alone: $280 \pm 30\%$ of ADP-alone aggregation ($p < 0.01$).

The multiple agonist approach was subsequently used to investigate effects of various anti-aggregatory agents. In blood samples from the patients, verapamil (an L-type calcium channel blocker), nitroglycerine (NTG, a stimulator of cGMP formation) and prostaglandin E_1 (PGE_1 , a stimulator of cAMP formation) inhibited platelet aggregation in vitro. With multiple agonists, the anti-aggregatory effects of NTG and PGE_1 were significantly increased in comparison with ADP alone. For example, inhibition of aggregation with 100 μ M NTG increased from $37 \pm 5\%$ with ADP alone to $86 \pm 13\%$

($p < 0.01$) with multiple agonists. Threshold effects of NTG were seen at 1 μM with ADP alone and 0.1 μM with multiple agonists; while threshold for PGE_1 was reduced from 0.1 to 0.01 nM. However, responses to verapamil were unchanged by multiple agonists, demonstrating that the potentiation of anti-aggregating effects utilising the multiple agonist approach is not a non-specific phenomenon.

The ability of the multiple agonist approach to enhance the anti-aggregating effects of some agents such as NTG and PGE_1 provides an in vitro experimental method mimicking the in vivo situation.

3.2 Introduction

As discussed in Chapter 1 the main physiological task of platelets within the circulation is to arrest the loss of blood when a blood vessel is damaged. This involves rapid adhesion of the platelets to the exposed subendothelium followed by platelet to platelet adherence (aggregation) which culminates in the formation of a platelet plug that temporally seals off the damaged vessel wall. In the pathological state of thrombosis, platelet plugs are formed in arterioles which may arrest the blood supply to nearby tissues thus causing local ischaemia. Platelet hyperaggregability has been documented in a number of cardiovascular disease states and has been proposed as a factor predisposing towards thrombotic events (Maseri. 1990; Schrader and Berk. 1990). To date, the development of clinically relevant strategies for limiting platelet aggregation has been hampered by the lack of appropriate in vitro models of aggregation.

Platelet aggregation can be studied in vitro, using different inductors of aggregation including ADP, adrenaline, serotonin, thrombin and vasopressin, usually in concentrations exceeding the physiological range (Grant and Scrutton. 1980; Owen and Le Breton. 1980; Huang and Detwiler. 1981; DiMinno, et al. 1982; Lanza, et al. 1986; Carty, et al. 1988; De Clerck. 1988; Olbrich, et al. 1989; Vanags, et al. 1992). However, in vivo platelets are exposed to more than one agonist at any one time; pro-aggregants can act together to exert either normal physiological action (in haemostasis) or pathological effects (in thrombosis and atherosclerosis) (Alarayyed, et al. 1995). Previous in vitro experiments have shown that the platelet response to a subthreshold

concentration of one agonist can be potentiated by the addition of a subthreshold concentration of another agonist (O'Brien. 1964; Ardlie, et al. 1966; Michal and Motamed. 1976; Grant and Scrutton. 1980; Huang and Detwiler. 1981; Alarayyed, et al. 1995). This phenomenon is of great interest, since usage of low concentrations of agonists in vitro experiments may mimic the physiological conditions under which aggregation occurs in vivo. Furthermore, McAuliffe et al showed that serotonin also potentiates adrenaline-induced thrombus formation in vivo (McAuliffe, et al. 1993).

The above studies have concentrated on pairs of agonists. Only one study (Bushfield, et al. 1986) so far has investigated the response towards multiple (more than two) agonists; this study suggested potentiated aggregation with a combination of adrenaline, ADP, platelet activating factor and vasopressin. Unfortunately this study was based on an unconventional, indirect method for the registration of platelet aggregation (disappearance of single platelets), and the agonists tested were used in high, supra-physiological concentrations.

All other previously reported studies involving paired agonists, have used optical (turbidometric) techniques to assess platelet aggregation. Optical aggregometry requires the preparation of platelet rich-plasma which lacks red and white blood cells and some of the heavier platelets. These blood components are important modulators of platelet function in vivo, since they actively take up and release adenine nucleotides and platelet-active agents, such as serotonin and prostanoids (Holmsen. 1994). Therefore a

theoretically preferable in vitro technique would not involve platelet-rich plasma. Thus whole blood aggregometry was employed in the current study.

With the advent of anti-platelet therapy, which has become a useful means for preventing acute thrombotic arterial occlusion in cardiovascular diseases, there is a need to search for effective drugs (for review see Schror. 1995). Aspirin, a widely used antiplatelet agent, is currently generally regarded as the drug of choice for the long term prophylaxis of recurrent thrombotic events in patients with ischaemic syndromes. Aspirin is an irreversible inhibitor of platelet cyclo-oxygenase, causes long-lasting inhibition of platelet function and platelet thromboxane formation and is associated with a low incidence of adverse effects. However, aspirin is of limited efficacy in the short-term management of states of acute platelet aggregation, such as unstable angina pectoris (Theroux, et al. 1988).

Nitroglycerine (NTG), which was once thought to be only a vasodilator, also affects platelets, suppressing platelet aggregation (for review see Anderson, et al. 1994). NTG both inhibits (Loscalzo. 1985) and reverses (Chirkov, et al. 1992) in vitro aggregation in platelet-rich plasma. Diodati et al. investigated ex vivo effects of NTG on platelet aggregation using whole blood aggregometry and found that NTG significantly inhibited subsequent platelet aggregation caused by ADP or thrombin (Diodati, et al. 1995). These findings, together with the elegant in vivo experiments of Lam et al in an animal model, established that NTG exerts significant antiplatelet effects (Lam, et al. 1988). The major limitation to the efficacy of NTG in the management of cardiovascular

diseases is development of "nitrate tolerance", which has been shown with haemodynamic effects (for review see Mangione and Glasser. 1994) and with platelets (Chirkov, et al. 1997).

Verapamil, an L-type calcium channel blocker, has previously been shown to inhibit platelet aggregation in vitro (Ono and Kimura. 1981; Jones, et al. 1985; Strano, et al. 1985). The mechanism of the anti-aggregatory effect of verapamil and other calcium channel blockers is thought to be the calcium antagonistic effects on the platelet membrane (Ono and Kimura. 1981). However, detailed mechanistic studies have not been performed to date.

Prostaglandin E_1 (PGE_1) is a cyclooxygenase product which inhibits the adherence of platelets to foreign surfaces and damaged endothelium (Kerins, et al. 1991). Prostaglandin E_1 has previously been shown to inhibit in vitro platelet aggregation by increasing cyclic AMP levels through the activation of adenylate cyclase. Chirkov et al investigated the anti-aggregatory effect of PGE_1 (as a probe for the cAMP system) in normal subjects and patients with stable angina pectoris (Chirkov, et al. 1995). Stable angina patients demonstrated impaired responses to PGE_1 . However this did not correlate with the increased extent of aggregation in these patients. Therefore the authors concluded that the cyclic AMP-mediated pathway was not the only mechanism responsible for the abnormal platelet aggregability in patients with stable angina.

3.3 Objectives of the study

The main objective of the work described in the chapter was to test the hypothesis that utilisation of multiple agonists (ADP, adrenaline, serotonin and thrombin) in physiological concentrations rather than a single agonist (ADP) in high concentration, leads to potentiation of aggregation. Furthermore, the study set out to examine the platelet responsiveness of anti-aggregating agent under conditions of single and multiple agonist-induced aggregation. For the latter purpose, NTG, PGE₁ and verapamil were utilised as inhibitors of platelet aggregation.

3.4 Experimental protocol

3.4.1 Development of the multiple agonist model

3.4.1.1 Patients Studied

Subjects studied were patients (n=27) undergoing routine diagnostic coronary angiography for investigation of stable angina pectoris. Patients were of both sexes (16 men and 11 women); age range from 43 to 77. Nineteen patients were receiving low dose aspirin. No other patient had taken any medication known to affect platelet aggregation during 2 weeks prior to study.

3.4.1.2 Blood Sampling

Blood samples (20 ml) were collected as described in Chapter 2. The time interval between collection of blood samples and platelet aggregation studies was 5-10 min in all experiments.

3.4.1.3 Platelet Aggregation studies

Platelet aggregation responses towards ADP (0.05-0.5 μ M), thrombin (0.006-0.01 U/ml), serotonin (10 nM to 10 mM) and adrenaline (0.1-1 μ M) were determined. The following pairs of agonists were investigated for potentiation of ADP-induced aggregation:- ADP + thrombin, ADP + serotonin and ADP + adrenaline. As platelets *in vivo* are exposed to more than two agonists at any one time (Alarayyed, et al. 1995), platelet responsiveness towards all four agonists was determined. All four agonists were added simultaneously and the following concentrations were examined for the extent of potentiation of ADP-alone induced aggregation; ADP (0.1-0.5 μ M), adrenaline (1-10 nM), serotonin (1-300 nM) and thrombin (0.005-0.01 U/ml).

3.4.2 Utilisation of the multiple agonist model

3.4.2.1 Patients Studied

Subjects studied (n=27) included cardiac patients undergoing routine diagnostic coronary angiography for investigation of stable angina pectoris. Patients were of both sexes (18 men and 9 women); age range from 44 to 76. Fourteen patients were

receiving low dose aspirin, no other patient had taken any medication known to affect platelet aggregation during 2 weeks prior to study. A cohort of normal subjects (4 men and 1 women) aged 22 to 71 were also studied.

3.4.2.2 Platelet aggregation studies

In experiments where ADP was used as a single pro-aggregate, ADP was utilised in concentrations (1-2 μ M) causing approximately a 7 Ohm response (see insert: Figure 3.5).

For multiple agonist studies, ADP concentrations (0.1 to 0.5 μ M) were chosen on the basis of a response just above threshold (in this case, approximately 2 Ohms). Platelet responses towards adrenaline, serotonin and thrombin were determined and subthreshold concentrations of these three pro-aggregants (adrenaline 1 nM, serotonin 1 nM and thrombin 0.005 U/ml) were utilised in multiple agonist studies. These concentrations fall within the range of concentrations for each agonist previously reported to exist within human plasma (Jabs, et al. 1978; Hitomi, et al. 1982; Siess. 1989; Coffman and Cohen. 1994). Adrenaline, serotonin and thrombin used individually or in combination (Figure 3.5) at these concentrations induced no detectable aggregation. However, when all four agonists were added simultaneously the resultant aggregation producing approximately a 7 Ohm response.

A cohort of normal subjects (n=5) was also examined to determine the degree of potentiation of ADP-induced aggregation.

3.4.2.3 *Inhibition of Aggregation*

Blood samples were preincubated with verapamil [5 min], NTG and PGE₁ [1 min] prior to induction of aggregation. Stock solutions of verapamil were made in physiological saline and subsequent dilution's were added to blood samples. NTG and PGE₁ were diluted from initial ethanol solutions using physiological (0.9 % NaCl) saline. Control studies with matched concentrations of ethanol showed no effect on platelet aggregation.

Inhibition of subsequent aggregation was evaluated as a percentage of maximal aggregation achieved in the absence of either agent.

3.5 Data Analysis

Potentiation of ADP-induced aggregation by adrenaline, serotonin and thrombin was calculated as increase in extent of aggregation, compared to that with ADP alone in the same concentration. Inhibition of aggregation by NTG, PGE₁ and verapamil was evaluated as percentage of maximal aggregation achieved in the absence of either agent.

Statistical assessment of the effects on potentiation of platelet aggregation was made utilising the paired *t* - test. Assessment of individual points on concentration-response curves was performed utilising Dunnett's *t* - test. Statistical significance was limited to $p < 0.05$. Results are expressed as mean $\pm$ SEM.

3.6 Results

3.6.1 Development of the multiple agonist model

3.6.1.1 Paired agonists

The potentiation of ADP-induced aggregation by another agonist (adrenaline, serotonin, thrombin etc) is a widely recognised phenomenon in platelet-rich plasma (O'Brien. 1964; Ardlie, et al. 1966; Michal and Motamed. 1976; Grant and Scrutton. 1980; Huang and Detwiler. 1981; Alarayyed, et al. 1995). However, potentiation of ADP-induced aggregation utilising whole blood aggregometry has received very little attention. Only one study (Boyd and Davis. 1988) has demonstrated the potentiation of aggregation by ADP and adrenaline. Boyd and Davis also reported that threshold levels of aggregation occur at lower concentrations in whole blood when compared to platelet-rich plasma, the authors concluded that whole blood provide a sensitive system with a more physiological environment than other in vitro systems (Boyd and Davis. 1988). Therefore, the following experiments were conducted to demonstrate that potentiation of ADP-induced aggregation occurs when aggregometry is performed in whole blood.

Potentialiation of aggregation was examined utilising ADP in combination with either adrenaline, serotonin or thrombin. Potentialiation of aggregation was defined as the extent of aggregation which is greater than the addition of the individual extents of aggregation produced by each agonist (Ardlie, et al. 1966; Alarayyed, et al. 1995).

When thrombin (0.006-0.01 U/ml, n=5) was added to whole blood simultaneously with ADP (0.04-0.1 μ M) the resultant aggregation was 411 ± 148 % of ADP alone aggregation (Figure 3.1). This value was approximately 2 fold the response when the individual extents of aggregation for ADP and thrombin are added together ($p=0.051$). The above results demonstrate that thrombin in the concentrations utilised has the ability to potentiate the aggregation induced by low concentrations of ADP.

Similarly, serotonin (1-10 μ M, n=2) or adrenaline (0.1-1 μ M, n=2) added to ADP (0.05 to 0.5 μ M) produced resultant aggregation of 246 ± 47 (Figure 3.2) and 415 ± 108 % (Figure 3.3) of ADP alone aggregation, respectively. These values were greater than the addition of the individual extents of aggregation for each agonist, thus demonstrating potentiation of ADP-induced aggregation by serotonin and adrenaline.

From the above results it has been established that these agonists in pairs produced significant potentiation of ADP-induced aggregation in whole blood. These results are consistent with previous observations regarding pairs of agonists (O'Brien. 1964; Ardlie, et al. 1966; Bushfield, et al. 1986; Bushfield, et al. 1987; Alarayyed, et al. 1995). However, the concentrations utilised for adrenaline, serotonin and thrombin are well

above threshold concentrations for each agonist, as demonstrated by the individual response of each agonist on their respective figures.

In an attempt to produce a more physiological model of aggregation adrenaline, serotonin and thrombin were added simultaneously with ADP and the extent of potentiation of ADP-induced aggregation was determined. The concentrations of adrenaline (1-10 nM), serotonin (1-30 nM), thrombin (0.01-0.005 U/ml) and ADP (0.1-0.5 μ M) utilised for these experiments ranged from previously reported sub-threshold concentrations to their physiological concentration (Hitomi, et al. 1982; Siess. 1989; Coffman and Cohen. 1994).

Marked potentiation of ADP-induced aggregation by the combination of adrenaline, serotonin and thrombin is demonstrated in Figure 3.4. Adrenaline, serotonin and thrombin did not produced aggregation individually or in combination at any of the agonist concentrations examined. The three combinations of agonist concentrations illustrated in Figure 3.3 indicated that potentiation occurred to a similar extent in each group (Table 1). As the study was designed to investigate the potentiation of aggregation by physiologically relevant concentrations of agonists, the combination of ADP (0.1-0.5 μ M), adrenaline (1 nM), serotonin (1 nM) and thrombin (0.005 U/ml) was chosen for subsequent experiments.

3.6.2 Utilisation of the multiple agonist model

3.6.2.1 *Potentiation of ADP-induced Aggregation*

Platelet aggregation was studied in whole blood obtained from patients with suspected stable angina pectoris. Both males and females were used in the study, although differences between males and females have previously been reported (Meade, et al. 1985), with platelet aggregation occurring to a greater extent in females than in males for an equivalent ADP concentration. However, in the present study, no differences between sexes in reference to responsiveness to ADP nor extent of the potentiation of ADP-induced aggregation or the inhibition of aggregation by verapamil, NTG or PGE₁ were observed. Therefore data obtained from both sexes were pooled.

The interaction between low concentrations of adrenaline, serotonin, thrombin and responsiveness to ADP is illustrated in Figure 3.5. With ADP alone, aggregation was concentration-dependent: ADP 0.2 μ M and 2 μ M produced a 2.5 and 7.5 Ohm response respectively. Adrenaline (1 nM), serotonin (1 nM) and thrombin (0.005 U/ml), which induced no detectable aggregation response alone or in combination, were utilised for potentiation experiments.

Whole blood from patients (n=27) exhibited increased extent of aggregation with ADP (0.1-0.5 μ M) plus adrenaline (1 nM), serotonin (1 nM) and thrombin (0.005 U/ml) compared with the aggregation produced by ADP alone in an equivalent concentration (Figure 3.6). There was a mean increase in aggregation of $280 \pm 30\%$ with multiple

agonists from ADP alone ($p < 0.01$). Adrenaline, serotonin and thrombin when added simultaneously to whole blood produced no detectable aggregation.

The above-described response of multiple agonists was also shown to occur in normal subjects ($n=5$); increase from ADP control aggregation was 258 ± 29 % ($p < 0.01$). Thus, there was no difference between patients and normal subjects with respect to the potentiation of ADP induced aggregation (Figure 3.6), demonstrating that the potentiation of ADP induced aggregation by adrenaline, serotonin and thrombin is a general phenomenon, which occurs irrespective of the subject population studied.

3.6.2.2 Anti-aggregatory Effects of Verapamil

Verapamil added to whole blood before induction of aggregation by ADP or multiple agonists caused inhibition of aggregation in a concentration-dependent manner. No difference was observed in the response of verapamil towards ADP alone or multiple agonists (Figure 3.7). Statistically significant suppression of aggregation was observed with verapamil concentrations > 0.2 nM; the concentration associated with 50 % inhibition of aggregation (C_{50}) was 100 μ M and total inhibition of aggregation occurred at 300 μ M verapamil.

3.6.2.3 Anti-aggregatory Effects of Nitroglycerine

When NTG was added to blood samples before induction of aggregation with ADP, it produced significant inhibition of platelet aggregation. 100 μ M NTG produced a 37 ± 5

% inhibition of aggregation, threshold effect being seen with 1 μ M (Figure 3.8). When aggregation was induced by multiple agonists, the concentration-response curve for the anti-aggregating effects of NTG was shifted to the left. 100 μ M NTG produced 86 ± 13 % inhibition of aggregation ($p < 0.01$ vs ADP-alone), threshold effects were detected at 0.1 μ M NTG.

3.6.2.4 Anti-aggregatory Effects of Prostaglandin E₁

PGE₁ added before induction of aggregation by ADP caused inhibition of aggregation in a concentration-dependent manner. Total inhibition of aggregation occurred at 0.1 μ M PGE₁; threshold concentration for inhibition of aggregation was 0.1 nM (Figure 3.9). C₅₀ for PGE₁ was 20 nM.

When aggregation was induced by multiple agonists, the slope of the concentration-response curve for the anti-aggregating effects of PGE₁ was shifted to the left. Total inhibition of aggregation occurred at 0.1 μ M, as with ADP alone. However, threshold concentration for inhibition of aggregation decreased from 0.1 nM with ADP alone to 0.01 nM. C₅₀ decreased from 20 to 3 nM ($p < 0.01$ vs ADP alone).

3.7 Discussion

The two major findings of this study are: (1) adrenaline, serotonin and thrombin in subthreshold concentrations potentiate platelet aggregation responses to ADP, and (2)

aggregation induced by this combination of agonists is more sensitive to inhibition by NTG (cGMP stimulator) and PGE₁ (cAMP stimulator), but not by verapamil (an L-type Ca²⁺ channel blocker), than that induced by ADP alone.

The observed potentiation of ADP responses by the combination of adrenaline, serotonin and thrombin observed in the current study was not unexpected. Previously it has been reported that agonists in concentrations too low to cause aggregation individually enhance the platelet response to each other (Ardlie, et al. 1966; O'Brien. 1964; Huang and Detwiler. 1981; Bushfield, et al. 1986; Alarayyed, et al. 1995). Experiments conducted in platelet-rich plasma utilising optical aggregometry, undertaken with the objective of investigating potentiation of aggregation response to pairs of agonists have generally utilised agonist concentrations exceeding the physiological range of concentrations for each agonist (O'Brien. 1964; Ardlie, et al. 1966; Michal and Motamed. 1976; Grant and Scrutton. 1980; Huang and Detwiler. 1981). Therefore, the use of paired agonists to examine antiplatelet effects of drugs has received little attention as an in vitro model of physiological aggregation. Only one study (Bushfield, et al. 1986) so far has investigated the response towards multiple agonists; this study suggested potentiated aggregation with a combination of adrenaline, ADP, platelet activating factor and vasopressin. Unfortunately this study used agonists in supra-physiological concentrations.

In the present study, aggregation was induced at physiologically relevant concentrations of the agonists. Aggregation is a complex process involving a vast number of

mechanisms. Potentiation of the platelet response to the combination of inducers of aggregation, documented is likely to reflect the activation of several biochemical pathways, independently leading to aggregation as a final, integrated physiological effect. The biochemical mechanism of potentiation was not directly addressed in the current study but remains of potential interest, particularly in view of the results of the experiments with inhibitors of aggregation (see below).

This study is the first to demonstrate that sensitivity to some inhibitors of aggregation is increased when aggregation is induced by multiple, as distinct from single, agonists. There is considerable clinical evidence to support the concept that some calcium channel antagonists, such as verapamil, may exert anti-aggregatory effects. For example, verapamil in therapeutic concentration of 0.1 to 0.4 μM (Powell, et al. 1988) has been shown to suppress symptoms in patients with unstable angina pectoris (Opie. 1996), and to reduced the risk of re-infarction after initially small myocardial infarction (Hansen. 1991).

Verapamil has previously been shown to inhibit platelet aggregation in vitro (Ono and Kimura. 1981; Jones, et al. 1985; Strano, et al. 1985). Inhibition of aggregation by verapamil has only been documented at concentrations, which are well above those estimated after in vivo administration (Jones, et al. 1985; Beaughard, et al. 1995). In the current study, the inhibitory effect of verapamil was unchanged when aggregation was induced by the "multiple agonist" approach (Figure 3.7).

NTG, which was once thought to be only a vasodilator, also affects platelets, suppressing platelet aggregation (Loscalzo. 1985; Lam, et al. 1988; Chirkov, et al. 1992; Anderson, et al. 1994; Diodati, et al. 1995;). Inhibition of platelet aggregation by NTG in vitro occurs only in high concentrations (Mehta and Mehta. 1980; Fitzgerald, et al. 1984; Schrader and Berk. 1990; Karlberg, et al. 1992). The physiological action of NTG is related to its thiol-mediated bioconversion to nitric oxide which stimulates soluble guanylate cyclase, with a resultant increase in intracellular concentrations of cGMP, which is responsible for both the vasodilatory (Anderson, et al. 1994) and antiplatelet effects (Chirkov, et al. 1993; Moncada and Higgs. 1993). In the current study, when NTG was tested in the presence of a single inductor of aggregation, inhibition of aggregation was observed with NTG concentrations above those achievable therapeutically (Figure 3.8). When aggregation was induced by multiple agonists, effects of NTG were more marked; the NTG concentration-response curve was shifted 1 log unit towards lower concentrations (Figure 3.8). Thus, the multiple agonist approach is a more sensitive method for evaluation of anti-aggregating effects of NTG. On the other hand, it has previously been demonstrated that the apparent lack of responsiveness of platelets to nitric oxide (NO) donors as inhibitors of aggregation is that platelet soluble guanylate sensitivity to these agents fluctuates, reaching a transient peak just after the onset of aggregation (Chirkov, et al. 1991). It remains to be determined whether the use of multiple agonists is associated with a further increase in platelet responsiveness to NTG in a model of reversal (Chirkov, et al. 1992) rather than inhibition, of aggregation.

PGE₁ is a cyclooxygenase product which inhibits platelet aggregation by increasing cyclic AMP levels through activation of adenylate cyclase (Kerins, et al. 1991). Many studies have investigated the inhibitory effect of PGE₁ on platelets (Wilsoncroft, et al. 1985; Fisher, et al. 1987; Kaiya. 1991; Katzenschlager, et al. 1991; Kahn, et al. 1992; Fisch, et al. 1995). PGE₁ has previously never been investigated utilising a multiple agonist approach to induction of aggregation. In the current study, PGE₁ exhibited significant antiplatelet effects at extremely low concentrations. When aggregation was induced by ADP alone, inhibition of aggregation by PGE₁ occurred with a threshold of effect at 0.1 nM (Figure 3.9). With the multiple agonist approach, PGE₁ produced significant antiplatelet effect at concentrations described by a leftwards shift in the slope of the concentration-response curve. This produced a threshold of PGE₁ effects at >0.01 nM (Figure 3.9).

The results of this study therefore demonstrate that “potentiated” ADP-induced aggregation represents a basis for increased platelet responsiveness to inhibitors of aggregation. Potentiation of the platelet response to the combination of inductors of aggregation is likely to reflect the involvement of several biochemical pathways, independently leading to aggregation as a final, integrated physiological effect. Inductors of aggregation induce their effects via specific receptors coupled with G-proteins (Brass, et al. 1993). Different subgroups of G-proteins can be involved in the transduction of the signal from agonist-activated receptors to intraplatelet effector systems to physiological response (Siess. 1989; Brass, et al. 1993).

For example, stimulatory G-protein (G_s -proteins) which activate adenylate cyclase with a subsequent rise in cAMP, can be coupled to β -adrenoceptors, prostacyclin and adenosine receptors. Inhibitory G-proteins (G_i -proteins) are coupled to α -adrenoceptors and mediate attenuation of adenylate cyclase with a subsequent decrease in cAMP production. Phospholipid related G-proteins (G_p -proteins), coupled to thrombin, ADP and serotonin receptors, are involved in the activation of phospholipase C (PL-C) which generates inositol 1,4,5-triphosphate, a mobiliser of intracellular calcium. Furthermore, some of the thrombin-coupled G_p -proteins activate phospholipase-A₂ (PL-A₂), which releases arachidonic acid (intraplatelet source of thromboxane A₂) (Siess. 1989) and some of the G_i -proteins coupled to α -adrenoceptors also stimulate PL-C. Therefore, when applied simultaneously, ADP, adrenaline, serotonin and thrombin bind to their respective receptors, and presumably activate all the G-protein subgroups. It is speculated that the interplay within the biochemical pathways controlled by these G-proteins may well underlie the observed phenomenon of potentiation. However, the precise mechanism of potentiation remains unclear and was not directly addressed in the current study.

Multiple agonist induced aggregation compared with ADP alone facilitated manifestation of the inhibitory effects of NTG and PGE₁. However, platelet responses to verapamil were unchanged. Although the reason for this distinction has not been delineated, this might imply that verapamil (as an L-type calcium channel blocker) has only one "target" within the biochemical cascade involved in platelet aggregation, this could be calcium -influx, which stimulates calcium release from intraplatelet stores

(Fleckenstein, 1971). Calcium release is a nonspecific response to all pro-aggregants (Siess, 1989) and therefore cannot be potentiated by the combination of agonists. NTG and PGE₁, activating cGMP and cAMP systems, might interfere with different pathways controlled by different G-proteins coupled to the receptors for the agonists used in the multiple agonist approach. For example, formation of cGMP and cAMP leads to: 1) sequestration of intraplatelet calcium, 2) stimulation of calcium efflux, 3) inhibition of glycoprotein IIb/IIIa mediated platelet-platelet contacts, 4) inhibition of PL-C, which generates inositol 1,4,5-trisphosphate, and 5) indirect inhibition of PL-A₂, which releases arachidonic acid (intraplatelet source of thromboxane A₂) (Siess, 1989; Kerins, et al. 1991; Anderson, et al. 1994). All these effects would contribute to inhibition of aggregation. Therefore the involvement of multiple biochemical sites of effect may also imply that NTG and PGE₁ inhibit aggregation via additional mechanisms when aggregation is induced by multiple agonists rather than by ADP alone. Analogously, it is proposed that, unlike NTG and PGE₁, verapamil (as an L-type calcium channel blocker) has only one biochemical "target"; and therefore, cannot be potentiated by the combination of agonists.

Although the experiments described provide data supporting multiple agonist potentiation of ADP-induced aggregation, there are a number of possible limitations of the study. These of course include an absence of biochemical data, which as explained above, are relevant to mechanistic understandings of the phenomena which has been characterised. As regards multiple agonist potentiation of aggregation, it might be suggested that the multiple agonist approach evaluated was only one of a very large

number of possible permutations, and it is therefore eminently possible that a greater degree of potentiation of single agonist effects might have been observed if alternative combinations of agonists were tested. Finally, as regards inhibition of multiple agonist induced aggregation, a significant concern is that responsiveness to NTG, while markedly potentiated, is still not equivalent to that inferable from *ex vivo* studies (Chirkov, et al. 1992; Diodati, et al. 1995).

3.8 Conclusion

The described ability of adrenaline, serotonin and thrombin to potentiate ADP-induced aggregation, and to enhance the anti-aggregating activity of antiplatelet drugs provides an *in vitro* experimental method mimicking the *in vivo* situation. This has possible clinical relevance because we are now equipped with a more physiological model of platelet aggregation.

These results, while of both physiological and clinical interest, do not yet include demonstration of the biochemical mechanisms underlying these phenomena. The elucidation of these mechanisms is an obvious priority for further experiments.

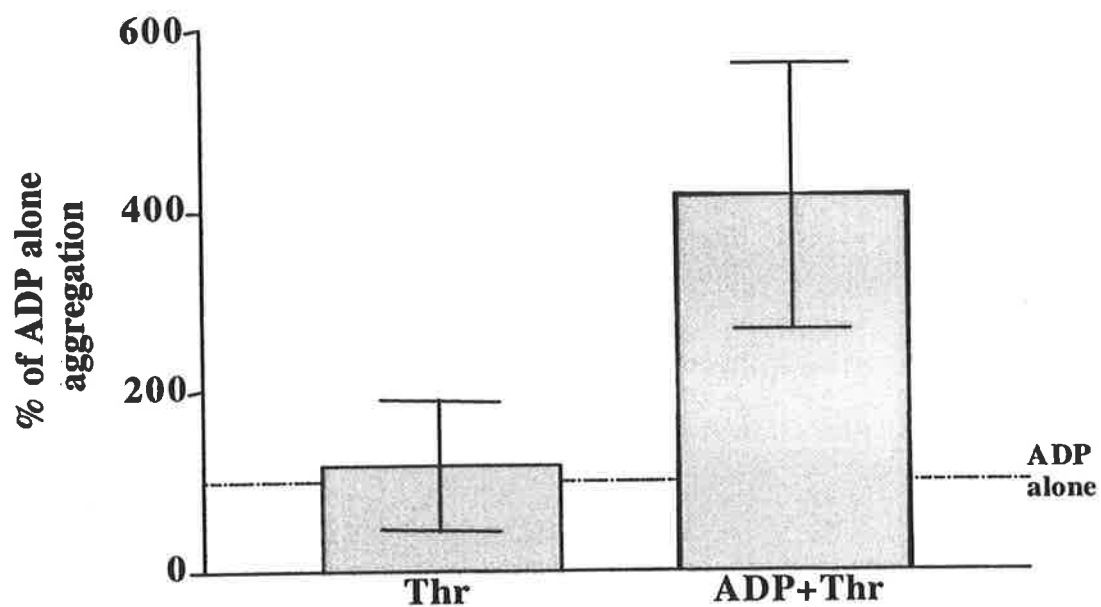


Figure 3.1.

Potentialiation of ADP-induced aggregation by thrombin (Thr). Thrombin (0.01-0.006 U/ml) added simultaneously with ADP (0.04 - 0.1 μ M) produced 411 $\pm$ 148 % of ADP alone aggregation.

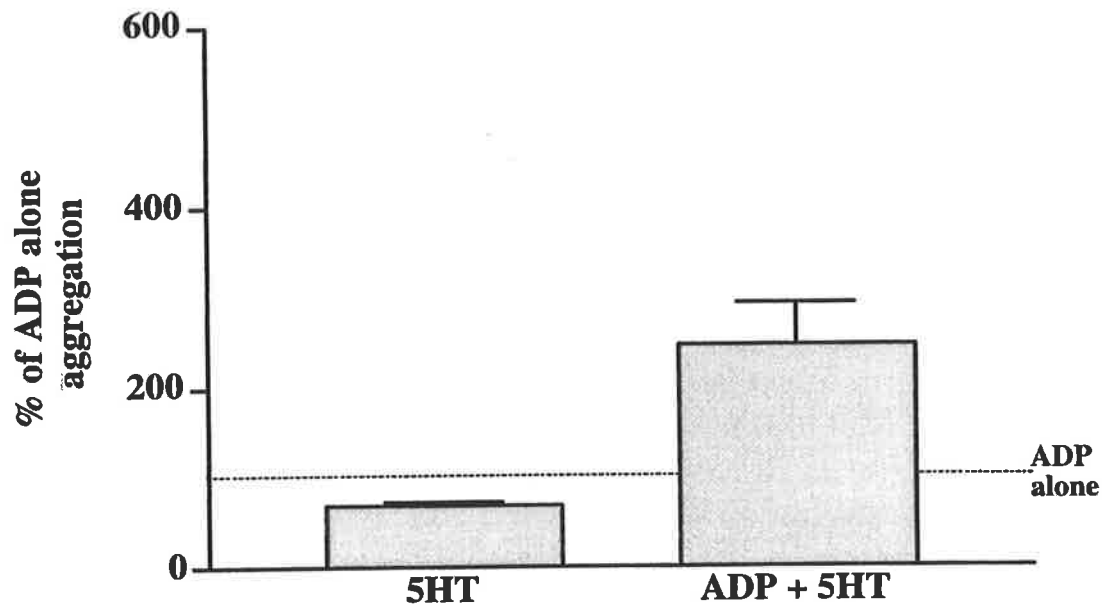


Figure 3.2.

Potentiation of ADP-induced aggregation by serotonin (5HT). Serotonin (1-10 μ M) added with ADP (0.05 - 0.5 μ M) produced 246 $\pm$ 47 % of ADP alone aggregation.

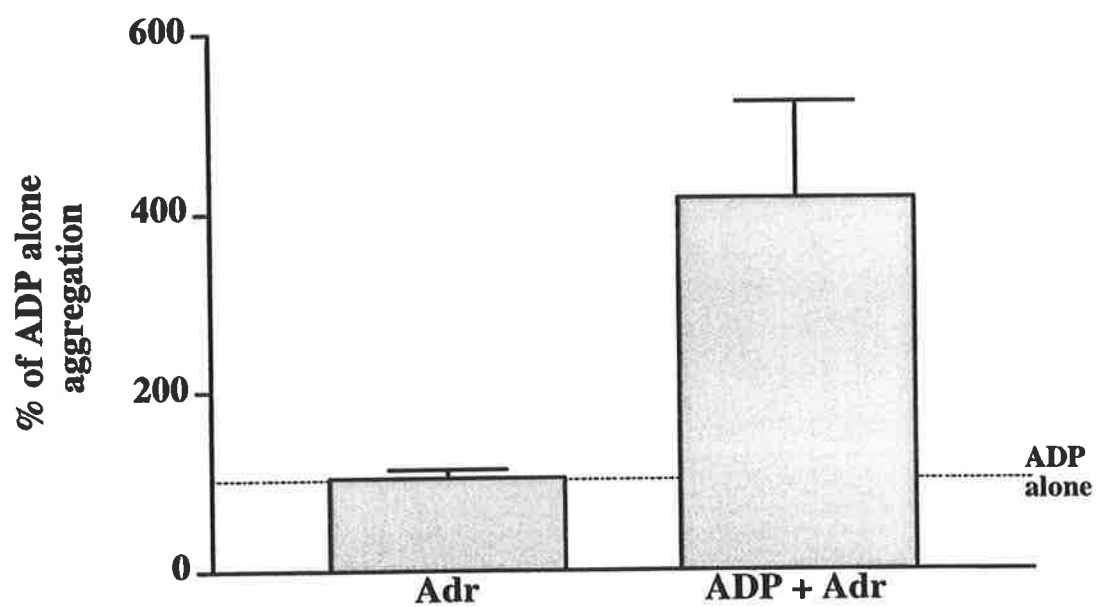


Figure 3.3.

Potentialiation of ADP-induced aggregation by adrenaline (Adr). Adrenaline (0.1-1 μ M) added with ADP (0.05 - 0.5 μ M) produced 415 $\pm$ 108 % of ADP alone aggregation.

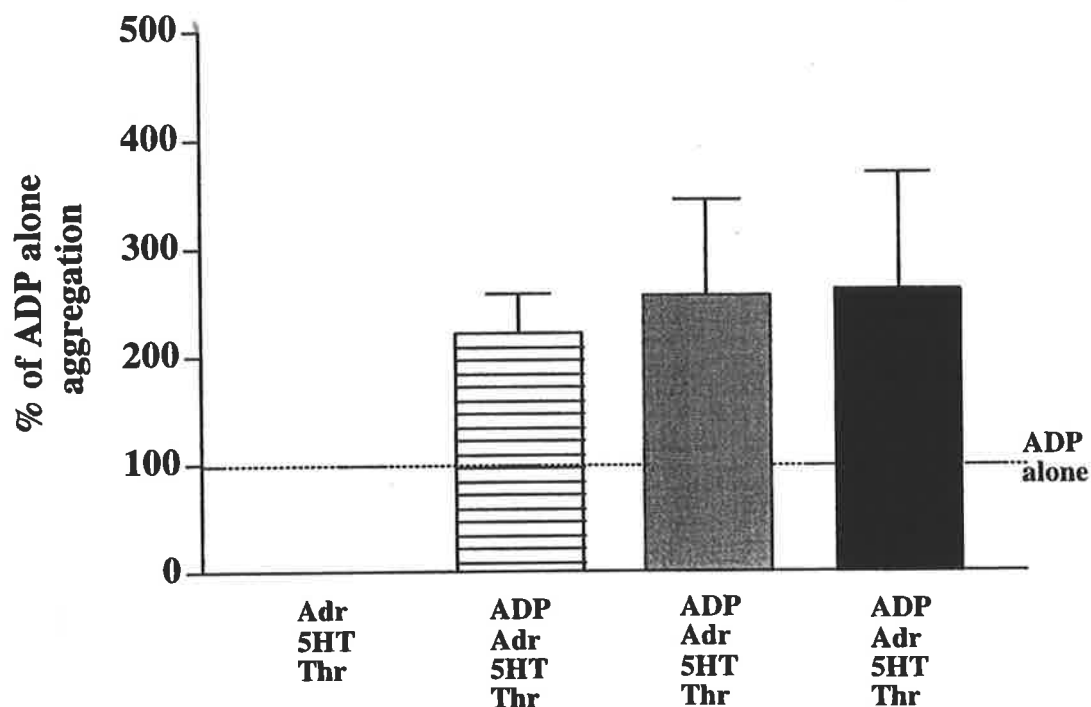


Figure 3.4.

Potentiation of ADP-induced aggregation by adrenaline (Adr), serotonin (5HT) and thrombin (Thr). Adrenaline (1-10 nM), serotonin (1-10 nM) and thrombin (0.01-0.005 U/ml) when added to blood sample in combination did not produced detectable aggregation. Adrenaline (10 nM), serotonin (10 nM) and thrombin (0.01 U/ml) [lined bar] when added simultaneously with ADP (0.1-0.5 μ M) produced 222 ± 36 % of ADP alone aggregation. Adrenaline (1 nM), serotonin (3 nM) and thrombin (0.005 U/ml) [shaded bar] when added to ADP produced 257 ± 88 % of ADP alone aggregation. Adrenaline (1 nM), serotonin (1 nM) and thrombin (0.005 U/ml) [black bar] when added to ADP produced 261 ± 110 % of ADP alone aggregation.

Table 3.1.

Potentialiation of ADP-induced aggregation by adrenaline (Adr), serotonin (5HT) and thrombin (Thr), individually and in combination.

Agonist Concentration				% of ADP alone aggregation
ADP [μ M]	Adr [nM]	5HT [nM]	Thr [U/ml]	
0.05-0.5	-	-	-	100
0.05-0.5	100-1000	-	-	415 $\pm$ 108
0.05-0.5	-	1000-10000	-	246 $\pm$ 47
0.05-0.5	-	-	0.01-0.006	411 $\pm$ 148
-	1-10	1-10	0.01-0.005	0
0.1-0.5	10	10	0.01	222 $\pm$ 36
0.1-0.5	1	3	0.005	257 $\pm$ 88
0.1-0.5	1	1	0.005	261 $\pm$ 110

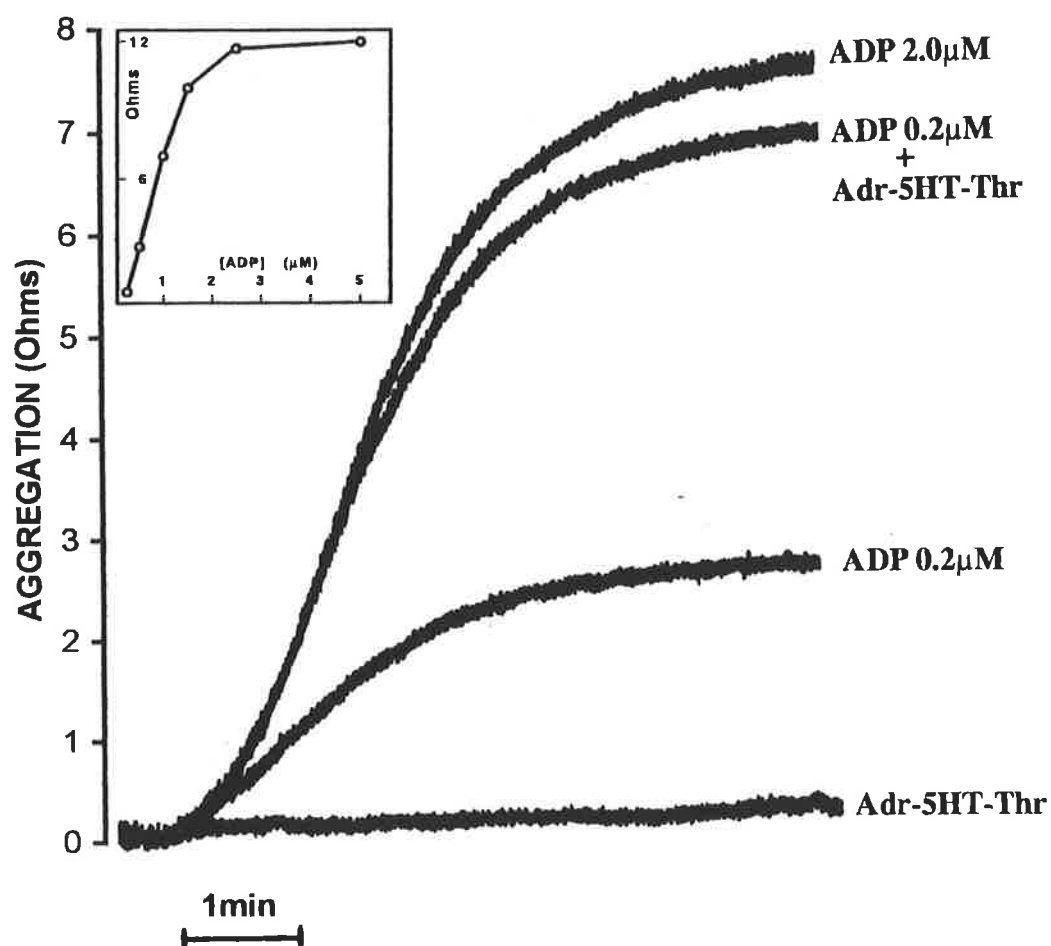


Figure 3.5.

Typical aggregograms for induction of platelet aggregation in whole blood by ADP (2 μM) alone and ADP (0.2 μM) in combination with adrenaline (1 nM), serotonin (1 nM) and thrombin (0.005 U/ml) [Adr-5HT-Thr]. Insert: ADP concentration-response curve for ADP alone.

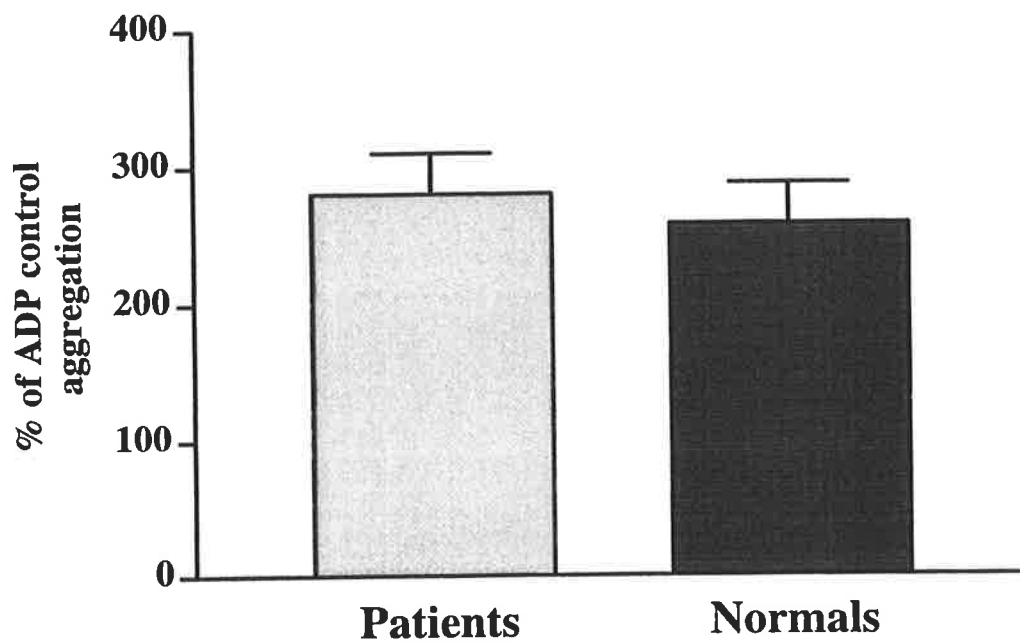


Figure 3.6.

Comparison of the potentiation of ADP-induced aggregation by the multiple agonist approach between patients and normal volunteers. No significant difference in response was observed.

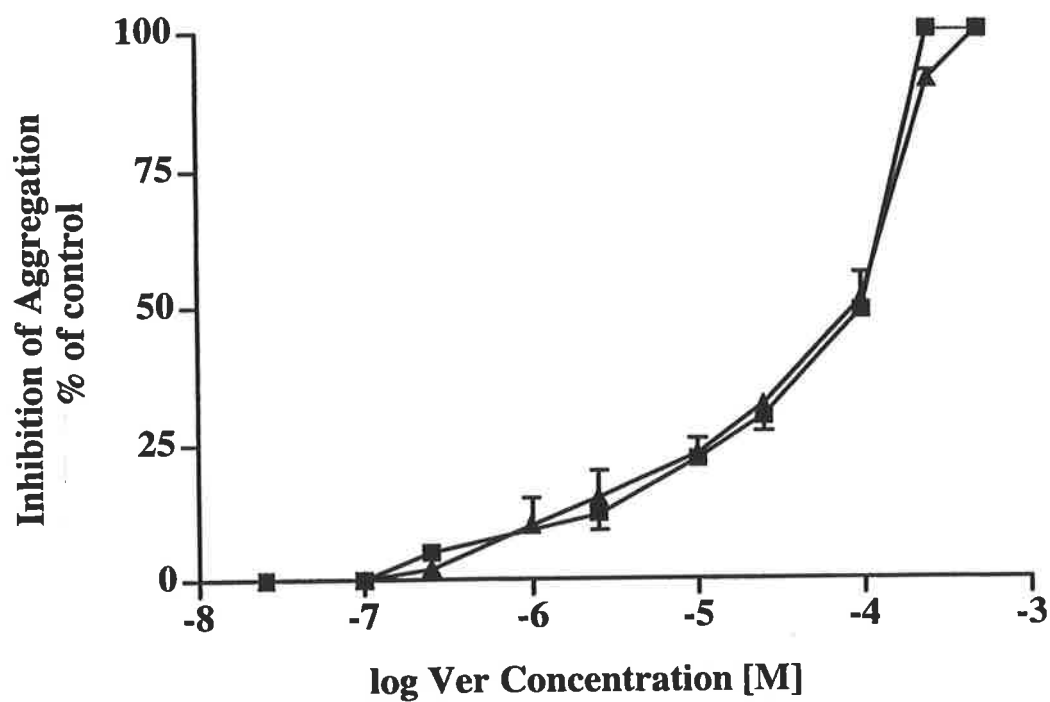


Figure 3.7.

Inhibition by Verapamil (Ver) of ADP (▲, n=5) and multiple agonists (■, n=5) - induced aggregation in whole blood samples from patients with stable angina. Error bars not indicated fall within the symbols used.

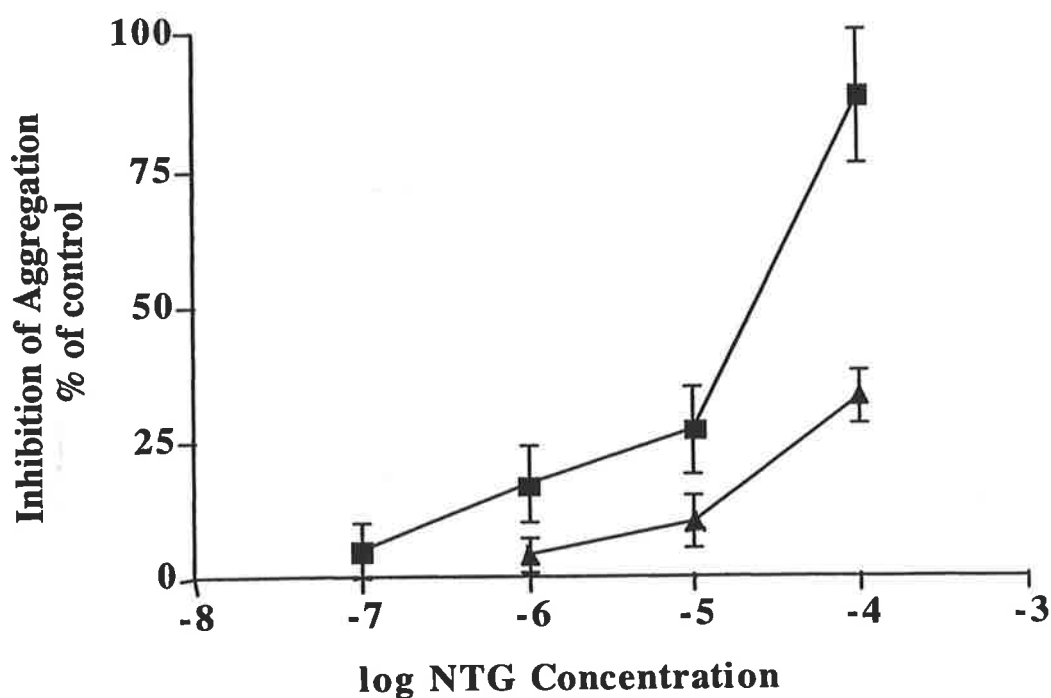


Figure 3.9.

Inhibition by Nitroglycerine (NTG) of ADP ($\blacktriangle$, $n=5$) and multiple agonist ($\blacksquare$, $n=5$) - induced aggregation in whole blood samples from patients with stable angina. Responses to all concentrations of NTG were significantly greater for aggregation induced by multiple agonists rather than for ADP alone ($p<0.05$ with 1 and 10 μ M NTG, $p<0.01$ for other concentrations of NTG).

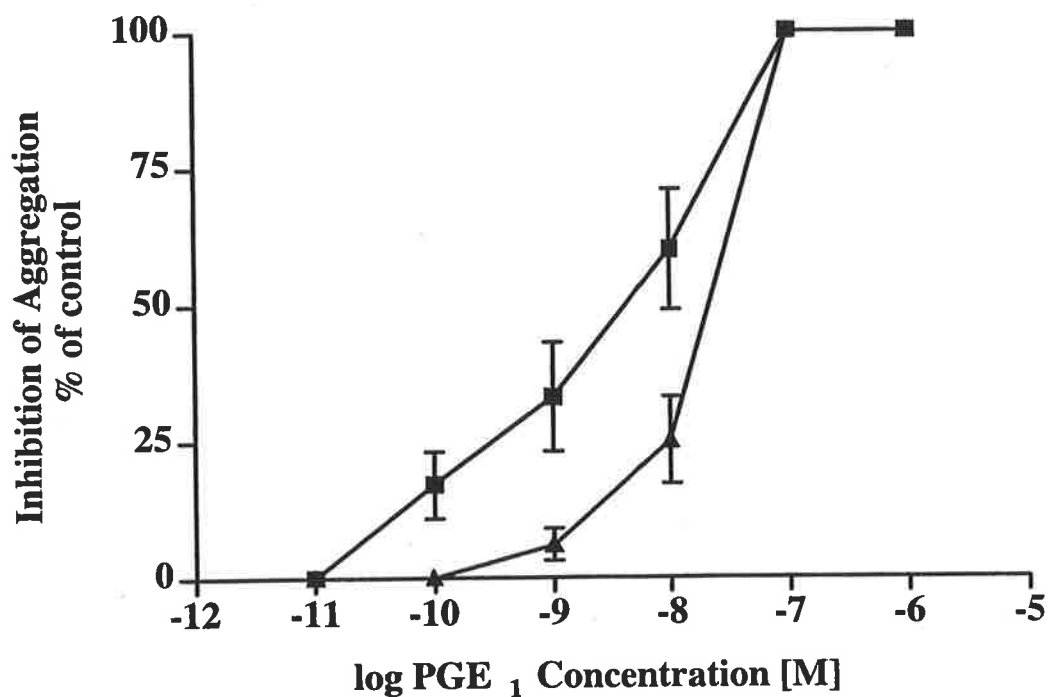


Figure 3.9.

Inhibition by Prostaglandin E₁ (PGE₁) of ADP (▲, n=5) and multiple agonist (■, n=5) -induced aggregation by PGE₁ in whole blood samples from patients with stable angina. Responses to concentrations of PGE₁ below 0.1 μM were significantly greater for aggregation induced by multiple agonists rather than for ADP alone (p<0.01 for all concentrations). Error bars not indicated fall within the symbols used.

Chapter 4

EFFECTS OF PERHEXILINE ON PLATELET AGGREGATION IN VITRO.

4 Chapter 4: Effects of Perhexiline on Platelet Aggregation In Vitro

4.1 Summary

Perhexiline has been utilised in the management of stable severe angina. Recent data suggests that therapeutic concentrations (0.5 - 2.0 μM) of perhexiline are effective in suppressing symptoms in patients with unstable angina pectoris. Perhexiline is an L-type calcium channel blocker at high concentrations. Suppression of platelet aggregation by perhexiline has been observed previously in human platelet-rich plasma, but has not been perceived as a therapeutically relevant observation. In the current study, with patients undergoing routine coronary angiography, we examined interactions between perhexiline and platelet aggregation in vitro, utilising impedance aggregometry in whole blood. Platelet aggregation studies were conducted utilising both single (ADP) and multiple pro-aggregants: ADP (0.1-0.5 μM), in conjunction with adrenaline (1 nM), serotonin (1 nM) and thrombin (0.005 U/ml).

In blood samples from patients with haemodynamically significant coronary artery disease (n=16) perhexiline inhibited ADP-induced aggregation. The perhexiline concentration-response curve was sigmoidal, and the concentration producing 50% inhibition of aggregation (C_{50}) for perhexiline was $24 \pm 3 \mu\text{M}$ with threshold at 1 μM . Individuals without significant coronary artery disease (n=12) had similar C_{50} values for perhexiline as those with coronary artery disease (26 ± 4 vs $24 \pm 3 \mu\text{M}$). Pretreatment with aspirin and/or verapamil did not affect platelet responses to perhexiline. When

aggregation was induced with multiple pro-aggregants the concentration-response curve for perhexiline became triphasic, with a local maximum occurring at $0.3 \pm 0.2 \mu\text{M}$; threshold perhexiline concentration for inhibition of aggregation was 1 nM. Perhexiline induced no significant changes in intraplatelet cGMP content. However, a small increase ($143 \pm 8\%$ of baseline) occurred in 10 out of 18 subjects. The nitric oxide scavenger oxyhaemoglobin (HbO_2) did not modify the anti-aggregating and cGMP-elevating effects of perhexiline. Similar results were obtained for inhibitors of nitric oxide synthase (L-NAME) and cyclic nucleotide phosphodiesterase (IBMX). Perhexiline did not affect intraplatelet cAMP content.

The current study also investigated the effect of perhexiline and verapamil on the mobilisation of intraplatelet calcium. Perhexiline was more potent than verapamil in inhibiting intraplatelet calcium mobilisation from washed platelets, and this effect was accentuated in the absence of extracellular calcium. For washed platelet aggregation, perhexiline was a far more potent inhibitor than verapamil, and its potency was independent of extracellular calcium concentration.

Conclusions: Perhexiline, in therapeutically achievable concentrations, inhibits platelet aggregation in whole blood in vitro. The anti-aggregating effects of perhexiline are: (a) disproportionate with its reported potency as a calcium channel blocker; (b) not accounted for by changes in cGMP or cAMP; (c) not mediated via NO-synthase or cyclic nucleotide phosphodiesterase; and (d) demonstrably due to at least 2 mechanisms (by virtue of the triphasic concentration-response curve). However, the current study

does not permit definitive evaluation of any putative effect of perhexiline on platelet aggregability *in vivo*.

4.2 Introduction

As outlined in the Chapter 1, the prophylactic anti-anginal agent, perhexiline, has been in clinical use for over 25 years. However, its use has steadily decreased due to a poor understanding of its mechanisms of efficacy and a high incidence of toxicity. Perhexiline was originally proposed to be a vasodilator (Hudak, et al. 1970) and then an L-calcium channel antagonist (Fleckenstein-Grun and al. 1978). However, these postulated mechanisms are inconsistent with its lack of hypotensive, vasodilator or negative inotropic effects at clinical doses (Pepine. 1973; Barry, et al. 1985; Silver, et al. 1985).

Data from one recent study provides evidence that perhexiline may also suppress symptoms in patients with unstable angina pectoris (Stewart, et al. 1996), a syndrome associated with activation of platelet aggregation. This suggests that perhexiline may have a clinically relevant anti-aggregatory effect, although it is possible that perhexiline might be effective in unstable angina pectoris via inhibition of the mitochondrial enzyme carnitine palmitoyltransferase-1. Inhibition of this enzyme produces a shift from fatty acid to glucose metabolism Kennedy, et al. 1996, this subsequently produces an oxygen sparing effect.

Furthermore, perhexiline (> 1 mM) has previously been shown to inhibit in vitro platelet aggregation induced by ADP, adrenaline and collagen (Ono and Kimura. 1981). This study was undertaken to compare the anti-aggregatory efficacy of a

number of L-channel calcium antagonists (diltiazem, nifedipine, perhexiline and verapamil). Perhexiline, the weakest calcium antagonist used when classified by vasodilator action, exhibited the greatest anti-platelet affect. This observation was left without any further exploration, and has not been followed up to date.

4.3 Objective of the study

The objective of the present study was to assess the anti-aggregatory effect of perhexiline in whole blood when aggregation was induced by either a single agonist (ADP) or the multiple agonist approach (as described in Chapter 3). It also examined the role of the cyclic nucleotides, cGMP and cAMP, in the anti-aggregatory mechanism of perhexiline.

4.4 Methods

4.4.1 Subjects studied

Subjects studied were cardiac patients (n=55) undergoing routine diagnostic coronary angiography for investigation of stable angina pectoris. Patients were of both sexes (37 men and 18 women); age range 43 to 80 years. Twenty three patients were receiving low dose aspirin. No other patient had taken any medication known to affect platelet aggregation during 2 weeks prior to study.

A cohort of normal volunteers (6 men and 5 women; aged from 23 to 51 years) were also examined for the effect of perhexiline on intraplatelet calcium mobilisation and the inhibition of washed platelet aggregation. No normal volunteer had taken any medication known to affect platelet aggregation or calcium release.

4.4.2 Blood sampling

Blood samples were collected as described in Chapter 2. The time interval between collection of blood samples and platelet aggregation studies was 5-10 min in all experiments.

4.4.3 Platelet aggregation studies

Single and multiple agonist methodology is described in Chapter 3.

4.4.4 Inhibition of aggregation

Blood samples were preincubated with perhexiline for 5 min prior to induction of aggregation. Perhexiline was diluted from an initial ethanol solution using physiological saline (0.9% NaCl). Control experiments with matched concentrations of ethanol produced no effect on platelet aggregation.

Inhibition of subsequent aggregation was evaluated as a percentage of maximal aggregation achieved in the absence of perhexiline.

4.4.5 Intraplatelet calcium concentration and washed platelet aggregation

Intraplatelet calcium concentration was determined in washed platelets, according to the method described in Chapter 2. Thrombin was utilised as the inductor of calcium mobilisation and platelet aggregation. Perhexiline and verapamil were investigated for their ability to inhibit both intraplatelet calcium mobilisation and washed platelet aggregation.

4.4.6 Intraplatelet cGMP/cAMP content

Intraplatelet cGMP/cAMP content was measured according to the method described in Chapter 2. The effects of the nitric oxide scavenger oxyhaemoglobin (HbO_2) and a cyclic nucleotide phosphodiesterase inhibitor (IBMX) on cGMP content were examined. See Chapter 2 for details of HbO_2 and IBMX preparation.

4.4.7 HbO_2 experiments in whole blood

HbO_2 was prepared according to the method described in Chapter 2. HbO_2 was added to whole blood samples ($n=2$) simultaneously with perhexiline and subsequent aggregation was measured.

4.4.8 L-NAME experiments in whole blood

L-NAME, an inhibitor of nitric oxide synthase was prepared according to method outlined in Chapter 2. L-NAME (0.1 mM) was added to whole blood (n=2) either before or simultaneously with perhexiline (1 to 10 μ M).

4.5 Data analysis

Potential of ADP-induced aggregation by adrenaline, serotonin and thrombin was calculated as increase in extent of aggregation, compared to that of ADP alone in the same concentration. Comparisons were made utilising Students 2-tailed paired t-test. Data involving multiple comparisons were analysed utilising Dunnett's test. Inhibition of aggregation by perhexiline was evaluated as percentage of maximal aggregation achieved in the absence of the drug.

For intraplatelet calcium experiments data is presented as the percentage of thrombin induced calcium mobilisation in the absence of either perhexiline or verapamil. Statistical significance was limited to $p < 0.05$. Results are expressed as mean $\pm$ SEM, unless otherwise stated. Comparisons between patient groups were made utilising Students unpaired t-test.

4.6 Results

4.6.1 Potentiation of ADP-induced aggregation

Whole blood from patients (n=16) exhibited increased extent of aggregation with ADP (0.1-0.5 μ M) plus adrenaline (1 nM), serotonin (1 nM) and thrombin (0.005 U/ml) compared with the aggregation produced by ADP alone in an equivalent concentration. There was a mean increase in aggregation of 270 ± 25 % with multiple agonists from ADP alone ($p < 0.01$). Adrenaline, serotonin and thrombin when added simultaneously to whole blood produced no detectable aggregation. These results are consistent with those of Chapter 3.

4.6.2 Anti-aggregatory Effects of Perhexiline

4.6.2.1 ADP-induced aggregation

Perhexiline added to the whole blood of patients with haemodynamically significant coronary artery disease (n=16) 5 min before induction of aggregation by ADP, caused inhibition of aggregation in a concentration-dependent manner (Figure 4.1). Five minutes of pre-incubation with perhexiline was found to be optimal for anti-aggregating effects in preliminary experiments (data not shown). Statistically significant suppression of ADP-induced aggregation was observed with perhexiline concentrations > 0.1 μ M; total inhibition of aggregation occurred at 100 μ M perhexiline. The concentration producing 50% inhibition of aggregation (C_{50}) was 24 ± 3 μ M. The concentration-response curve for inhibition of aggregation by perhexiline in this model was sigmoidal.

Blood samples from patients without haemodynamically significant coronary artery disease (n=12) were also examined for inhibition of aggregation by perhexiline (Figure 4.1). The concentration-response curve for perhexiline in this group of patients was similar to that obtained in patients with significant disease (C_{50} : 26 ± 4 vs 24 ± 3 μM). Patient medication, for example aspirin or verapamil, did not affect the platelet responses to perhexiline in either group of patients.

4.6.2.2 Multiple agonist model

When perhexiline was added to blood samples prior to induction of aggregation by multiple agonists the concentration-response curve for perhexiline effects was no longer sigmoidal as it was for ADP alone (Figure 4.1); a local maximum was detected in the range of perhexiline concentrations from 0.01 to 1 μM (Figure 4.2). The position of this local maximum varied from individual to individual. In Figure 4.3 four representative individual concentration-response curves are shown.

The mean values for the position (within the concentration range) and amplitude of the local maximum were calculated from 16 individual concentration-response curves obtained from patients (Figure 4.2). Statistically significant suppression of multiple agonist-induced aggregation was observed with perhexiline concentrations >1 nM ($p < 0.01$ vs ADP alone). The concentration-response curve for inhibition of multiple agonist-induced aggregation by perhexiline was triphasic, with an average local

maximum occurring at approximately 0.3 μM (Figure 4.4). However, the C_{50} concentration for multiple agonist induced aggregation did not differ significantly from that obtained with ADP-alone induced aggregation (C_{50} : 20 ± 4 vs 24 ± 3). Furthermore, the perhexiline concentration associated with total inhibition of aggregation was 100 μM , thus corresponding to the perhexiline responsiveness when aggregation was induced by ADP-alone.

The finding that with multiple agonist-induced aggregation, the response of perhexiline is triphasic suggested that there are multiple molecular mechanisms of interaction between perhexiline and intraplatelet systems controlling the process of aggregation.

4.6.3 Intraplatelet cGMP/cAMP content

The effects of perhexiline (0.01-100 μM) on intraplatelet cGMP was examined in 18 patients. Perhexiline produced no consistent changes in cGMP content at any concentration examined. Marked interindividual variation was evident, with the pattern of responsiveness resembling that of the multiple agonist model. Peak perhexiline effects occurred in the range of concentrations 1 to 10 μM . Results are summarised in Table 4.1. Perhexiline (10 μM) produced no significant changes in intraplatelet cGMP content overall (113 ± 7 % of control, $p=0.08$). Perhexiline (1 μM , a concentration close to the observed multiple agonist peak) also produced no significant changes in cGMP content (105 ± 8 % of control). However, in 10 out of the 18 patients there was a definite increase in cGMP content (143 ± 8 % of control, $p<0.05$).

Intraplatelet cGMP content reflects both generation of cGMP by guanylate cyclase and hydrolysis of cGMP by cyclic nucleotide phosphodiesterases. These two aspects were investigated in the present study. Guanylate cyclase is activated by nitric oxide, therefore it is possible that intraplatelet cGMP content is being affected by an interaction of perhexiline with nitric oxide. To examine this issue the effect of HbO₂ (nitric oxide scavenger) was examined in 4 patients. HbO₂ did not affect intraplatelet cGMP content (97 ± 4 % of control) when added simultaneously with perhexiline (1 μ M). As nitric oxide is not affecting intraplatelet cGMP content another possibility exists for the lack of cGMP effect, that is, generated cGMP may be rapidly hydrolysed by cyclic nucleotide phosphodiesterases. This was examined using the phosphodiesterase inhibitor, IBMX (n=3). No change in intraplatelet cGMP content was observed when IBMX was added to platelets together with 1 μ M perhexiline (113 ± 18 % of control, Table 4.1).

In 15 patients the effect of perhexiline on cAMP were also examined. Perhexiline (10 μ M) produced no change in intraplatelet cAMP content (98 ± 5 % of control). Similar results were obtained with 1 μ M perhexiline (103 ± 8 % of control, Table 4.1).

4.6.4 Whole blood aggregation

HbO₂ and the nitric oxide synthase inhibitor, L-NAME were also examined for their effect on perhexiline's ability to inhibit whole blood aggregation. HbO₂ (10 μ M) and L-

NAME (100 μ M) did not inhibit ADP-induced platelet aggregation. When used in conjunction with perhexiline (10 μ M), HbO₂ did not alter the extent of inhibition of aggregation produced (32 ± 18 vs 32 ± 19 % of control, $n=2$). L-NAME also had no effect on extent of inhibition produced by perhexiline (26 ± 3 vs 19 ± 12 % of control, $n=2$).

4.6.5 Intraplatelet calcium concentration

The effects of perhexiline on intraplatelet calcium were investigated in a cohort of normal ($n=27$) volunteers and compared to the L-type calcium channel blocker, verapamil. Normal volunteers have been used extensively in the characterisation of calcium mobilisation by agonists and the inhibitory response of antagonists (Hallam and Rink. 1985; Johnson, et al. 1985; Johnson, et al. 1985; Ware, et al. 1985; Johnson, et al. 1989).

Intraplatelet calcium mobilisation was investigated utilising the photoprotein aequorin. Aequorin was chosen in preference to the commonly used fluorphores quin2 or Fura2 as aequorin is more sensitive to local calcium transients (Johnson, et al. 1985; Ware, et al. 1986; Ware, et al. 1989; Rink and Sage. 1990). Aequorin has also a greater sensitivity to low levels of cytoplasmic ionised calcium concentrations. In a study by Ware et al the difference between several platelet agonists on calcium mobilisation in the presence of either aequorin or quin2 was investigated. The authors concluded that aequorin-indicated calcium concentrations appear to be more closely related to both stimulatory

and inhibitory changes in aggregation than rises in quin2 detected calcium concentrations (Ware, et al. 1986).

Thrombin was chosen as the agonist for this series of experiments as the amount of internal calcium discharged by thrombin is virtually the same as that discharged by calcium inophores (Rink and Sage. 1990) which suggests that all the readily mobilised intraplatelet calcium is accessible to the thrombin receptor-mediated pathway. Although ADP has been utilised for experiments in whole blood to examine perhexiline's inhibitory effect on platelets, it was not chosen to investigate intraplatelet calcium mobilisation. The reasons for this decision were 1) the concentration of ADP required to elicit a calcium response tend to be 5 to 10 fold higher than the concentrations utilised in whole blood experiments (Ware, et al. 1986) and 2) in a washed platelet preparation (see method Chapter 2) platelets usually lose their responsiveness to ADP over very short periods of time (Hallam and Rink. 1985) therefore making experiments difficult.

Thrombin (10 U/ml) produced transient increases in cytoplasmic calcium concentrations of $7.7 \pm 0.3 \mu\text{M}$ ($n=27$).

Perhexiline produced a concentration-dependent inhibition of the thrombin-induced rise in cytoplasmic calcium (Figure 4.5). Statistically significant inhibition by perhexiline was recorded at 10 μM (12.2 ± 5.1 % inhibition from control, $p < 0.05$, $n=9$); with 100 μM perhexiline there was a 100 ± 0 % inhibition ($p < 0.05$, $n=3$). Verapamil was less potent than perhexiline as an inhibitor of cytoplasmic calcium mobilisation (Figure 4.6).

Statistically significant inhibition by verapamil was recorded at 100 μM (22.4 ± 5.7 % inhibition from control, $p < 0.05$, $n=8$) and 200 μM (40.3 ± 5 % inhibition from control, $p < 0.05$, $n=3$).

Chelation of extracellular calcium with EGTA reduced but did not eliminate rises in aequorin-indicated calcium concentration. Therefore any measured calcium was mobilised from internal platelet stores. Both perhexiline and verapamil exerted greater effects on calcium transients in the absence of extracellular calcium (Figures 4.5 and 4.6). For perhexiline, statistically significant inhibition of internal calcium mobilisation was observed at 1 μM (19.6 ± 6.5 % inhibition from control, $p < 0.05$, $n=3$), 10 μM (43.4 ± 8.3 % inhibition from control, $p < 0.05$, $n=6$) and 100 μM (100 ± 0 % inhibition from control). For verapamil, significant inhibition was observed only at 100 μM (78.2 ± 15.5 % inhibition from control, $p < 0.05$, $n=5$) and 200 μM (82.5 ± 17.5 % inhibition from control, $p < 0.05$, $n=3$).

The aggregation of washed platelets was also examined in the presence and absence of external calcium. Perhexiline was far more potent as an inhibitor of washed platelet aggregation than verapamil; indeed verapamil exerted minimal effects, significant only in the absence of external calcium (Figure 4.7). The effects of perhexiline in this model were not modified by the removal of external calcium by EGTA (Figure 4.7).

The anti-aggregatory effects of perhexiline are therefore similar when examined in whole blood and washed platelets. Thus, it appears that the inhibitory responses to

perhexiline, at least at concentrations above the therapeutic range (that is concentrations corresponding to the right-hand side of the perhexiline concentration-response curve when aggregation is induced by either ADP alone or by multiple agonists), are associated with inhibition of intraplatelet calcium release. However, this inhibition of calcium release does not explain the peak observed with the multiple agonist approach (Figure 4.4).

4.7 Discussion

The major finding of the current study is that perhexiline inhibits whole blood aggregation induced by either ADP alone or by multiple agonists. The unique (triphasic) nature of the concentration-response curve for perhexiline when aggregation was induced by multiple agonists implies that perhexiline has a complex mechanism of action which does not involve significant interaction with intraplatelet cGMP or cAMP pathways, inhibition of nitric oxide synthase or the scavenging of nitric oxide. However, especially with high concentrations of perhexiline ($>1 \mu\text{M}$) mobilisation of intraplatelet calcium appears to be ultimately responsible for the inhibition of aggregation. Nevertheless this result does not account for the peak observed with the multiple agonist approach, nor does it explain interference with signal transduction mechanisms culminating in changes in calcium mobilisation.

Perhexiline has previously been shown to have antiplatelet effects in vitro, but only in high concentrations (Ono and Kimura, 1981). In the current study perhexiline exhibited

significant antiplatelet effects at concentrations achievable therapeutically. When aggregation was induced by ADP alone, inhibition of aggregation by perhexiline occurred with threshold for perhexiline effects at 0.1 μM (Figure 4.1). With the multiple agonist approach, perhexiline produced significant antiplatelet effects described by a triphasic concentration-response curve. A local maximum occurred at approximately 0.3 μM perhexiline; with a threshold at 1 nM (Figure 4.4). This triphasic response curve has never been reported previously, and has only become evident by virtue of the employment of multiple agonist induced aggregation. Hence a multiple agonist approach gives perhexiline an additional “window of opportunity” to exert its inhibitory effects and elicit greater effects than for ADP alone. Furthermore, the triphasic nature of the perhexiline concentration-response curve implies that perhexiline has at least two mechanisms of action, that is, one acting as a physiological antagonist of ADP, with threshold effects at approximately 0.1 μM perhexiline, and another more sensitive mechanism, at least partially involving interactions with the other agonists.

Intraplatelet cGMP-mediated inhibition of aggregation is one of the major controlling mechanisms of platelet activation (Siess, 1989). Therefore the present study examined the effect of perhexiline on intraplatelet cGMP content. Perhexiline produced no consistent changes in cGMP content. However, some interindividual variation was evident. This is further supported by the small increase in cGMP content in 10 out of the 18 patients studied. Thus implying that perhexiline affects cGMP content indirectly or only in a proportion of the patient population.

Other possible explanation for the lack of change in intraplatelet cGMP content are: 1) its generation from guanylate cyclase is impaired or 2) its being degraded as soon as its formed. These issues were addressed in the present study utilising HbO_2 , a nitric oxide scavenger and IBMX, a cyclic nucleotide phosphodiesterase inhibitor. Both HbO_2 and IBMX had no effect on cGMP content. HbO_2 and IBMX were also examined for their effects on inhibition of aggregation. Again no effect was observed. These results suggest that the anti-aggregatory effects of perhexiline are not mediated by nitric oxide generation or by cyclic nucleotide phosphodiesterase activity, at least when examined acutely in vitro.

Platelet aggregation is also under the negative control of the intraplatelet cAMP system, therefore effects of perhexiline on cAMP content were also examined. Perhexiline did not modify cAMP content at any concentration investigated.

The lack of overall effect of perhexiline on intraplatelet cGMP or cAMP content implies that the anti-aggregatory effects of perhexiline are not accounted for by these pathways, although it is possible that perhexiline may have an indirect effect on the cGMP pathways, the nature of the effect is yet to be elucidated.

One of the early proposed mechanism of perhexiline's therapeutic efficacy was attributed to inhibition of L-type calcium channels (see Chapter 1), although these effects were observed at concentrations well above the therapeutic range (Barry, et al. 1985); perhexiline was shown to be a less potent L-type calcium channel blocker than

the commonly used agents such as nifedipine, diltiazem and verapamil (Barry, et al. 1985). Verapamil for example, has well-established, although not particularly potent, anti-aggregatory effects (Ono and Kimura. 1981; Jones, et al. 1985; Strano, et al. 1985) which provide a partial basis for its clinical use after non-Q-wave myocardial infarction (Hansen. 1991). Although perhexiline is indeed a L-type calcium channel blocker this mechanism has been ruled out as its primary mechanism of action. However, the question still remains as to the relative importance of perhexiline's calcium channel blocking effects in platelets.

The effects of perhexiline and verapamil on intraplatelet calcium mobilisation was examined in the present study. The results are summarised as follows:-

- (1) Perhexiline was somewhat more potent than verapamil in inhibiting intraplatelet calcium mobilisation, and this effect was accentuated in the absence of extracellular calcium.
- (2) In washed platelets, perhexiline was far more potent than verapamil in inhibiting platelet aggregation, and its potency was independent of reduction in extracellular free calcium concentration. Therefore, perhexiline did not act as an L-type calcium channel antagonist in this preparation.

Hence perhexiline is likely to affect calcium transfer between the dense tubular system and cytoplasm, although the precise mechanism whereby this occurs has not been elucidated. Further investigations to elucidate the mechanism might have included:-

- a) Inhibitors of inositol 1,4,5-trisphosphate (IP_3) -induced calcium release. Platelet IP_3 inhibitors include cinnarizine and flunarizine (Seiler, et al. 1987), and
- b) Inhibitors of intraplatelet calcium release, ryanodine and thapsigargin.

The current study therefore leaves unanswered a fundamental question:- what is the precise mechanism by which perhexiline inhibits platelet aggregation? However, as discussed in Chapter 1, perhexiline has many known mechanisms of action other than calcium antagonism. Recently, substantial interest has focused on agents which alter the energy metabolism and subsequently the supply of oxygen to the myocardium (Ferrari, et al. 1998; Lopaschuk. 1998; Mody, et al. 1998; Schelbert. 1998; Taegtmeyer, et al. 1998). Perhexiline has been shown to inhibit carnitine palmitoyltransferase-1 in the mitochondria which produces a shift from fatty acid to glucose metabolism (Kennedy, et al. 1996) this subsequently produces an oxygen sparing effect. As platelets require energy and oxygen the inhibition of carnitine palmitoyltransferase-1 may be responsible for perhexiline's inhibition of aggregation at therapeutic concentrations. Although this was not examined in the current study, it is the focus of Chapter 5.

4.8 Conclusions

Although the precise mechanism(s) whereby perhexiline inhibits platelet aggregation and intraplatelet calcium mobilisation is / are not elucidated to date, the finding of inhibition of aggregation at concentrations as low as 0.1 μM is likely to be clinically

relevant. The therapeutic range for perhexiline is 0.15 - 0.60 $\mu\text{g/L}$. Thus, although perhexiline is rarely used as monotherapy, the current results provide theoretical support for the concept that inhibition of platelet aggregation is a significant component of perhexiline's therapeutic spectrum of actions.

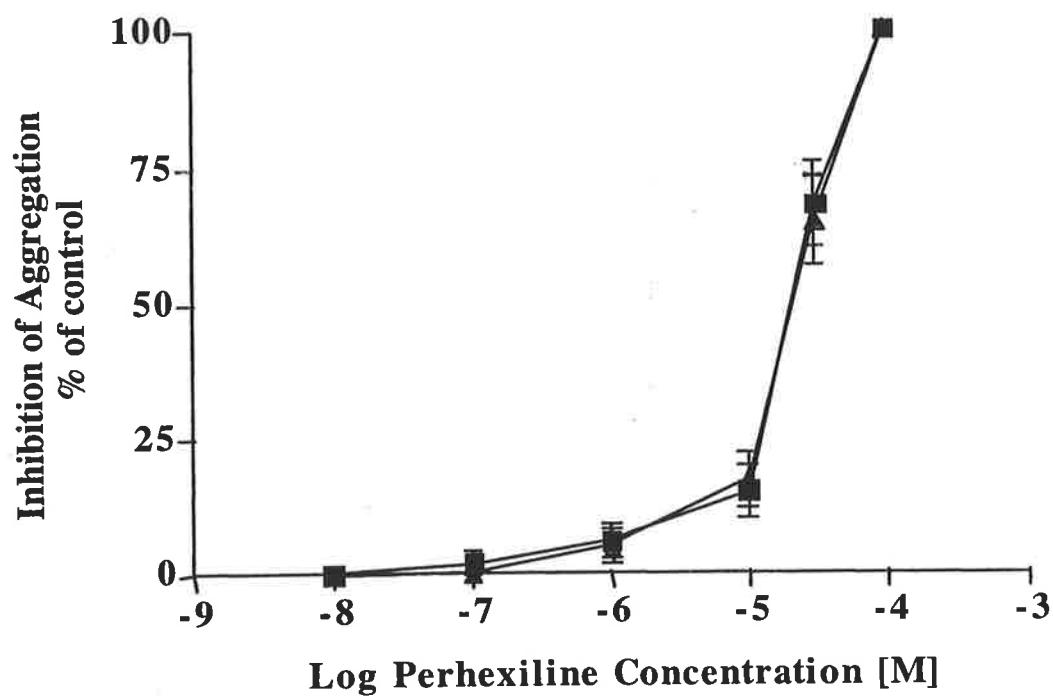


Figure 4.1.

Inhibition of ADP-induced whole blood aggregation by perhexiline in blood samples from patients with (■, n=16) and without (▲, n=12) significant coronary artery disease.

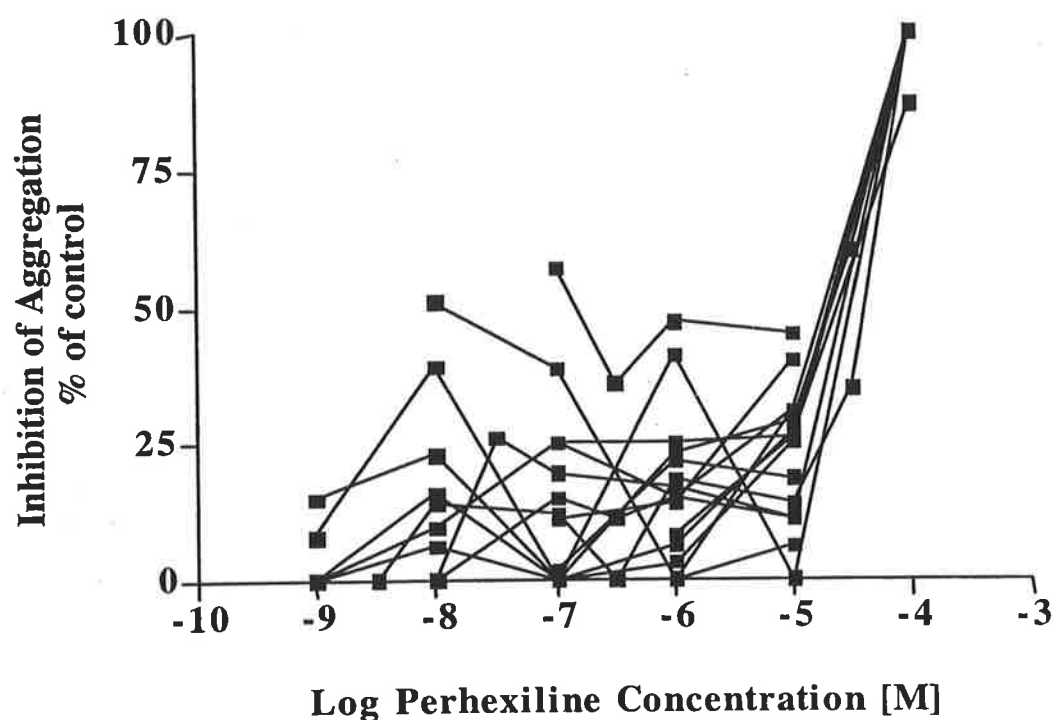


Figure 4.2.

Inhibition of multiple agonist-induced aggregation by perhexiline (n=16). The perhexiline concentration-response curve is no longer sigmoidal as it was with ADP alone. The curve is highly variable and frequently local maxima occur. Perhexiline effects on multiple agonist induced aggregation demonstrate a large degree of interindividual variability. Maximum inhibition of aggregation occurs at 100 μ M as with ADP alone, but threshold perhexiline effects occur at 1 nM.

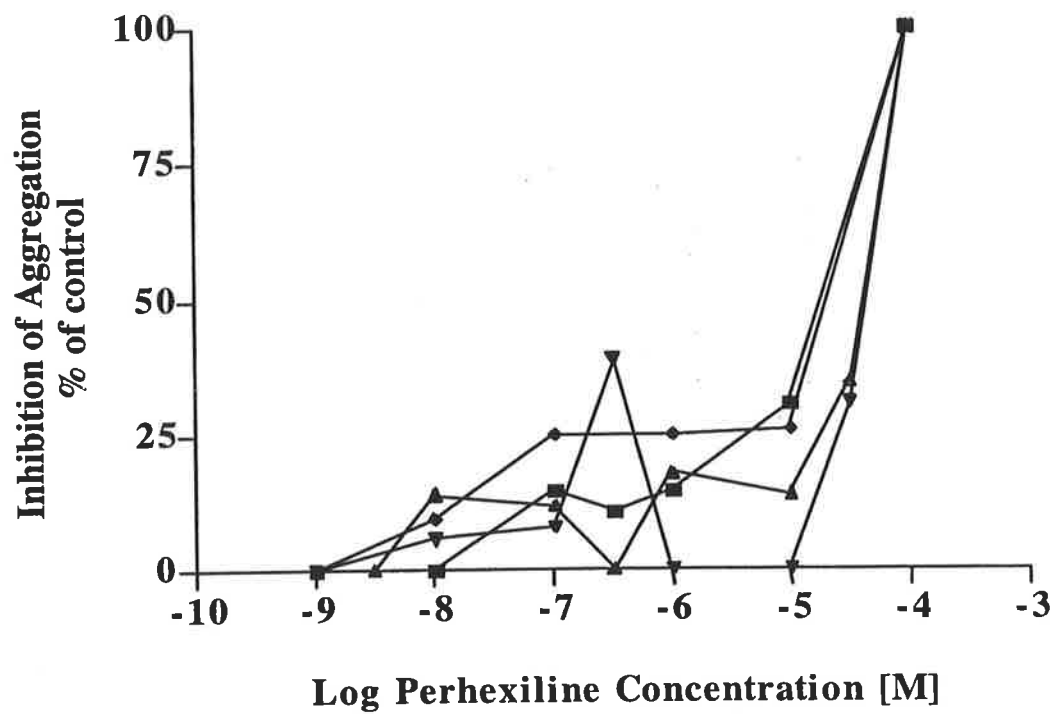


Figure 4.3.

Inhibition of multiple agonist-induced aggregation by perhexiline. Representative concentration-response curves for 4 individuals.

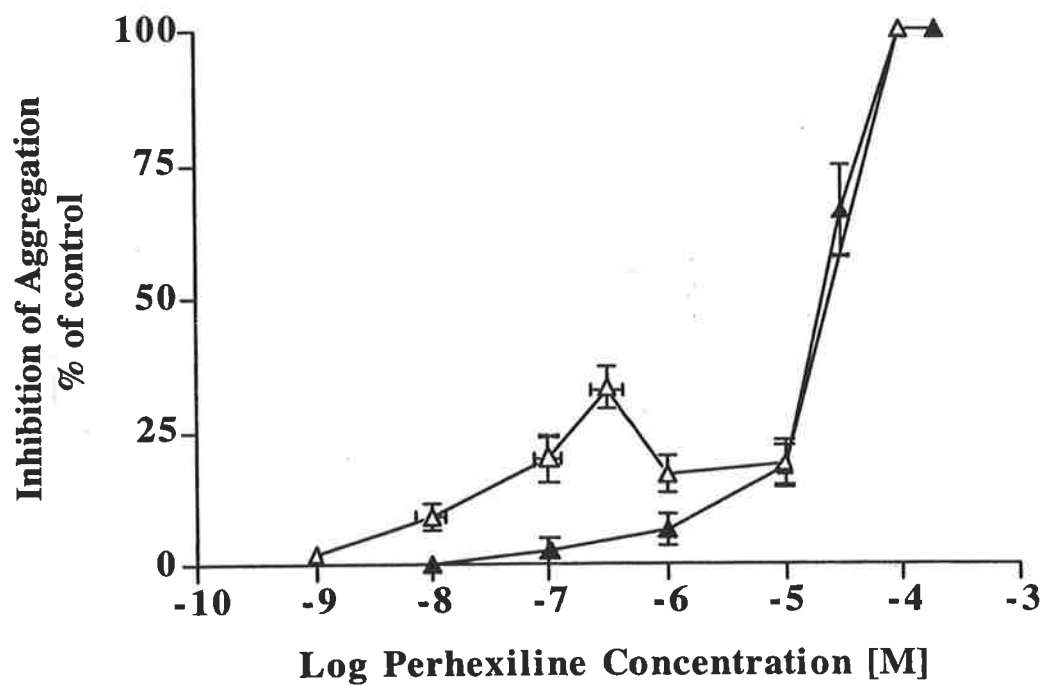


Figure 4.4.

Inhibition of ADP alone (▲) and multiple agonist (Δ) -induced aggregation by perhexiline.

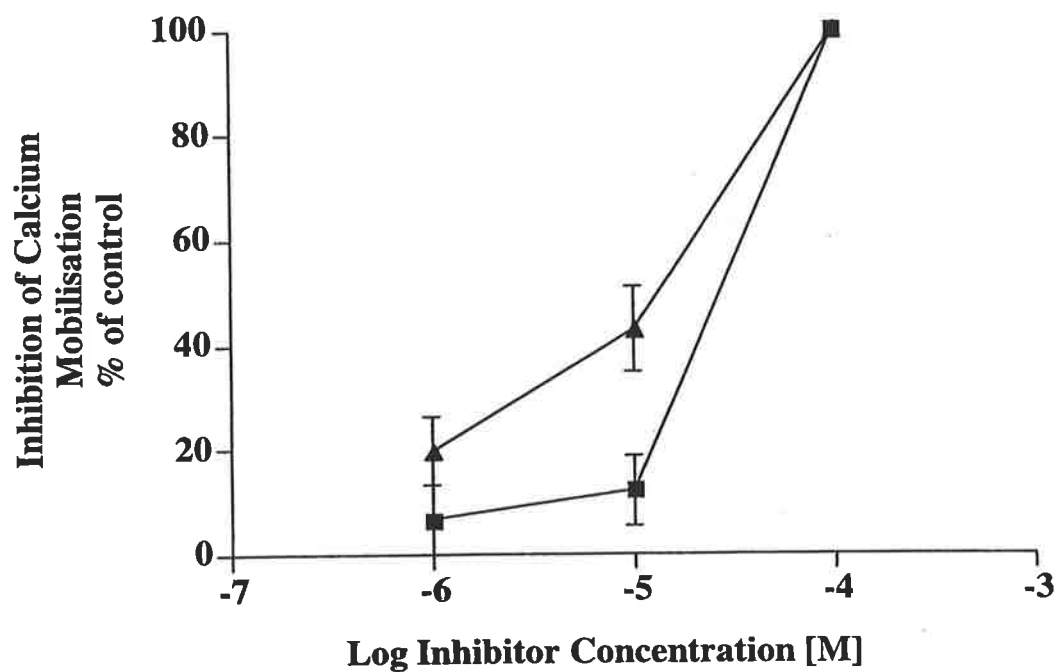


Figure 4.5.

Inhibition of intraplatelet calcium mobilisation by perhexiline in the presence (■) and absence (▲) of extracellular calcium.

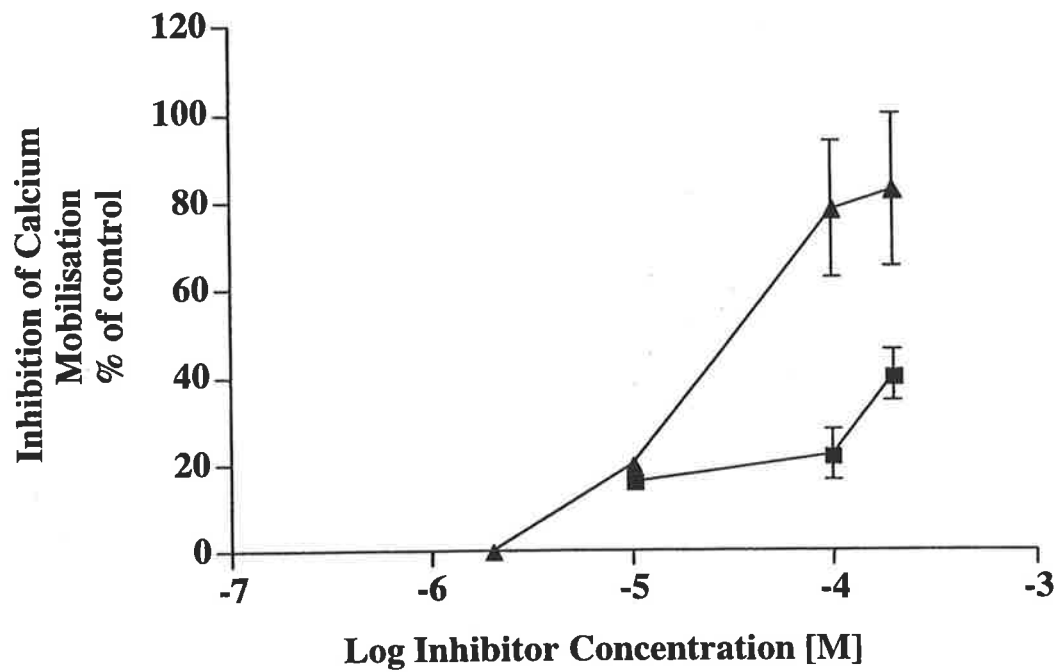


Figure 4.6.

Inhibition of intraplatelet calcium mobilisation by verapamil in the presence (■) and absence (▲) of extracellular calcium.

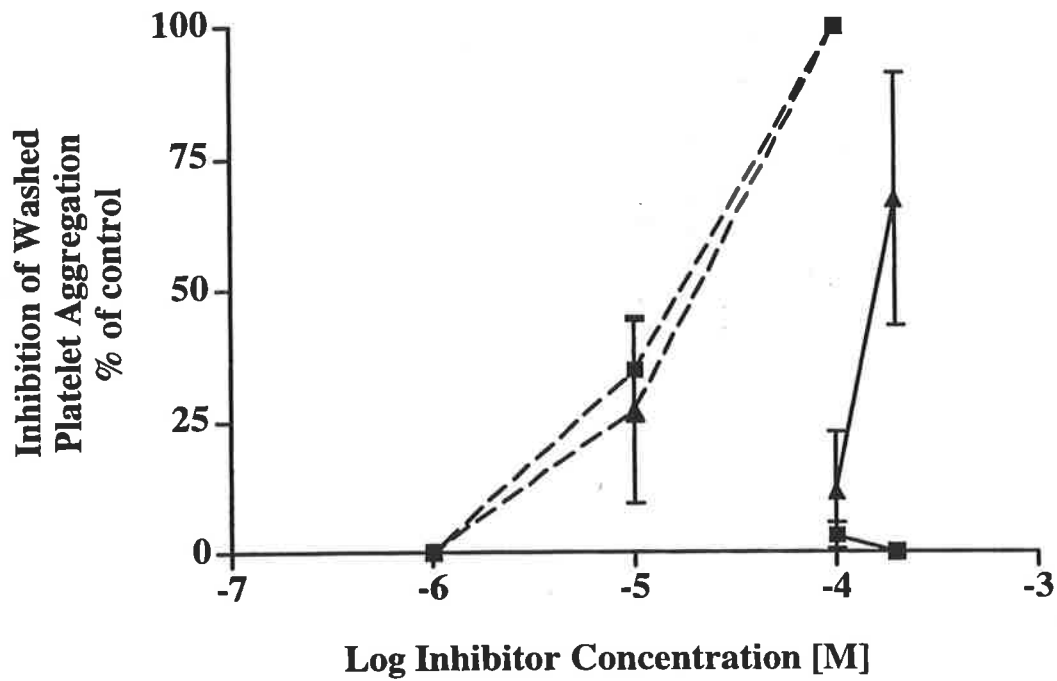


Figure 4.7.

Inhibition of washed platelet aggregation by perhexiline [dotted line] verapamil [solid line] in the presence (■) and absence (▲) of extracellular calcium.

Table 4.1. Perhexiline (Pex) effects on intraplatelet cGMP and cAMP contents

Experiment	cGMP % of Control	cAMP % of Control
Pex 10μM	113$\pm$7	98$\pm$5
Pex 1μM	105$\pm$8	103$\pm$8
Pex 1μM §	143$\pm$8	†
IBMX	113$\pm$18	†
HbO₂	97$\pm$4	†

§ Perhexiline results in 10 out of 18 patients ($p < 0.05$ vs control). † not examined.

Chapter 5

ANTI-AGGREGATORY EFFECTS OF PERHEXILINE ARE NOT MODULATED BY THE INHIBITION OF CARNITINE PALMITOYLTRANSFERASE-1.

5 Chapter 5: Anti-Aggregatory Effects of Perhexiline are not Modulated by the Inhibition of Carnitine Palmitoyltransferase-1.

5.1 Summary

A number of anti-anginal agents (perhexiline, amiodarone, trimetazidine) have been shown to inhibit myocardial carnitine palmitoyltransferase-1, which controls access of long-chain fatty acids to mitochondrial sites of β -oxidation. In view of clinical data suggesting that perhexiline improves symptomatic status in unstable angina pectoris, and the known role of perhexiline in mitochondrial β -oxidation, we compared the platelet carnitine palmitoyltransferase-1 inhibitory and putative anti-aggregatory effects of perhexiline, amiodarone and trimetazidine with those of specific carnitine palmitoyltransferase-1 inhibitors: etomoxir and hydroxyphenylglyoxylate in both normal subjects and patients with stable angina.

All of the compounds examined inhibited platelet carnitine palmitoyltransferase-1 activity; rank order of potency etomoxir > malonyl-CoA > hydroxyphenylglyoxylate > amiodarone $\geq$ perhexiline > trimetazidine. However, only perhexiline, amiodarone and trimetazidine inhibited platelet aggregation.

We conclude that (a) the carnitine palmitoyltransferase-1 inhibitors perhexiline, amiodarone and trimetazidine exert significant anti-aggregatory effects which may be

therapeutically relevant and (b) these effects are independent of carnitine palmitoyltransferase-1 inhibition.

Introduction

The known interactions of perhexiline with cellular metabolic processes have been discussed in Chapter 1. In brief, perhexiline has been shown to inhibit the mitochondrial membrane enzyme carnitine palmitoyltransferase-1 in rat heart and liver; inhibition of carnitine palmitoyltransferase-1 in heart provides a basis for the proposed beneficial effects of perhexiline on myocardial energetics and thus is likely to explain much of the anti-anginal efficacy of perhexiline together with its effects in aortic stenosis (Unger, et al. 1997). Carnitine palmitoyltransferase-1 is a widely distributed enzyme, being directly relevant to ATP production in lung, kidney and skeletal muscle as well as formed elements of blood. It is therefore possible that carnitine palmitoyltransferase-1 inhibition by perhexiline may have significant extracardiac implications, including those involving platelet function.

Mitochondrial carnitine palmitoyltransferase-1 exists in two distinct isoforms: 1) liver-type and 2) muscle-type, this distinction is based upon the tissues originally identified as expressing these isoforms. When examined in intact mitochondria (McGarry, et al. 1983) the two isoforms can be distinguished as they exhibit a 100-fold difference in sensitivity to malonyl-CoA (IC_{50} values of $\approx 2.7\mu M$ for liver and $0.03\mu M$ for muscle). The lung has previously been shown to contain the liver isoform of carnitine palmitoyltransferase-1, while the heart is predominantly skeletal muscle (McGarry, et al.

1983; Weis, et al. 1994; Weis, et al. 1994). It has been shown that the lung (liver isoform) is unsensitive to the inhibitory action of amiodarone, while the heart (muscle isoform) is sensitive to its action (personal communication Dr J Kennedy). This observation, as well as the results of the present study demonstrates that platelets contain the muscle isoform of carnitine palmitoyltransferase-1 (see results section). Although two isoforms of carnitine palmitoyltransferase-1 occur only one isoform of carnitine palmitoyltransferase-II has been identified (Woeltje, et al. 1990; McGarry, et al. 1991).

Platelets also require energy, which is utilised for routine maintenance and for the performance of special functions related to the maintenance of haemostasis, such as aggregation, release reactions and clot retraction (Mustard and Packham. 1975; Majerus and Miletich. 1978). Energy production in platelets has been shown to be dependent on the oxidation of fatty acids, in addition to glycolysis and the oxidation of carbohydrates (Donabedian and Nemerson. 1971; Akkerman. 1978; Iida, et al. 1991). Detwiler and Zivkozic suggested that respiration occurred at the expense of substrates (eg long-chain fatty acids) rather than glucose and glycogen (Detwiler and Zivkozic. 1970). Thus, glucose and glycogen appear to be the main energy sources of platelets. This observation is supported by Heijnen et al who demonstrated that in human platelets, increased energy metabolism may be precisely coupled to the platelet activation response by means of the translocation of the platelet glucose transport by regulated secretion of alpha-granules (Heijnen, et al. 1997). The data of Heijnen et al shows that glucose uptake is upregulated by five to six times during in vivo activation of platelets (Heijnen, et al. 1997).

Deficiencies in the carnitine palmitoyltransferase-1 enzyme although very rare have been reported, with the affected enzyme being the liver isoform (Demaugre, et al. 1991; Britton, et al. 1995). Therefore any clinical symptomatology displays liver involvement, for example hypoketotic hypoglycaemia (Bougnères, et al. 1981). Carnitine palmitoyltransferase-II has only one isoform therefore deficiencies exist in the liver, heart and platelets (Angelini, et al. 1981; Trevisan, et al. 1984; Nosadini, et al. 1987). Individuals with carnitine palmitoyltransferase-II deficiencies usually exhibit recurrent myoglobinuria (Angelini, et al. 1981; Trevisan, et al. 1984) and during prolonged fasting the level of serum free fatty acids increase. However, there has been no reported cases of platelet dysfunction (ie, increased bleeding events) in this group of individuals. Deficiencies in the muscle isoform of carnitine palmitoyltransferase-1 have never been reported.

Recently, Iida et al have characterised carnitine palmitoyltransferase-1 in saponin-permeabilized rat platelets, demonstrating that the activity of the enzyme is increased by streptozotocin-induced diabetes and decreased by insulin in vitro, and that this enzyme performs an important role in the regulation of long-chain fatty acid oxidation in platelets (Iida, et al. 1991). Moreover increased carnitine palmitoyltransferase-1 activity in diabetic rat platelets is associated with increased oxygen consumption and increased ADP- and thrombin-induced aggregation in washed platelets (Iida, et al. 1993). Increased carnitine palmitoyltransferase-1 activity facilitates the entry of long-chain acyl-CoA's into the mitochondria hence the energy derived from fatty acid oxidation is

increased suggesting that carnitine palmitoyltransferase-1 may be involved in the control of platelet function.

In support of this possibility, Lascu et al demonstrated that in vitro incubation with long chain acyl-CoA's inhibited ADP or thrombin-induced aggregation of gel-filtered platelets (Lascu, et al. 1988). Further support is supplied by Ishikura et al (Ishikura, et al. 1992) demonstrated that in vivo treatment of rats with the irreversible carnitine palmitoyltransferase-1 inhibitor, 2-tetradecylglycidic acid, reduced the ex vivo oxygen consumption, ATP concentration, ATP/ADP ratio and maximum aggregation rate of platelets in response to ADP, thrombin and Ca^{2+} ionophore, A23187. The complete inhibition of mitochondrial carnitine palmitoyltransferase-1 activity observed in platelets from 2-tetradecylglycidic acid treated rats implied that no energy was supplied to these platelets via long-chain fatty acid oxidation, and that they were thus, largely dependent on glycolysis for energy production.

Thus there is some evidence to suggest that carnitine palmitoyltransferase-1 activity may modulate platelet aggregability. On the other hand, it is possible that this is not a specific cause and effect phenomenon: fatty acid oxidation is stimulated after the addition of thrombin to human platelets in vitro (Donabedian and Nemerson. 1971).

5.2 Objective of the study

Since the main objective of the current body of research is to characterise the anti-aggregatory effects of perhexiline, an obvious issue arises; is the known biochemical effects (carnitine palmitoyltransferase-1 inhibition) linked to perhexiline's observed inhibition of aggregation? Thus the aim of the present study was to compare the effects of perhexiline, amiodarone and trimetazidine on carnitine palmitoyltransferase-1 activity and aggregation in human platelets in vitro in order to determine whether the two effects were associated. A comparison was also made with the well-characterised carnitine palmitoyltransferase-1 inhibitors, hydroxyphenylglyoxylate and etomoxir.

5.3 Experimental Protocol

5.3.1 Subjects/patients

Subjects studied included normal volunteers (9 men and 19 women aged 44 ± 12.4 (S.D.) years, range 24-75), and patients undergoing routine diagnostic coronary angiography for investigation of stable angina pectoris (31 men and 13 women aged 61 ± 8.1 (S.D.) years, range 25-76). 27 patients were receiving either low dose aspirin, nitrates or verapamil; no other patient had taken any medication known to affect platelet function during 2 weeks prior to study.

5.3.2 Blood Sampling

Blood sample for platelets from patients were withdrawn through a femoral arterial sheath during cardiac catheterisation.

5.3.3 Platelet carnitine palmitoyltransferase-1 activity

Carnitine palmitoyltransferase-1 activity was measured by the method described in Chapter 2.

5.3.4 Platelet aggregation studies

ADP was utilised as a single pro-aggregant and platelet aggregation was measured by the method described in Chapter 2. Unless otherwise stated, blood samples were preincubated with either perhexiline, amiodarone, trimetazidine, hydroxyphenylglyoxylate or etomoxir for 5 min prior to induction of aggregation.

5.3.5 Data Analysis

The data are presented as mean $\pm$ SEM unless otherwise indicated. Inhibition of aggregation by perhexiline, amiodarone, trimetazidine, hydroxyphenylglyoxylate and etomoxir were evaluated as a percentage of maximal aggregation achieved in the absence of either agent. IC₅₀ was defined as the concentration of drug inhibiting carnitine palmitoyltransferase-1 activity and platelet aggregation by 50 %, while IC₃₀ was defined as the concentration of drug inhibiting carnitine palmitoyltransferase-1 activity by 30 %. The IC₅₀ and IC₃₀ data are presented as geometric means with 95 %

confidence limits. The data were analysed by non-paired t-test, and a critical value of $p=0.05$ was used throughout.

5.3.6 Chemicals

Dithiothreitol, fatty acid-free bovine serum albumin, malonyl-CoA lithium salt, oxfenicine, palmitoyl-CoA free acid, and saponin were obtained from Sigma (St. Louis, MO, USA). [^3H] 1-Carnitine (specific activity 77 Ci/mmol) was obtained from Amersham International (Aylesbury, Bucks, U.K.).

5.4 Results

5.4.1 Platelet carnitine palmitoyltransferase-1 activity

The endogenous carnitine palmitoyltransferase-1 inhibitor, malonyl-CoA, produced 91 ± 2 % ($n=6$) reduction of palmitoylcarnitine formation in permeabilized, washed human platelets, indicating that the majority of the activity measured was due to carnitine palmitoyltransferase-1. Perhexiline ($n=7$), amiodarone ($n=4$) and trimetazidine ($n=4$) inhibited platelet carnitine palmitoyltransferase-1 in a concentration-dependent manner (Figure 5.1). A full concentration-response curve could not be obtained for amiodarone due to difficulties in solubilising the drug at high concentrations in the incubation medium. However, the 45 ± 2 % inhibition of carnitine palmitoyltransferase-1 at 200 μM amiodarone suggests that it has a slightly higher potency than perhexiline which had a IC_{50} of 300 μM (Table 5.1). Trimetazidine was the least potent of these

agents as a platelet carnitine palmitoyltransferase-1 inhibitor. Oxfenicine (n=5) produced no inhibition of platelet carnitine palmitoyltransferase-1 up to a concentration of 2 mM, but its active metabolite, hydroxyphenylglyoxylate (n=11), inhibited carnitine palmitoyltransferase-1 in a concentration-dependent manner. Etomoxir (n=5) was the most potent of these agents with an IC_{50} of 0.11 μ M. Thus, the rank order of potency for carnitine palmitoyltransferase-1 inhibition in platelets was etomoxir > malonyl-CoA > hydroxyphenylglyoxylate > amiodarone $\geq$ perhexiline > trimetazidine (Table 5.1).

5.4.2 Platelet aggregation

Perhexiline, amiodarone and trimetazidine added to blood samples 5 min prior to induction of aggregation by ADP (1 μ M), produced inhibition of aggregation in a concentration-dependent manner (Figure 5.2). Threshold concentrations for inhibition of aggregation by both perhexiline (n=16) and amiodarone (n=13) were approximately 1 μ M, while for trimetazidine (n=5) threshold was 100 μ M. Perhexiline was the most potent inhibitor of platelet aggregation on a molar basis (Table 5.1). The inhibitory effects of perhexiline, amiodarone and trimetazidine were unaffected in patients on either aspirin, nitrates or verapamil. The well-characterised carnitine palmitoyltransferase-1 inhibitors hydroxyphenylglyoxylate (n=5) and etomoxir (n=5) did not inhibit ADP-induced aggregation in concentrations up to 800 μ M and 100 μ M respectively, which were in excess of those which produced inhibition of platelet carnitine palmitoyltransferase-1 activity (Figure 5.1). More prolonged pre-incubation of platelets (30 min) with etomoxir or hydroxyphenylglyoxylate also did not produce

inhibition of aggregation. Therefore, the rank order of potency of inhibition of ADP-induced aggregation was perhexiline > amiodarone > trimetazidine.

5.4.3 Comparison between normal subjects and patients

5.4.3.1 *Carnitine palmitoyltransferase-1 activity*

Potential variability in potency of carnitine palmitoyltransferase-1 inhibitors between normal subjects and patients with angina pectoris was investigated for perhexiline and etomoxir. In the case of perhexiline potency could be compared only in terms of IC_{30} , which was slightly but significantly ($P=0.047$) less for patients [117 μ M (72-191 μ M)] than for normal volunteers [200 μ M (140-280 μ M)]. While with etomoxir IC_{50} values in normals [0.11 μ M (0.05-0.21 μ M)] were significantly ($p=0.04$) lower than in patients with angina [0.36 μ M (0.16-0.83 μ M)] (Figure 5.3).

5.4.3.2 *Platelet aggregation*

There was no difference in regards anti-aggregatory effects of perhexiline between normals and patients (Figure 5.4).

5.5 Discussion

The major findings of this series of experiments was that the in vitro anti-aggregatory effects of perhexiline are not mediated by the inhibition of human platelet carnitine

palmitoyltransferase-1 under the conditions of the experiment. This also raises the question of the importance of fatty acids in the regulation of platelet aggregability.

Platelets, like other mammalian cells take up long-chain fatty acids and metabolise them to either CO₂ (via mitochondrial β -oxidation) or to lipid esters, a process primarily controlled by carnitine palmitoyltransferase-1. The carnitine palmitoyltransferase-1 enzyme has previously been shown to exist in rat platelet mitochondria (Iida, et al. 1991), while palmitoylcarnitine formation has been demonstrated in human platelet mitochondria (Vollset and Farstad. 1979) suggesting the involvement of carnitine palmitoyltransferase-1. However, the importance of carnitine palmitoyltransferase-1 to platelet energy metabolism is largely unknown, beyond the previously reported effects of carnitine palmitoyltransferase-1 inhibition with 2-tetradecylglycidic acid on platelet energetics (Ishikura, et al. 1992). The IC₅₀ for malonyl-CoA obtained for human platelets in the present study was of the same order of magnitude as that obtained by Iida et al for rat platelets (3.6 μ M compared with 0.9 μ M respectively) using similar incubation conditions (Iida, et al. 1991). Oxfenicine, an inhibitor of carnitine palmitoyltransferase-1 in other tissues (Higgins, et al. 1981; Bielefeld, et al. 1985) produced no inhibition of human platelet carnitine palmitoyltransferase-1 up to a concentration of 2 mM, but its active metabolite, hydroxyphenylglyoxylate, was inhibitory with a IC₅₀ of 45 μ M (Table 5.1). These data suggest that human platelets do not metabolise oxfenicine to hydroxyphenylglyoxylate as readily as does cardiac tissue (Stephens, et al. 1985).

Thus, as previously demonstrated in rat myocardium (Kennedy, et al. 1996), those inhibitors like perhexiline, amiodarone and trimetazidine whose carnitine palmitoyltransferase-1 binding site is destroyed by nagarse treatment are less potent carnitine palmitoyltransferase-1 inhibitors than the nagarse-sensitive agents hydroxyphenylglyoxylate and etomoxir in human platelets.

Despite the marked inhibition of human platelet carnitine palmitoyltransferase-1 by the irreversible inhibitor, etomoxir, and by the reversible inhibitor, hydroxyphenylglyoxylate, neither agent inhibited ADP-induced aggregation in whole blood. Hence, the present study has failed to provide evidence for a functional role of carnitine palmitoyltransferase-1 in platelet aggregation in vitro. The reason for the discrepancy between the present data and the inhibitory effect of tetradecylglycidic acid on rat platelet aggregability observed by Ishikura et al (Ishikura, et al. 1992) is not clear from these results. However, Ishikura et al treated rats in vivo with tetradecylglycidic acid (Ishikura, et al. 1992): it is possible that the metabolic consequences of carnitine palmitoyltransferase-1 inhibition in circulating platelets are more significant than those of in vitro application of carnitine palmitoyltransferase-1 inhibitors, this possibility could not be examined in the current study.

Although Lascu et al demonstrated that long chain acyl-CoA's inhibit ADP or thrombin-induced aggregation in gel-filtered platelets, considerably higher concentrations (20 fold) of acyl-CoA's were required to inhibit ADP-induced platelet-rich plasma (Lascu, et al. 1988). The authors accounted for this by presuming that acyl-CoA's bind to plasma

proteins such as serum albumin, therefore decreasing their activity. Thus the ability of acyl-CoA's or free fatty acids remains uncertain. It also raises the question as to whether inhibition of aggregation would have been demonstrated if Ishikura et al investigated whole blood or platelet-rich plasma aggregation instead of washed platelets (Ishikura, et al. 1992).

The finding that perhexiline inhibits aggregation of human platelets in whole blood extends the previous observations of Ono and Kimura in platelet-rich plasma (Ono and Kimura. 1981. Intriguingly a recent study by Stewart et al suggested that perhexiline was effective in suppressing symptoms in patients with otherwise refractory unstable angina (Stewart, et al. 1996), an observation consistent with the observed anti-aggregatory effect of perhexiline (Figure 5.2). In the present study the threshold concentrations for anti-aggregatory effects of perhexiline (1 μ M) correspond approximately to its therapeutic plasma concentrations (Stewart, et al. 1996; Unger, et al. 1997) when aggregation is induced by ADP-alone.

Amiodarone inhibits in vitro platelet aggregation in whole blood taken from patients with angina pectoris (Figure 5.2). Significant suppression of platelet aggregation was produced by concentrations greater than 20 μ M. Amiodarone was less potent as an inhibitor of platelet aggregation than perhexiline on a molar basis. Trimetazidine has previously been shown to inhibit human platelet aggregation in vitro (Devynck, et al. 1993; Astarie-Dequeker, et al. 1994). Threshold effects in the present study were seen at 100 μ M, this concentration is consistent with that obtained by Devynck et al., (Devynck,

et al. 1993). The concentrations of trimetazidine utilised in vitro are considerably greater than those occurring in vivo in humans (0.1-1 μ M) and therefore the relevance of the observed inhibition of aggregation to the in vivo effects of trimetazidine might be questioned (Devynck, et al. 1993). However, trimetazidine has previously been shown to reduce ex vivo platelet aggregation in patients with ischaemic heart disease (Higgins, et al. 1981) and to decrease the cyclic flow variation (platelet-rich thrombus formation) in stenosed canine coronary arteries (Belcher, et al. 1993). Both these observations suggest that trimetazidine has a clinically relevant anti-aggregatory effect and raise the possibility that the anti-aggregatory effects of trimetazidine are enhanced in vivo.

There are several limitation to the current study. Firstly, of the 72 subjects studied, only 10 had both carnitine palmitoyltransferase-1 inhibition and platelet aggregation studies. Secondly, we did not measure platelet energetics in this study. Given that Ishikura et al was able to demonstrate changes in both maximal extent of aggregation and platelet energetics (ATP/ADP ratio, oxygen consumption and lactate production) when utilising an irreversible carnitine palmitoyltransferase-1 inhibitor in vivo (Ishikura, et al. 1992), changes in platelet energetics after in vitro exposure could have been expected. However, this issue is essentially answered by the results of the present study: etomoxir while inhibiting carnitine palmitoyltransferase-1 activity at very low concentrations was unable to inhibit whole blood aggregation at incubation times up to 30 min. Therefore, under the conditions of this study platelet energetics are not critical to aggregability. And thirdly, the finding that carnitine palmitoyltransferase-1 inhibition per se (by hydroxyphenylglyoxylate or etomoxir) is not associated with detectable effects on

platelet aggregability has only been explored at this stage in normoxic platelets. It is unknown whether during or after hypoxia in vivo, effects on relative glucose vs long chain fatty acid metabolism may have greater consequences.

5.6 Conclusion

The conclusions from the current study are: 1) Perhexiline inhibits carnitine palmitoyltransferase-1 in platelets about to the same extent as in myocardium (this observation is not without practical interest, ie could platelet carnitine palmitoyltransferase-1 inhibition ex vivo be used as a means of monitoring perhexiline effect in the body eg. Myocardium); 2) There is definitely at least one anti-aggregatory mechanism beyond carnitine palmitoyltransferase-1 inhibition; and 3) carnitine palmitoyltransferase-1 inhibition appears irrelevant to platelet aggregation, as far as we can see. With the caveats mentioned above, the results of the present study suggest that perhexiline, amiodarone and trimetazidine have anti-aggregatory effects reflecting a different, as yet unidentified, pharmacological effect. In view of the recent observations suggesting a clinical role for perhexiline in unstable angina pectoris (Stewart, et al. 1996) this additional effect is likely to be of therapeutic significance.

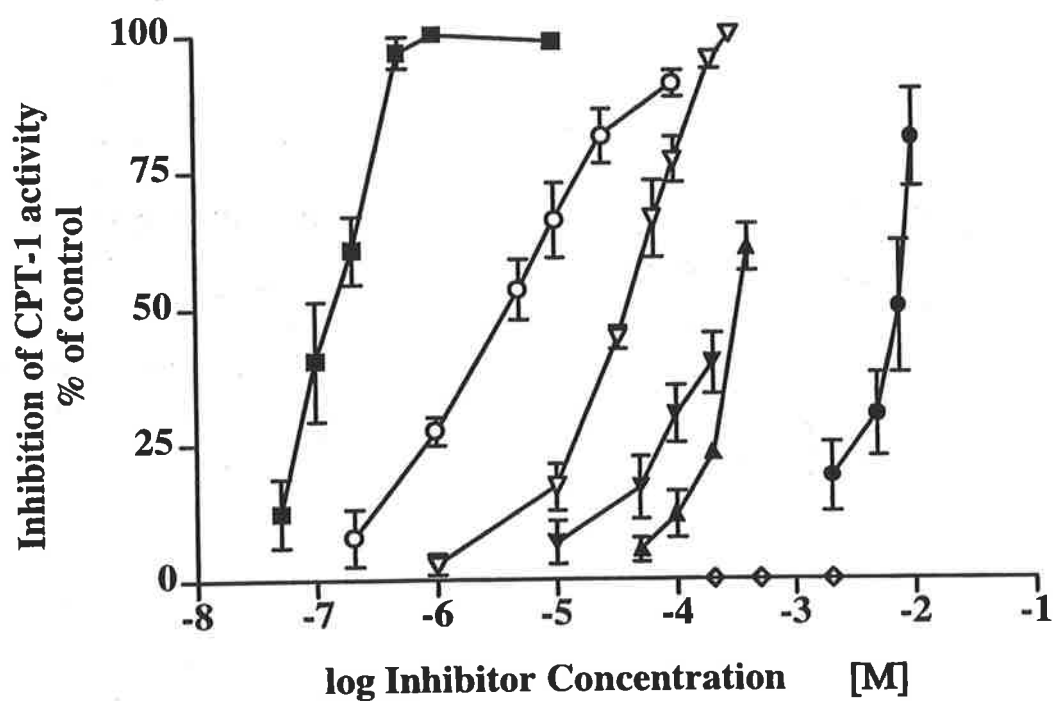


Figure 5.1.

Inhibition of carnitine palmitoyltransferase-1 in human platelets by etomoxir (■), malonyl-CoA (○), hydroxyphenylglyoxylate (▽), oxfenicine (◇), amiodarone (▼), perhexiline (▲) and trimetazidine (●).

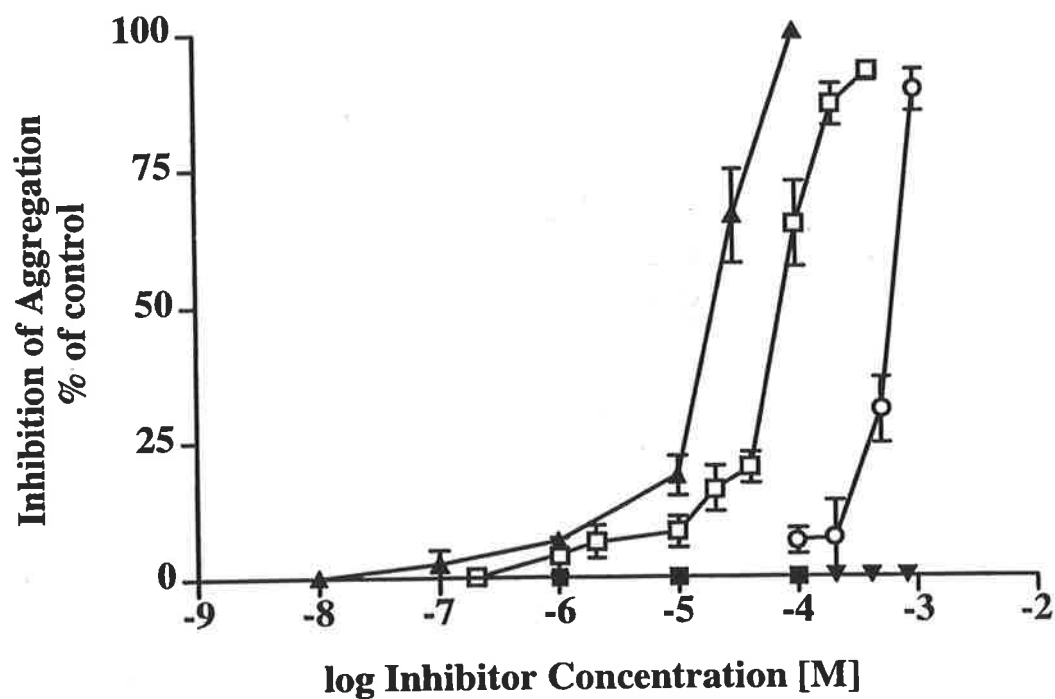


Figure 5.2.

Inhibition by perhexiline ($\blacktriangle$), amiodarone ($\blacktriangledown$) and trimetazidine ($\bullet$) of ADP-induced platelet aggregation in whole blood taken from patients with stable angina. Hydroxyphenylglyoxylate (∇) and etomoxir ($\blacksquare$) did not inhibit aggregation.

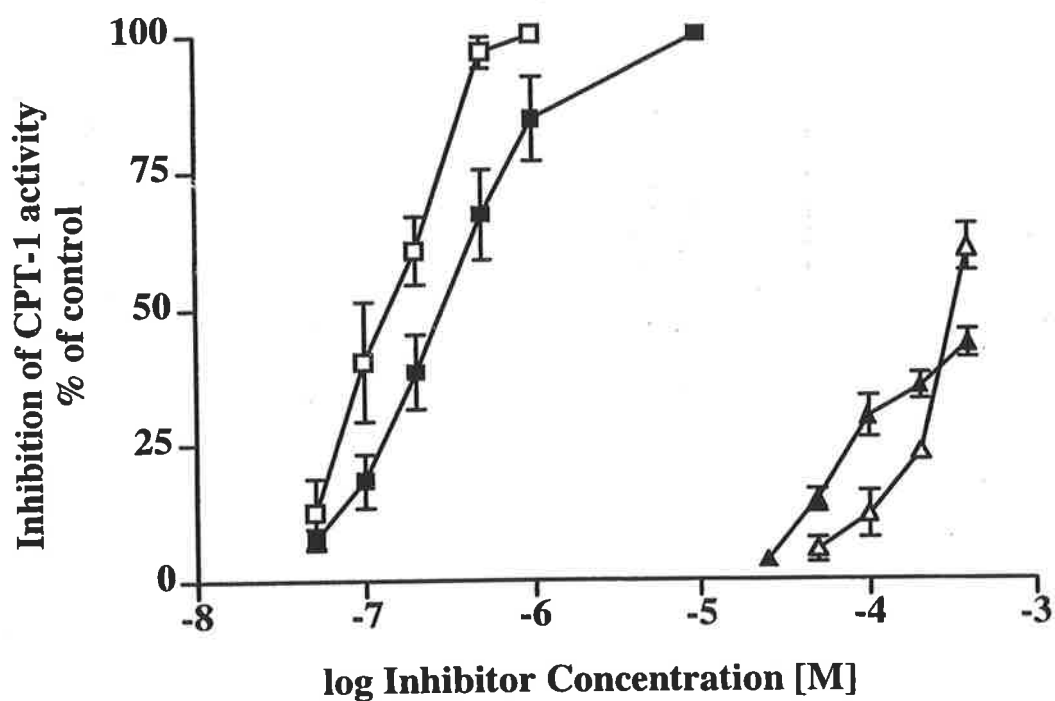


Figure 5.3.

Inhibition of carnitine palmitoyltransferase-1 in human platelets from normal volunteers (open symbols) and patients (closed symbols), by etomoxir (■) and perhexiline (▲).

Table 5.1.

IC₅₀ values for platelet aggregation and carnitine palmitoyltransferase-1 (CPT-1) activity.

Agents	Platelet aggregation	CPT-1 activity
	IC ₅₀ (μM)	IC ₅₀ (μM)
Perhexiline	21(18-26)	300 (70-1400)
Amiodarone	62 (44-87)	^a
Trimetazidine	614 (396-954)	6500 (5212-8375)
Hydroxyphenylglyoxylate	^b	45 (30-67)
Etomoxir	^b	0.11 (0.05-0.21)
Malonyl-CoA	^c	3.6 (1.7-7.6)

IC₅₀ values are expressed as mean ± 95 % confidence limits. ^a in washed platelets amiodarone (200 μM, n=4) inhibit carnitine palmitoyltransferase-1 activity by 45±2 %. Amiodarone was insoluble in the assay medium above 200 μM. ^b Hydroxyphenylglyoxylate and etomoxir did not inhibit platelet aggregation at any concentration tested (Figure. 2). ^c Malonyl-CoA was not utilised in whole blood experiments.

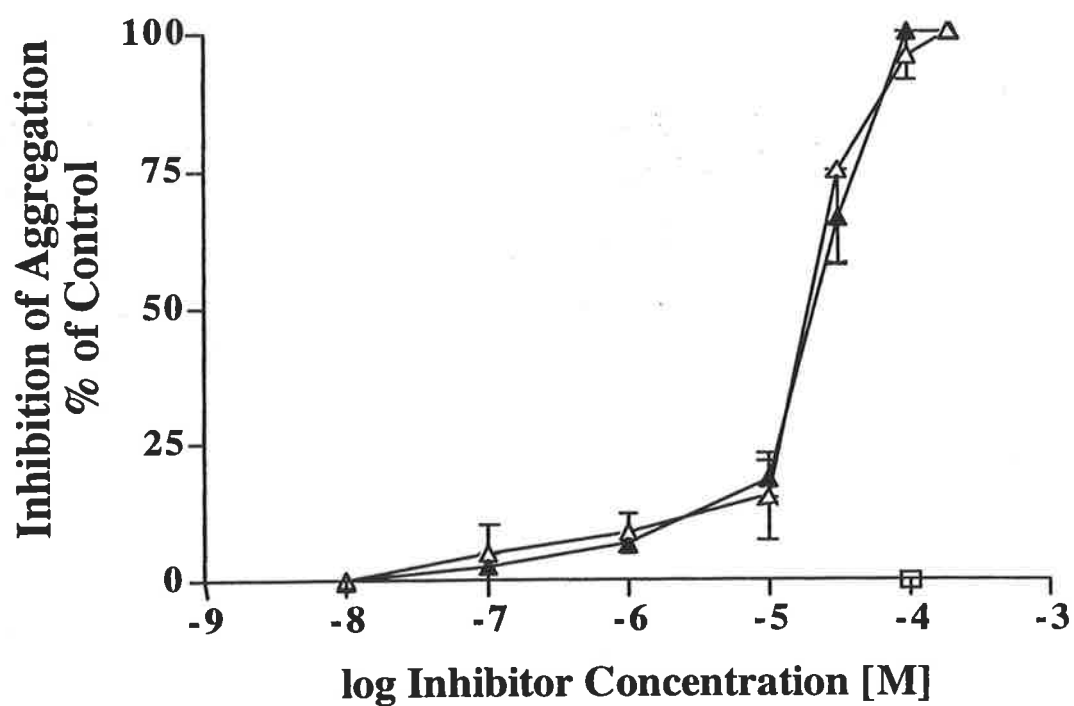


Figure 5.4.

Inhibition of ADP-induced aggregation by perhexiline in whole blood taken from patients (▲) and normal volunteers (Δ). Etomoxir did not inhibit ADP-induced aggregation in blood sample from either patients or normal volunteers.

Chapter 6

EX VIVO EFFECTS OF PERHEXILINE: PLATELET AGGREGABILITY AND NITRIC OXIDE RESPONSIVENESS.

6 Chapter 6: Ex vivo effects of Perhexiline: Platelet aggregability and Nitric Oxide Responsiveness

6.1 Summary

Platelets from patients with stable and unstable angina exhibit de novo resistance to the anti-aggregating effects of nitric oxide donors. From previous clinical and in vitro data perhexiline exerts a relatively weak anti-aggregatory effect, but appears to accelerate resolution of symptoms in unstable angina pectoris. We examined the effects of perhexiline, in vitro and ex vivo, on platelet aggregation and response to the nitric oxide donor sodium nitroprusside (SNP) in blood samples from stable angina pectoris and acute coronary syndrome (unstable angina pectoris or Non-Q-wave myocardial infarction) patients. Acute coronary syndrome (n=50) and stable angina (n=30) patients were studied before and after addition of perhexiline to prior anti-anginal therapy. Inhibitory effects of SNP (10 μ M) on ADP (1 μ M) -induced platelet aggregation in whole blood were determined. Patients with acute coronary syndromes not receiving perhexiline (n=12) and normal subjects (n=24) provided comparative data in order to exclude aggregability changes associated with efflux of time with acute coronary syndromes, and to establish a normal range of responses respectively.

At baseline, acute coronary syndrome patients had a trend towards increased platelet aggregability in comparison to normals ($p=0.10$). Anti-aggregatory effects of SNP were reduced in patients: $51\pm4\%$ inhibition of aggregation in normals; $35\pm5\%$ and $29\pm3\%$

inhibition in stable angina ($p<0.05$) and acute coronary syndrome ($p<0.01$) patients, respectively.

No significant changes in response to ADP were observed in either group of patients relative to normals. However, in the presence of perhexiline, response to SNP increased to $39\pm4\%$ in acute coronary syndrome and $53\pm4\%$ in stable angina patients ($p<0.01$ vs baseline in both cases). In patients with acute coronary syndromes resolution of symptoms of ischaemia within 3 days of perhexiline therapy ($n=29$) was associated with significantly greater ($p<0.01$) increases in platelet responses to SNP than non-resolution ($n=11$). Plasma perhexiline concentration varied widely: for the stable angina patients, mean 0.68 ± 0.15 $\mu\text{g/L}$ (range 0.00 - 2.94 $\mu\text{g/L}$), while for the acute coronary syndrome patients, mean 0.60 ± 0.07 $\mu\text{g/L}$ (range 0.05 - 2.23 $\mu\text{g/L}$). In acute coronary syndrome patients not receiving perhexiline, there were no serial changes in SNP response.

Acute coronary syndrome patients were also examined for the effect of perhexiline on whole blood superoxide content utilising lucigenin-based chemiluminescence. Perhexiline therapy did not significantly affect superoxide content ($n=11$). Similarly, superoxide content did not fluctuate in patients who did not receive perhexiline ($n=3$). Similarly, superoxide dismutase (SOD), in conjunction with catalase, did not alter the SNP response of acute coronary syndromes patients either receiving ($n=6$) or not receiving ($n=4$) perhexiline.

The interaction between perhexiline and nitric oxide was further examined using the

selective soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) in patients with acute coronary syndromes (n=10). Prior to the commencement of perhexiline SNP response was 27.5 ± 7 % inhibition of aggregation in this group of patients. ODQ inhibited the anti-aggregatory effect of SNP by 7.1 ± 3.1 %, demonstrating that the majority of SNP response is mediated by guanylate cyclase-independent mechanisms in this group of patients. After perhexiline therapy, the SNP response increased to 43.9 ± 9.2 % inhibition of aggregation, while ODQ inhibited the SNP response by 12.0 ± 3.6 %. These data therefore suggest that perhexiline therapy increased both the guanylate cyclase-independent and guanylate cyclase-dependent components of nitric oxide effect.

In vitro, perhexiline (1-10 μ M) had no significant effect on platelet responsiveness to SNP.

Conclusions: In acute coronary syndrome and stable angina pectoris patients, perhexiline, in doses which have no detectable incremental anti-aggregatory effect in the presence of background anti-platelet therapy, improves (towards normal) platelet responses to the anti-aggregatory effects of SNP. This normalization of response towards exogenous nitric oxide donors implies a better response to endogenous nitric oxide (EDRF); this may contribute to the anti-anginal efficacy of perhexiline. The mechanism of the change in nitric oxide responsiveness is not established by the current data. However, the current series of experiments permit exclusion of changes in superoxide content as a mechanism, while suggesting that perhexiline potentiates both

NO- dependent and -independent components of the anti-aggregatory effect. The results suggest that these changes may contribute to the therapeutic effects of perhexiline in acute coronary syndromes, and possibly to prevention of acute coronary events in patients with stable angina.

6.2 Introduction

As discussed in Chapter 1, during the early development of perhexiline a large number of controlled studies examined the efficacy of perhexiline as either monotherapy or in combination with a limited number of prophylactic agents in the management of stable angina pectoris. These studies provided evidence of perhexiline's clinical efficacy as regards improved exercise tolerance and reduced anginal frequency (Armstrong. 1973; Gitlin. 1973; Gitlin and Nellen. 1973; Linquette, et al. 1973; Pilcher. 1973; Pilcher, et al. 1973; Souza. 1973; Warembourg, et al. 1973; Barraine, et al. 1974; Pepne, et al. 1974; Brown, et al. 1976; Hitchcock, et al. 1977; Pilcher. 1978; Horgan, et al. 1981).

Perhexiline has been demonstrated to provide incremental benefits in the presence of other anti-anginal agents among patients who remain symptomatic despite being treated with β -adrenoceptor blockers (Morgans and Rees. 1973; Armstrong, et al. 1974; White and Lowe. 1983) and combinations of other (nitrates, β -adrenoceptor blockers and calcium channel antagonists) anti-anginal agents (Horowitz and Mashford. 1979; Horowitz, et al. 1986; Cole, et al. 1990). Cole et al evaluated the clinical efficacy of perhexiline in a double-blind placebo controlled crossover study among 17 patients with angina refractory to maximal anti-anginal therapy including nitrates, β -adrenoceptor blockers and a calcium channel antagonist. Anginal frequency and severity was reduced in 11 patients receiving perhexiline compared to no patients receiving placebo. A marked improvement in exercise tolerance was also demonstrated in the patients receiving perhexiline (Cole, et al. 1990).

More recently, perhexiline has been utilised in the treatment of unstable angina pectoris (Horowitz and Henry. 1987; Stewart. 1995; Stewart, et al. 1996), although controlled clinical trial data are lacking. Stewart et al studied 40 patients admitted to a coronary care unit for treatment of unstable angina (Stewart, et al. 1996). The majority (31 of 40) patients had remained symptomatic despite combined use of aspirin, heparin, intravenously infused nitroglycerine (NTG) and verapamil. Furthermore, in 16 patients, N-acetylcysteine (NAC) was co-infused with NTG (Horowitz, et al. 1988; Horowitz, et al. 1988) without elimination of symptoms. Perhexiline therapy was administered with an initial loading dose, which consists of 400mg/day of perhexiline for 3 days (Horowitz, et al. 1986). This regimen is associated with progressive but very variable increases in plasma perhexiline concentrations, with therapeutic [0.15-0.60 µg/L (Horowitz, et al. 1986; Cole, et al. 1990; Horowitz, et al. 1995)] concentrations being present in most cases within 3 days. Angina resolved within 72 hours in 28 of 40 patients (Stewart, et al. 1996). The results suggested ($p=0.055$) a direct relationship between resolution of anginal symptoms and plasma perhexiline concentration. While these data are therefore not conclusive, they provide some support for the hypothesis that perhexiline may be effective in otherwise intractable cases of unstable angina. On the other hand, the mechanism of such an effect was not evaluated.

Platelet aggregation is central to the pathogenesis of arterial thrombosis. Apart from the effects of plaque fissure and rupture in providing a stimulus for local platelet adhesion and aggregation, platelet hyperaggregability plays a role in acute coronary syndromes

(Opie. 1980; Mehta and Mehta. 1981; Meade, et al. 1985; Fitzgerald, et al. 1986; Frishman and Miller. 1986; Willerson, et al. 1986; Conti and Mehta. 1987; Bashour, et al. 1988; Kroll and Schafer. 1989; Maseri. 1990; Schrader and Berk. 1990; Diodati, et al. 1992; Fuster, et al. 1992; Badimon, et al. 1993; Cahill and Newland. 1993; Diodati, et al. 1994; White. 1994; Frishman, et al. 1995; Kamat and Kleiman. 1995; Farstad. 1998; Puri and Colman. 1997). The use of anti-aggregatory agents has become routine in the management of myocardial infarction and unstable angina (Frishman and Miller. 1986; Frishman, et al. 1995; Alexander and Harrington. 1998; Cannon and Smith. 1999). As perhexiline inhibits in vitro platelet aggregation, the possibility exists that a component of its ability to provide anti-anginal effects incremental to those of conventional therapy is due to a direct or indirect anti-platelet effect. The results of the study by Stewart et al , in unstable angina provide some support for this hypothesis (Stewart, et al. 1996). However, the magnitude of the effect of perhexiline in this regard would have to be considerable in order to be detectable beyond the combined anti-ischaemia and anti-aggregatory effects of the usual pharmacotherapy used in the management of such patients. On the other hand, perhexiline might be effective in both stable and unstable angina primarily via changes in efficiency of myocardial oxygen utilisation, predicted on carnitine palmitoyltransferase-1 inhibition (Kennedy, et al. 1996).

Platelets contain both constitutive and inducible nitric oxide synthase (Mehta, et al. 1995; Chen and Mehta. 1996). Unstable angina pectoris is associated with apparently decreased activity of platelet nitric oxide synthase (Sase and Michel. 1995), potentially reducing platelet concentrations of, and anti-aggregatory effect of nitric oxide.

Freedman et al demonstrated that platelets from patients with unstable angina pectoris produce less nitric oxide than patients with stable angina pectoris (Freedman, et al. 1998), and normal volunteers (Freedman, et al. 1997). Furthermore, Chirkov et al have recently demonstrated that platelets from patients with stable angina are relatively resistant to nitric oxide (Chirkov, et al. 1999).

“Nitrate resistance” at the vascular level is well documented in congestive heart failure (Armstrong, et al. 1980; Magrini and Niarchos. 1980; Elkayam, et al. 1985; Packer, et al. 1986; Armstrong. 1987; Elkayam, et al. 1987; Roth, et al. 1987; Kulick, et al. 1988; Abrams. 1991) and has been suggested to be present in up to 50% of congestive heart failure patients with severe left ventricular failure (Elkayam, et al. 1985; Armstrong. 1987). Typically these patients, in the absence of prior nitrate therapy exhibit a primary or “de novo” resistance to effects of nitrates on preload (ie the venodilator effects of nitrates) for example via failing to significantly reduce pulmonary capillary wedge pressure despite infusion of very high concentrations of NTG (Armstrong, et al. 1980). The mechanisms and specificity of “nitrate resistance” in blood vessels have not been subjected to detailed examination to date.

It is also unclear to what extent de novo “nitrate resistance” occurs in patients with ischaemic heart disease in the absence of systolic heart failure. Recent studies have emphasised that “nitrate resistance” may also occur as a component of the oxidant response to hypertriglyceridemia (Lundman, et al. 1997). Furthermore, “normal” black adults are hypo-responsive to nitric oxide relative to Caucasians (Cardillo, et al. 1999).

As regards platelet aggregability, previous work by Chirkov et al has demonstrated that patients with stable angina, in comparison with normal volunteers, exhibited not only increased platelet aggregability but also reduced sensitivity to the anti-aggregatory effects of both organic nitrates (NTG) and the non-nitrate nitric oxide donor sodium nitroprusside (SNP) (Chirkov, et al. 1996; Chirkov, et al. 1995; Chirkov, et al. 1993). The anti-aggregatory effects of NTG and other vasodilators are mediated primarily via formation of nitric oxide, which activates platelet guanylate cyclase, leading to generation of cGMP (for review see Anderson, et al. 1994). While the effects of NTG are mediated primarily by thiol-dependent bioconversion to nitric oxide (Noack and Feelisch. 1991), SNP is a more direct nitric oxide donor (Anderson, et al. 1994; Feelisch and Noack. 1987; Bates, et al. 1991); therefore, reduced sensitivity to both NTG and SNP suggests a reduction in responsiveness to nitric oxide, rather than purely an impairment of enzymatic denitration of organic nitrates.

Recently, Chirkov et al has extended the above observation by examining the phenomenon of "nitrate resistance" in blood samples from normal volunteers and patients with stable angina pectoris (Chirkov, et al. 1999). The authors demonstrated that platelets from patients with ischaemic heart disease manifested increased sensitivity to ADP as an inductor of aggregation. On the other hand, the anti-aggregatory and cGMP-stimulating effects of NTG and SNP were markedly reduced in platelets from patients relative to normal subjects, thus representing de novo "nitrate resistance" at the platelet level. This result is consistent with their previous findings (Chirkov, et al. 1996).

The decreased platelet response to NTG and SNP in ischaemic patients appears to be associated (inter alia) with defect(s) in the nitric oxide/cGMP pathway. In this respect, Chirkov et al demonstrated that with NTG and SNP, effects of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, inhibitor of nitric oxide-stimulated guanylate cyclase activity) are significantly less pronounced in patients than in normals (Chirkov, et al. 1999). Concentrations of superoxide in blood were elevated in the presence of "nitrate resistance". This result suggests that "nitrate resistance" is produced by either a decrease in nitric oxide-sensitivity of platelet guanylate cyclase, and/or decreased availability of NO for activation of guanylate cyclase because of clearance of nitric oxide by superoxide (Iuliano, et al. 1997). Furthermore, nitrate resistance involves an increased component of guanylate cyclase-independent inhibition of aggregation by nitric oxide donors.

Chirkov et al demonstrated that superoxide scavenger, superoxide dismutase (SOD) in conjunction with catalase inhibited ADP-induced aggregation and enhanced the anti-aggregatory effects of SNP in patients with ischaemic heart disease (Chirkov, et al. 1999). This implies that superoxide contributes to the diminished platelet responsiveness to nitric oxide donors and is partly responsible for the phenomenon of "nitrate resistance" [or more correctly, "nitric oxide resistance"]. The authors also noted that decreased platelet responsiveness to exogenous sources of nitric oxide implies diminution of responsiveness to endogenous sources of nitric oxide (endothelium-derived relaxing factor and S-nitrosothiols).

Despite previous *in vitro* studies, perhexiline (although demonstrated in this (see Chapter 4) and one previous (Ono and Kimura. 1981) study to inhibit *in vitro* platelet aggregation) has not been investigated for possible effects on *ex vivo* platelet aggregation. Therefore, a study of the effects of the acute perhexiline therapy on *ex vivo* platelet aggregation and platelet responsiveness to nitric oxide (and nitric oxide donors) was conducted in patients with stable angina pectoris and acute coronary syndromes. However, a problem inherent in such investigations is the lack of routine perhexiline use as monotherapy. In general, perhexiline is added to the conventional therapies of NTG, calcium antagonists (and β -blockers), which have well-documented anti-aggregatory actions. Hence it was recognised that perhexiline effects on aggregation might be obscured by those of concomitant pharmacotherapy.

6.3 Objective of the study

This study was designed to examine the effect of perhexiline maleate therapy on *ex vivo* platelet aggregation and platelet SNP responsiveness in the following groups of cardiac patients: 1) patients with stable angina pectoris and 2) patients with acute coronary syndromes.

6.4 Methods

6.4.1 Subjects

6.4.1.1 *Normal subjects*

24 healthy volunteers participated in the study (11 males and 13 females; mean age: 38 ± 3 years). No volunteer had taken any medication known to affect platelet aggregation in the two weeks prior to blood sampling. Normal subjects were utilised to compare baseline extent of ADP-induced aggregation and SNP responsiveness with those of the stable and acute coronary syndrome patients.

6.4.1.2 *Stable angina pectoris*

30 consecutive patients (see Table 1 for summary) attending an outpatient clinic of The Queen Elizabeth Hospital for follow-up management of previously diagnosed stable angina pectoris were enrolled in the study. Patients were eligible for inclusion in the study if perhexiline maleate therapy was to be initiated because patients remained symptomatic, despite being treated with combinations involving aspirin, oral nitrates, β -adrenoceptors and Ca^{2+} channel blockers. Patients were excluded if there was any suggestion of cognitive or communication deficiency likely to affect data collection.

6.4.1.2.1 *Perhexiline maleate therapy*

The majority of patients received a loading regimen of perhexiline maleate which consisted of 200 mg twice daily for 3 days (72 hours) and 100 mg twice daily thereafter (Horowitz, et al. 1986).

6.4.1.3 *Acute coronary syndromes*

50 consecutive patients (see Table 2 for summary) presented to the Coronary Care Unit of The Queen Elizabeth hospital with a diagnosis of either unstable angina pectoris or non-Q-wave myocardial infarction. Unstable angina pectoris (n=33) was diagnosed on the basis of typical ischaemic chest pain occurring at rest with associated ST segment depression on echocardiography (ECG). Non-Q-Wave myocardial infarction (n=17) was diagnosed by the presence of typical chest pain, ischaemic ECG changes (and a diagnostic elevation of creatine kinase concentration) without subsequent development of pathological Q waves. Patients were eligible for inclusion in the study if perhexiline maleate was initiated because frequent episodes of chest pain persisted at rest, despite maximal conventional therapy. Conventional therapy consisted of 1) aspirin 150 mg/day, 2) intravenous unfractionated heparin, comprising an initial bolus of 5000 units followed by an infusion of 1000 units/hour titrated thereafter to maintain an APTT of 60-90 seconds, 3) an intravenous infusion of NTG, initial rate 5-10 µg/minute titrates to a maximum of 20 µg/minute to achieve amelioration of symptoms and maintain systolic blood pressure of >90 mmHg. In refractory cases NTG was co-infused with intravenous NAC 10g/24 hours (Horowitz, et al. 1988) and 4) a bolus intravenous dose of verapamil

(1-5 mg over 5 minutes) followed by oral verapamil (240 mg) or an oral β -adrenoceptor blocker. Deviations from the above conventional pharmacotherapy were made on the following basis:- 1) NTG withheld or dose reduced due to persistent hypotension and 2) verapamil withheld because of evidence of severe left ventricular systolic dysfunction. Patients were excluded if there was any suggestion of cognitive or communication deficiency likely to affect data collection. Chronic symptomatology potentially able to be confused with acute perhexiline maleate toxicity (ie nausea/dizziness) was also a criterion for exclusion.

6.4.1.3.1 *Perhexiline maleate therapy*

All patients with acute coronary syndromes received a loading regimen of perhexiline maleate which consisted of 200 mg twice daily for 3 days (72 hours) and 100 mg twice daily thereafter (Horowitz, et al. 1986).

6.4.1.4 *Acute coronary syndrome patients not receiving perhexiline*

A cohort of patients (n=12; see Table 2 for summary) admitted to the Coronary Care Unit of The Queen Elizabeth Hospital for the diagnosis of unstable angina pectoris (n=4) or non-Q-wave myocardial infarction (n=8) who were not treated with perhexiline during their first 3 days of hospitalisation were assessed in order to examine the effects of efflux of time on platelet responsiveness to SNP. It was recognised that the severity of symptoms at baseline for this cohort was less than that of the patients receiving

perhexiline; hence these patients in one important respect do not constitute a true “control” group.

6.4.2 Symptomatic status

A subset of patients with stable angina pectoris (n=22) completed an anginal diary documenting the number of ischaemic episodes experienced per day. All patients with acute coronary syndromes (including “control” acute coronary syndrome patients) answered a questionnaire on days 2 and 3 of perhexiline therapy regarding their symptomatic profile in the previous 24 hours. This information was correlated with information from medical records, drug charts, ECG’s and arrhythmia monitoring data. Symptomatic status was then categorised on each day post perhexiline maleate initiation according to the following: 1) angina absent or present and 2) nausea and/or dizziness (symptoms consistent with “short-term” toxic effects of perhexiline (Stewart, et al. 1996)) absent or present.

Symptomatic status in all cases was classified by interviewers blinded to the results of simultaneous plasma perhexiline concentration.

6.4.3 Blood sampling

Blood samples were collected as described in Chapter 2. Blood samples from patients were obtained immediately prior to commencement of perhexiline maleate therapy and after 1-2 weeks of therapy in the case of stable angina patients or 3 days for unstable

angina patients. At the second collection blood was also withdrawn to determine the plasma perhexiline concentration. Plasma perhexiline concentration was measured according to a modification (Morris, et al. 1992) of a standard HPLC assay (Horowitz, et al. 1981) which has a threshold sensitivity for perhexiline of 0.05 µg/ml.

Perhexiline has been shown to induce falls in blood sugar levels in diabetes (Roger, et al. 1975; Dally, et al. 1977; Cousteau, et al. 1978; Fournier, et al. 1978; Luccioni, et al. 1978; Schlienger, et al. 1978; Erhart, et al. 1981) this may reflect changes in responsiveness to insulin. Because of previous studies showing that vascular responsiveness to NO may be altered in the presence of increased insulin and / or decreased blood glucose concentrations, changes in blood sugar levels post introduction of perhexiline were documented in all diabetic subjects receiving either insulin or sulphonylureas (n=17). Furthermore, responses to perhexiline were subsequently compared in diabetic and non-diabetic individuals. Blood sugar levels were also documented in the diabetic patients who did not receive perhexiline (n=4).

6.4.4 Platelet aggregation studies

ADP (1 µM) was utilised as a single pro-aggregant, and platelet aggregation was measured by the method described in Chapter 2. Blood samples were preincubated with sodium nitroprusside (SNP 10 µM) for 1 min prior to induction of aggregation.

6.4.5 Chemiluminescence assay for superoxide

In order to examine possible correlation between perhexiline therapy and changes in whole blood superoxide concentrations, superoxide content was assayed via lucigenin chemiluminescence (see Chapter 2 for methodology) before and 3 days after introduction of perhexiline (n=11). In patients not treated with perhexiline (n=3) paired superoxide assays were performed to exclude changes associated with resolution of ischaemia.

6.4.6 Superoxide dismutase (SOD) / catalase

In order to test the hypothesis that changes in nitric oxide responsiveness in the presence of perhexiline were induced by increased tissue generation of superoxide / hydrogen peroxide, the effects of SOD / catalase on response to SNP in the presence (n=6) and absence (n=4) of perhexiline were compared. In both subgroups, ADP and SNP responses were determined at admission and after 3 days.

6.4.7 Guanylate cyclase studies

To further examine the phenomenon of “nitrate resistance” the effects of perhexiline on nitric oxide/soluble guanylate cyclase interactions were investigated in a subset of acute coronary syndrome patients receiving perhexiline (n=10) using the potent and selective guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μ M).

6.4.8 Effects of perhexiline on SNP responses in vitro

In order to examine the possibility of a direct effect of perhexiline on platelet responses to SNP, a cohort of patients (n=15) undergoing routine diagnostic coronary angiography for investigation of stable angina pectoris were studied. Platelet aggregation was measured as described above. Blood samples were preincubated with perhexiline (5 min) and SNP (1 min). Aggregation was induced by either ADP alone (1 μ M, n=11) or by the multiple agonist approach (n=4). Responsiveness of perhexiline (1-10 μ M) and SNP (10 μ M) individually and in combination was determined. Inhibition of aggregation was evaluated as a percentage of maximal aggregation achieved in the absence of either agent. Potentiation in the presence of both agents was defined on the basis of nett inhibition greater than the sum of the individual effects.

6.4.9 Statistical analysis

Inhibition of aggregation by SNP was evaluated as percentage of maximal aggregation in the absence of the agent. Comparison of baseline ADP-induced aggregation and SNP responsiveness was examined using analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Changes in extent of aggregation and SNP responsiveness produced by perhexiline therapy were examined using paired t-tests. The relationship between changes in SNP responsiveness, extent of ADP-induced aggregation and plasma perhexiline concentration was examined using linear regression. Results are expressed throughout as mean $\pm$ SEM, for normally distributed data.

6.5 Results

6.5.1 Baseline responsiveness

Both males and females were used in the study, although differences between males and females have previously been reported (Meade, et al. 1985), with platelet aggregation occurring to a greater extent in women than in men for an equivalent ADP concentration. However, in the present study, neither baseline differences between sexes for ADP responsiveness nor the inhibition by SNP were observed; therefore those data were pooled.

6.5.1.1 *Extent of ADP-induced aggregation*

Blood samples obtained from normal volunteers, stable angina and acute coronary syndrome patients were compared for baseline extent of platelet aggregation induced by ADP (Figure 6.1). The baseline clinical characteristic of the 30 stable angina patients and 50 acute coronary syndrome patients are summarised in Table 6.1 and Table 6.2 respectively. As discussed in the introduction, platelet hyperaggregability has been documented in a number of coronary artery disease states, and proposed to be a factor predisposing towards thrombotic events. Interpretation of the results of the study is potentially complicated by the fact that anti-platelet therapy (eg. aspirin, nitrates and calcium antagonists; see Chapter 1) among the patient population was variable. However, as the study was designed to elucidate incremental anti-platelet effects of perhexiline beyond existing therapies, patient medications were not altered.

Comparison of the baseline extent of aggregation between normals, stable angina and acute coronary syndrome patients produced no significant differences (ANOVA, $F=1.55$, $p=0.21$, Figure 6.1). However, acute coronary syndrome patients exhibited a trend towards increased platelet aggregability induced by ADP when compared with normal subjects (12.5 ± 0.9 vs 10.6 ± 0.7 Ohms, $p=0.10$). In contrast to previous observation (Chirkov, et al. 1999) no significant difference was observed for extent of ADP-induced aggregation between stable angina patient and normals (11.1 ± 0.9 vs 10.6 ± 0.7 Ohms, $p=0.65$).

6.5.1.2 Baseline SNP responsiveness

SNP inhibited platelet aggregation in whole blood samples from normal subjects, stable angina and acute coronary syndrome patients, but to differing extents (ANOVA, $F=9.30$, $p=0.0002$, Figure 6.2). A significant reduction in the degree of inhibition by SNP was observed for acute coronary syndrome patients versus normal subjects (29.4 ± 2.7 vs 50.5 ± 3.4 % inhibition of aggregation, $p<0.001$) and stable angina patients versus normal subjects (35.3 ± 4.8 vs 50.5 ± 3.4 % inhibition of aggregation, $p<0.05$).

6.5.2 Effect of perhexiline therapy on stable angina patients

The effects of short term (mean 8.0 ± 0.6 days) perhexiline therapy was examined in 30 patients with stable angina. After commencement of perhexiline therapy there was no significant difference in the extent of ADP-induced aggregation (10.8 ± 0.9 Ohms) when compared to baseline data (11.1 ± 0.9 Ohms, $p=0.7$). Therefore perhexiline did not

appear to have a “direct” anti-aggregatory effect when assessed ex vivo (Figure 6.3) in this group of patients.

However, there was a significant increase from baseline values in the responsiveness towards SNP after perhexiline therapy (35.3 ± 4.8 vs 51.7 ± 4.2 % inhibition of aggregation, $p < 0.001$, Figure 6.4). The SNP response in the presence of perhexiline was therefore similar to that observed in normal subjects.

There was no significant correlation between change in extent of aggregation and change in SNP response ($r^2 = 0.001$, $p = 0.98$) in this group of patients (Figure 6.5).

Prior to commencement of perhexiline therapy the anginal frequency in these patients was 6.7 ± 0.9 episodes per day [median 5], while after short-term therapy the anginal frequency had significantly decreased to 1.0 ± 0.3 episodes per day [median 0] ($p < 0.001$, Figure 6.6).

Plasma perhexiline concentration after short-term therapy in this group of patients (Figure 6.7) was 0.68 ± 0.15 $\mu\text{g/L}$ (range 0.00 - 2.94 $\mu\text{g/L}$), with 30% of patients having levels greater than the upper limit of the therapeutic range (ie $> 0.6 \mu\text{g/L}$) and 33 % having subtherapeutic levels ($< 0.15 \mu\text{g/L}$). Approximately 7% of the Caucasian population are slow hydroxylators (see Chapter 1), and consistent with this 3 patients (10 %) had very elevated plasma perhexiline levels. While many such patients ($n=6$)

developed toxic side-effects of nausea and dizziness, in no cases were these symptoms sufficiently severe to cause interruption or cessation of perhexiline therapy.

Data were also analysed to determine if perhexiline plasma concentration after short-term therapy was correlated with either the changes from baseline in SNP response (Figure 6.8) or extent of aggregation (Figure 6.10). Change in SNP responsiveness was weakly but significantly correlated with plasma perhexiline concentration ($r^2 = 0.13$, $p = 0.04$) as was reduction in response to ADP with plasma perhexiline concentration ($r^2 = 0.40$, $p = 0.02$). In view of the relatively high proportion of patients with perhexiline concentrations outside the therapeutic range, and the accentuation of nitric oxide-potentiating effect with high perhexiline concentrations, the effects of perhexiline on SNP responses were also assessed in those patients ($n = 21$) with therapeutic or lower plasma perhexiline concentrations (Figure 6.9). Mean increase in SNP response from baseline for this group of patients was 12.2 ± 4.2 % inhibition of aggregation ($p < 0.01$). Change in SNP responsiveness was within this subset significantly but inversely correlated with plasma perhexiline concentration ($r^2 = 0.33$, $p = 0.01$).

14 patients continued perhexiline therapy for more than 29.1 ± 1.9 days (4.2 ± 0.3 weeks), therefore we were able to assess the ex vivo effects of perhexiline over this time frame. After approximately 4 weeks of perhexiline therapy, the extent of aggregation was not significantly different from baseline (9.8 ± 1.2 vs 11.4 ± 1.4 Ohms, $p = 0.21$, Figure 6.11), or after 1-2 weeks of therapy (9.8 ± 1.2 vs 10.9 ± 1.4 Ohms, $p = 0.45$). The SNP response remained significantly increased from baseline (48.5 ± 5.2 vs 27.8 ± 5.1 % inhibition of

aggregation, $p < 0.05$, Figure 6.12). There was no difference between the extent of inhibition by SNP between 1-2 weeks and 4 weeks of therapy (45.3 ± 4.0 vs 48.5 ± 5.2 % of inhibition, $p > 0.05$). Therefore, it appears that perhexiline produces its effects within the first 1-2 weeks of therapy and this effect continues for at least 4 weeks.

6.5.3 Effect of perhexiline therapy on acute coronary syndrome patients

6.5.3.1 ADP responsiveness

After 3 days of perhexiline therapy there was no significant decrease in the extent of ADP-induced aggregation from baseline (12.5 ± 0.8 vs 12.9 ± 0.8 Ohms, $p = 0.8$, Figure 6.13). This result is consistent with the effects of perhexiline on ADP-induced aggregation after short-term therapy in stable angina patients.

6.5.3.2 SNP responsiveness

After 3 days of perhexiline therapy the SNP response was significantly greater than at baseline (43.0 ± 3.7 vs 29.4 ± 2.2 % inhibition of aggregation, $p < 0.001$, Figure 6.14). Perhexiline therefore restored SNP responsiveness in this group of patients towards that of normal subjects.

Of the 50 patients, 11 were still experiencing anginal symptoms at rest after 3 days of perhexiline therapy. When the SNP response was analysed according to change in symptomatic status (Figure 6.15), patients who continued to experience ischaemic

symptoms had no increase in response to SNP ($p < 0.001$ vs patients in whom symptoms resolved).

There was no significant correlation between change in extent of aggregation and change in SNP response ($r^2 = 0.01$, $p = 0.6$) in this group of patients (Figure 6.16).

Perhexiline plasma concentration after 3 days ranged from 0.05 to 2.23 $\mu\text{g/L}$ (mean $0.61 \pm 0.08 \mu\text{g/L}$). In contrast to data from stable angina patients, plasma perhexiline concentration was not correlated with either change in extent of aggregation ($r^2 = 0.02$, $p = 0.4$, Figure 6.17) or SNP response ($r^2 = 0.07$, $p = 0.1$, Figure 6.18). 19 of the 50 patients had "toxic" ($> 0.60 \mu\text{g/ml}$) plasma perhexiline concentrations after 3 days of perhexiline therapy (Figure 6.19), of these 9 developed nausea and/or dizziness; these two results were correlated ($p = 0.02$) and are consistent with the previous study by Stewart et al in a similar group of patients (Stewart, et al. 1996). Only one patient with plasma perhexiline concentrations of $< 0.6 \mu\text{g/ml}$ developed such toxic effects.

Fasting blood sugar levels were recorded for the 17 diabetic patients in the study. Perhexiline maleate therapy (72 hours) was associated with reduced mean blood sugar levels when compared to baseline values (5.9 ± 0.5 vs $10.1 \pm 1.3 \text{ mM}$, $p < 0.0001$, Figure 6.20). No correlation was apparent between extent of fall in blood sugar level and 3 day plasma perhexiline concentration. The decrease in blood sugar level by perhexiline maleate therapy is consistent with the study by Stewart et al (Stewart, et al. 1996). This cohort of diabetic patients did not differ significantly from the other patients in respect to

either extent of change in ADP-induced aggregation or change platelet responsiveness to SNP.

6.5.4 Acute coronary syndrome patients not receiving perhexiline

In order to examine whether the increase in SNP response was due to perhexiline therapy per se and not just associated with improvement in clinical status, a group of acute coronary syndrome patients (n=12) not receiving perhexiline as therapy were studied. Baseline SNP response was 33.4 ± 4.6 % inhibition of aggregation ($p=0.37$ vs baseline SNP response for patients receiving perhexiline), while after 3 days of conventional therapy the SNP response was unchanged at 33.1 ± 4.2 % inhibition of aggregation, $p=0.82$ (Figure 6.21). Within this group of patients, only one patient experienced angina within 72 hours of baseline measurements of SNP responsiveness. These data therefore suggest that perhexiline therapy rather than the efflux of time is responsible for the increase in SNP response.

Within this group of patients we also examined extent of ADP-induced aggregation. No significant reduction in extent of aggregation was observed over the 3 days follow-up period. At baseline, extent of aggregation was 11.9 ± 1.7 Ohms, while after 3 days it was 10.3 ± 1.9 Ohms, $p=0.13$ (Figure 6.22).

Fasting blood sugar levels were recorded for the 4 diabetic patients in this cohort. In contrast to the fall in blood sugar levels observed in the diabetic patients receiving perhexiline, fasting blood sugar levels were unchanged in this group of patients.

6.5.5 Mechanism of SNP response

6.5.5.1 Superoxide content

Superoxide content was directly measured in blood samples from acute coronary syndrome patients receiving perhexiline (n=11) via lucigenin-based chemiluminescence. After perhexiline therapy, there was no significant difference in superoxide when compared to baseline values (136.0 ± 41.7 vs 107.0 ± 27.0 mV, $p=0.20$, Figure 6.23).

Superoxide content was also examined in acute coronary syndrome patients not receiving perhexiline (n=3). After 3 days of conventional therapy the superoxide content was not significantly different from baseline (Figure 6.24).

6.5.5.2 Effect of SOD / catalase on SNP responsiveness

At baseline (prior to commencement of perhexiline therapy) there were no statistically significant effects of SOD plus catalase on either platelet aggregation ($p=0.09$ vs ADP control aggregation, Figure 6.25) or SNP response ($p=0.18$ vs baseline SNP response). After perhexiline therapy, there was no effect of SOD plus catalase on either the

inhibition of aggregation when compared with baseline values ($p=0.11$) or the increase in SNP response ($p=0.79$).

In those acute coronary syndrome patients not receiving perhexiline there was a significant effect of SOD plus catalase on inhibition of platelet aggregation ($19\pm1.6\%$ inhibition from ADP control, $p=0.01$, Figure 6.26), there was no effect on the SNP response ($p=0.11$ vs SNP baseline). After 3 days, SOD plus catalase did not affect the SNP response ($p=0.70$). In addition, there was no difference in the extent of inhibition of aggregation produced by SOD plus catalase from the baseline value ($p=0.33$).

6.5.5.3 Effect of ODQ on SNP responsiveness

The effect of the selective soluble guanylate cyclase inhibitor, ODQ was examined in a cohort of acute coronary syndrome patients receiving perhexiline ($n=10$). At baseline ODQ ($10\text{ }\mu\text{M}$) significantly decreased platelet responsiveness to SNP from 27.5 ± 7.0 to $20.4\pm4.4\%$ inhibition of aggregation ($p<0.05$, Figure 6.27). Although a significant decrease occurred, the proportion of ODQ-mediated inhibition was smaller than expected (approximately 26%), indicating that the majority of inhibition produced by SNP occurs via guanylate cyclase-independent mechanisms in this cohort of patients.

After perhexiline therapy there was a significant increase in SNP responsiveness from baseline in this subset of patients ($p<0.03$). ODQ significantly decreased SNP responsiveness from 43.9 ± 9.2 to $31.7\pm8.0\%$ inhibition of aggregation, $p<0.007$ (Figure

6.28). As at baseline, the majority of the inhibition produced by SNP was mediated by guanylate cyclase-independent mechanisms. However, when the data was analysed for the individual components of SNP mediated inhibition (ie guanylate cyclase-independent and -dependent mechanisms) it appeared that both components were increased after perhexiline therapy (Figure 6.29). The guanylate cyclase-independent component increased from 20.7 ± 4.4 to 31.7 ± 8.0 ($p=0.13$), while the guanylate cyclase-dependent component increased from 7.1 ± 3.1 to 12.0 ± 3.6 ($p=0.06$).

6.5.6 Acute effects of perhexiline on SNP responsiveness in vitro

In an attempt to reproduce the increase in SNP response observed after short term perhexiline therapy we examined the acute effects of *in vitro* perhexiline on SNP responsiveness in blood samples from stable angina patients utilising whole blood aggregation.

Two concentrations of perhexiline were investigated (Figure 6.30). Individually, after a 5 min pre-incubation perhexiline in concentrations of $1 \mu\text{M}$ ($n=8$) and $10 \mu\text{M}$ ($n=3$) produced 2.9 ± 1.6 and 18.5 ± 5.3 % inhibition of ADP-induced aggregation, respectively. SNP ($10 \mu\text{M}$, $n=11$), the same concentration utilised in *ex vivo* experiments, produced 29.9 ± 6.7 % inhibition of aggregation. When perhexiline ($1 \mu\text{M}$) and SNP ($10 \mu\text{M}$) were added to whole blood samples the resultant inhibition of aggregation was 28 ± 4.8 %. This value, which is smaller than the addition of the individual extents of inhibition for perhexiline and SNP indicates that no potentiation was observed.

Similarly, when perhexiline (10 μ M) and SNP (10 μ M) were added to blood samples the resultant inhibition of aggregation was 27.6 ± 7.7 %. This value is again less than the combined extents of inhibition for each agent.

The above experiment was repeated utilising the multiple agonist approach (see Chapter 3) for induction of aggregation. Perhexiline (1 μ M, n=4) was only examined using the technique, and produced 4.5 ± 4.5 % inhibition of aggregation. SNP (10 μ M, n=4) produced 31 ± 8.3 % inhibition of aggregation (Figure 6.31). When added together the resultant inhibition of aggregation was 33 ± 5.7 %. This value is again not greater than the arithmetic addition of the individual extents of inhibition.

Therefore, in vitro perhexiline does not significantly affect the inhibitory response of SNP when measured acutely.

6.6 Discussion

This is the first study to examine the effect of perhexiline maleate therapy on ex vivo platelet aggregation in patients with acute coronary syndromes. The major findings of this study are: 1) short-term perhexiline maleate therapy does not significantly change the extent of ex vivo ADP-induced platelet aggregation in patients with stable and acute coronary syndromes previously receiving conventional pharmacotherapy, 2) short-term perhexiline maleate therapy significantly increased platelet responsiveness to the nitric

oxide donor SNP, therefore, reversing the “nitric oxide resistance” observed at baseline in the these patients. However, the results provide evidence in support of both incremental effects in stable angina and in acute coronary syndromes, a circumstance for which there is little prior information on perhexiline.

The current investigation was not designed to evaluate clinical efficacy of perhexiline maleate in the management of stable angina pectoris and acute coronary syndromes resistant to conventional therapy. Patients with stable angina despite maximal conventional therapy demonstrated a marked decrease in anginal frequency after short-term perhexiline maleate therapy; no patients experienced an increase in symptoms. This result is consistent with the numerous studies who have demonstrated perhexiline’s effectiveness in the treatment of stable angina pectoris (Cherchi, et al. 1973; Liberti, et al. 1973; Morgans and Rees. 1973; Morledge. 1973; Teo, et al. 1983; Horowitz, et al. 1986 Cole, et al. 1990). Patients with acute coronary syndromes who were experiencing chest pain at rest despite maximal therapy also demonstrated a reduction in symptoms. 29 of the 46 patients studied were asymptomatic after 3 days of perhexiline maleate therapy. This result is consistent with the only other study of perhexiline maleate in acute coronary syndromes ([Stewart, 1996 #51]) and provides further evidence suggesting the usefulness of perhexiline maleate in the treatment of acute coronary syndromes. Indeed the data on changes in platelet response to SNP (see below) further support this contention.

The involvement of platelets in acute coronary syndromes is well documented (Diodati, et al. 1994; White. 1994; Frishman, et al. 1995; Kamat and Kleiman. 1995; Farstad. 1998; Puri and Colman. 1997). Surprisingly, prior to the commencement of perhexiline maleate therapy both stable angina and acute coronary syndrome patients demonstrated no significant increase in extent of ADP-induced platelet aggregation, when compared to normal subjects; these findings are at odds with our previous observations (Chirkov, et al. 1999). Nevertheless, patients with acute coronary syndromes exhibited a strong trend towards an increase from control (13.3 ± 0.9 vs 10.6 ± 0.7 Ohms, $p=0.07$). The basis for the lack of observed hyperaggregability to ADP, especially in the stable angina population, was not explored in the current study. However, it may reflect more intense background pharmacotherapy than would be the case in randomly selected patients with angina pectoris, such as were studied previously: nitrates and verapamil, for example inhibit ex vivo platelet aggregation (Johnson, et al. 1986; Diodati, et al. 1990; Chirkov, et al. 1993; Wallen, et al. 1995; Wolfram, et al. 1996; Knight, et al. 1997).

Analogously, in the present study the observation that perhexiline maleate therapy was not found to further decrease ex vivo platelet aggregation is therefore not entirely unexpected, and only implies absence of a profound effect on aggregation:- for example if perhexiline was indeed a potent anti-aggregatory agent (eg glycoprotein IIb/IIIa inhibitor) it may have produced detectable incremental effects. Nevertheless, the results do not show a significant reduction in extent of aggregation. On the otherhand, as it is desirable that agents administered to patients with acute coronary syndromes should not have adverse effects on platelet aggregation it was also important that perhexiline did not

demonstrate a pro-aggregatory effect. These results are consistent with the absence of documented bleeding events (indicating inhibitory) or cerebral events (indicating pro-aggregatory) in the literature, even in those studies where perhexiline was used as monotherapy (Brown, et al. 1976; Pilcher. 1978; Horgan, et al. 1981).

Therefore, perhexiline probably exerted no major effect on ex vivo platelet aggregation. However, the possibility may still exist that perhexiline possesses an anti-aggregatory component to its anti-anginal mechanism. Furthermore ADP-induced whole blood impedance aggregation is only one of many methods available for assessing platelet activity. Alternative methods might have included 1) different agonists, or 2) different techniques for aggregometry (see Chapter 1).

The anti-aggregatory effect of SNP was reduced at baseline in both stable angina and acute coronary syndrome patients when compared to normal subjects, thus representing the phenomenon of “nitric oxide resistance” at the platelet level. Patients with acute coronary syndromes exhibited the lowest anti-aggregatory response to SNP, although this result was not significantly different from the value obtained for stable angina patients. This observation is consistent with the findings of Chirkov et al regarding “nitric oxide resistance” in patients with stable angina pectoris (Chirkov, et al. 1999). The results of the present study raise the possibility that the extent of “nitric oxide resistance” is greater in acute coronary syndromes than in stable angina pectoris. The heterogenous effects of perhexiline on “nitric oxide resistance” support the contention that impairment in the anti-aggregatory efficacy of endogenous nitric oxide (EDRF)

plays an important role in the pathogenesis of both stable angina and acute coronary syndromes.

After short-term perhexiline maleate therapy patients with both stable angina and acute coronary syndromes demonstrated an increased responsiveness to SNP. This result, which restored the SNP response to approximately that of the normal subjects reversed the "nitric oxide resistance" initially observed in each group. The perhexiline plasma concentration was not correlated with this increased responsiveness in either case. This suggests that these changes may be induced by very low concentrations of perhexiline.

In order to determine that the increase in SNP response was not merely a result of efflux of time with progressive clinical improvement, a cohort of acute coronary syndrome patients, who did not receive perhexiline, were studied. These patients did not demonstrate any significant change in SNP response after three days of conventional therapy (Fig 6.21), thus suggesting that the improvement in SNP responsiveness is solely due to perhexiline therapy. However, this conclusion is less than definite, as the perhexiline-free patients had less severe ischaemia than those treated with perhexiline. On the other hand, improved platelet response to SNP in stable angina patients is likely to reflect a perhexiline effect.

The present study also confirmed that diabetic patients had a fall in blood sugar levels after 3 days of perhexiline maleate therapy. Insulin resistance or poor responsiveness to insulin has been reported in diabetic patients (Rizza, et al. 1981; Olefsky, et al. 1985;

Genuth. 1990; Joffe, et al. 1994; Sonnenberg and Kotchen. 1998; Turner and Clapham. 1998; Whitelaw and Gilbey. 1998). This is of particular interest to the current study as diabetic patients also have reduced production of endothelial nitric oxide. Insulin has been shown to stimulate endothelin and nitric oxide production and it has been postulated that insulin resistance provokes an imbalance between the two resulting in impaired vasoreactivity (Huszka, et al. 1997; Lekakis, et al. 1997). This observation suggests that perhexiline may mimic the effect of insulin, thus restoring endothelial function and responsiveness to nitric oxide.

Troglitazone, an insulin-sensitising agent, which produces metabolic shifts similar to those seen with carnitine palmitoyltransferase-1 inhibition (Horton, et al. 1998) has recently been shown to upregulate nitric oxide synthesis, and therefore nitric oxide production in vascular smooth muscle cells (Hattori, et al. 1999). Perhexiline's similarity with troglitazone raises the issue of whether the known effects of perhexiline in inhibiting platelet carnitine palmitoyltransferase-1 might be responsible for the observed changes in responsiveness to nitric oxide. Superficially the results of Chapter 5 suggest that perhexiline-induced carnitine palmitoyltransferase-1 inhibition in platelets occurring rapidly in vitro, but in the current experiments, in vitro perhexiline did not affect nitric oxide responses. However, the studies in Chapter 5 were performed in saponin-permeabilized platelets (and indeed the original studies of Kennedy et al were performed in broken cell preparation (Kennedy, et al. 1996)). Recent investigations (Kennedy and Unger, unpublished) have suggested that the onset of metabolic effects of perhexiline in intact cells is relatively slow; hence carnitine palmitoyltransferase-1

inhibition cannot be excluded as a mechanism. Analogous investigations with other “pure” carnitine palmitoyltransferase-1 inhibitors, such as hydroxyphenylglyoxylate might help to settle this issue.

Conversely, it might be argued, especially in diabetic subjects, that insulin may mediate the effects of perhexiline. While this was not explored, the increase in SNP response in diabetic subjects was similar to that in non-diabetics. While nondiabetic patients are not necessarily insulin-resistant, the possibility that insulin mediates perhexiline effects on SNP responses cannot be ruled out as the study by Petrie et al demonstrated in healthy volunteers a direct link between nitric oxide synthesis and insulin sensitivity (Petrie, et al. 1996).

Chirkov et al has recently demonstrated that “nitrate resistance” is produced by either a decrease in nitric oxide-sensitivity of platelet guanylate cyclase and/or decreased availability of nitric oxide for activation of guanylate cyclase because of clearance by superoxide (Chirkov, et al. 1999). The possible relevance of the latter mechanism to perhexiline effects was investigated in a subset of acute coronary syndrome patients. Whole blood superoxide content was assessed using lucigenin-based chemiluminescence and was unaffected by perhexiline therapy. In a small subset of acute coronary syndrome patients not receiving perhexiline superoxide content was also assessed, and did not fluctuate with time. To conclusively rule out the involvement of superoxide in the increased platelet responsiveness to SNP, effects of SOD were examined. SOD, through its metabolism of superoxide, prevents rapid inactivation of nitric oxide by superoxide.

In a group of patients with acute coronary syndromes, perhexiline-induced potentiation of SNP responses was independent of the presence/absence of SOD/catalase. Although kinetics of superoxide turnover and peroxynitrite formation were not measured in the current study, the *in vitro* experimental results imply that perhexiline does not potentiate nitric oxide responsiveness by a direct effect on superoxide kinetics.

The effect of the soluble guanylate cyclase inhibitor, ODQ was examined in acute coronary syndrome patients receiving perhexiline (n=10). Prior to the commencement of perhexiline therapy these patients demonstrated that a large component of their SNP response (approximately 75%) was mediated by guanylate cyclase-independent mechanisms. This observation suggests that patients with acute coronary syndromes have a decreased sensitivity of platelet guanylate to nitric oxide. Chirkov et al have recently demonstrated that the inhibition of the anti-aggregatory effects of NTG and SNP is significantly less pronounced in stable angina patients when compared to normal subjects (Chirkov, et al. 1999). However, the guanylate cyclase-dependent component of inhibition in these patients is approximately 50% of the SNP response. This is in contrast to the present data whereby guanylate cyclase-dependent inhibition comprises approximately 25% of the SNP response in patients with acute coronary syndrome. This phenomenon may be responsible for the somewhat greater degree of "nitrate resistance" observed in patients with acute coronary syndromes compared to stable angina patients. After perhexiline therapy there was an increase in the relative extent of inhibition by both guanylate cyclase-independent and -dependent components of the SNP responsiveness (Figure 6.30). Although the increase in both components was not

significant, the data suggest that perhexiline therapy increase nitric oxide responsiveness via multiple biochemical pathways.

We investigated if the potentiation of SNP responsiveness by perhexiline could be produced acutely in vitro. Perhexiline added to whole blood sample prior to induction of aggregation did not increase the concomitant inhibition produced by SNP. Overall the results suggest that the potentiation of SNP responsiveness by perhexiline occurs overtime (days) in vivo. However, a time course of effects was not the objective of the current study.

The current study has a number of limitations. There was no true control group for either stable angina or acute coronary syndrome patients: the acute coronary syndrome patients not receiving perhexiline would have constituted a lower risk population as regards ongoing ischaemia. The study results do not necessarily reflect accurately the extent of platelet resistance to nitric oxide (SNP and NTG) in vivo. Finally and most importantly, the potentiation of SNP responsiveness by perhexiline has not yet been demonstrated to improve nitric oxide mediated responses to vasomotor stimuli; that is improved endothelial function.

6.7 Conclusions

Perhexiline, in doses which have no detectable anti-aggregatory effect in the presence of background anti-platelet therapy, improves (towards normal) platelet responses to the

anti-aggregating effects of SNP in patients with stable angina and acute coronary syndromes. The precise mechanism of the change in nitric oxide responsiveness was not established in the current study. However, one of the postulated mechanisms for “nitrate resistance” (increased clearance of nitric oxide by superoxide) has been eliminated. The novel finding that perhexiline increases both guanylate cyclase-independent and -dependent components of SNP’s anti-aggregatory effect (and therefore that of nitric oxide) warrants further investigation.

Table 6.1. Clinical characteristics: Stable angina pectoris patients.

Age (yr $\pm$SEM)	65$\pm$2.1
Male:Female	20:10
hypertension	13 (43%)
diabetes	7 (23%)
hypercholesterolaemia	4 (13%)
current smoker	3 (10%)
family history of CAD	8 (27%)
previous MI	5 (17%)
previous PTCA/CABG	8 (27%)
Medications	
aspirin	28 (93%)
beta-blocker	7 (23%)
Ca²⁺ channel blocker	9 (30%)
oral nitrates	20 (67%)
ACE inhibitor	6 (20%)

Abbreviations: Coronary artery disease (CAD), myocardial infarction (MI), post transluminal coronary angioplasty (PTCA), coronary artery bypass graft (CABG).

Table 6.2. Clinical characteristics: acute coronary syndrome patients.

	Receiving PM (n=50)	Not receiving PM (n=12)
Age (yr±SEM)	68±1	57±3
Age range	42-84	42-76
Male:Female	31:20	9:3
UAP:Non-Q-wave MI	34:17	4:8
CAD (single:multi vessel)*	5:32	8:3
hypertension	22 (44%)	6 (50%)
hypercholesterolaemia	19 (38%)	6 (50%)
diabetes	17 (34%)	4 (33%)
current smoker	15 (30%)	4 (33%)
previous MI	24 (48%)	5 (42%)
<u>Medications</u>		
aspirin	36 (72%)	12 (100%)
IV Heparin	50 (100%)	12 (100%)
IV GTN	50 (100%)	12 (100%)
IV NAC	29 (58%)	6 (50%)
Ca²⁺ channel blocker	30 (60%)	10 (83%)
beta-blocker	11 (22%)	2 (17%)

* not all patient underwent coronary angiography

Abbreviations: Perhexiline maleate (PM), unstable angina pectoris (UAP), myocardial infarction (MI), coronary artery disease (CAD), intravenous (IV), nitroglycerine (GTN), N-acetylcysteine (NAC).

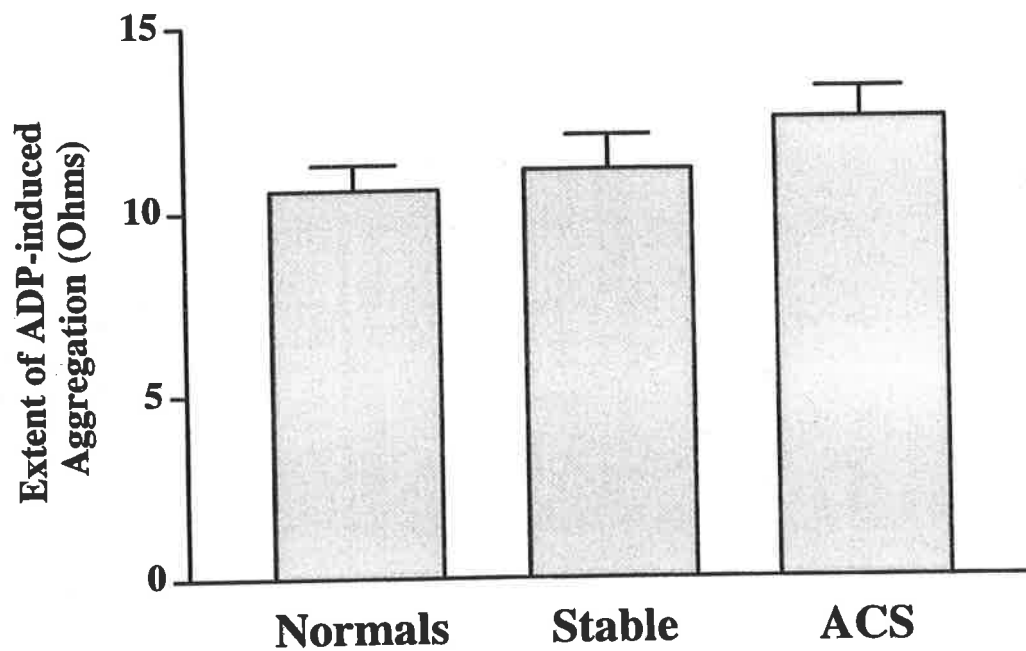


Figure 6.1.

Comparison of baseline extent of aggregation in response to 1 μ M ADP between normal subjects, stable angina and acute coronary syndrome (ACS) patients (ANOVA, $F=1.55$, $p=0.21$). No significant difference was observed for extent of aggregation between normal subjects and stable angina patients ($p=0.65$) or between normal subjects and acute coronary syndrome patients ($p=0.10$).

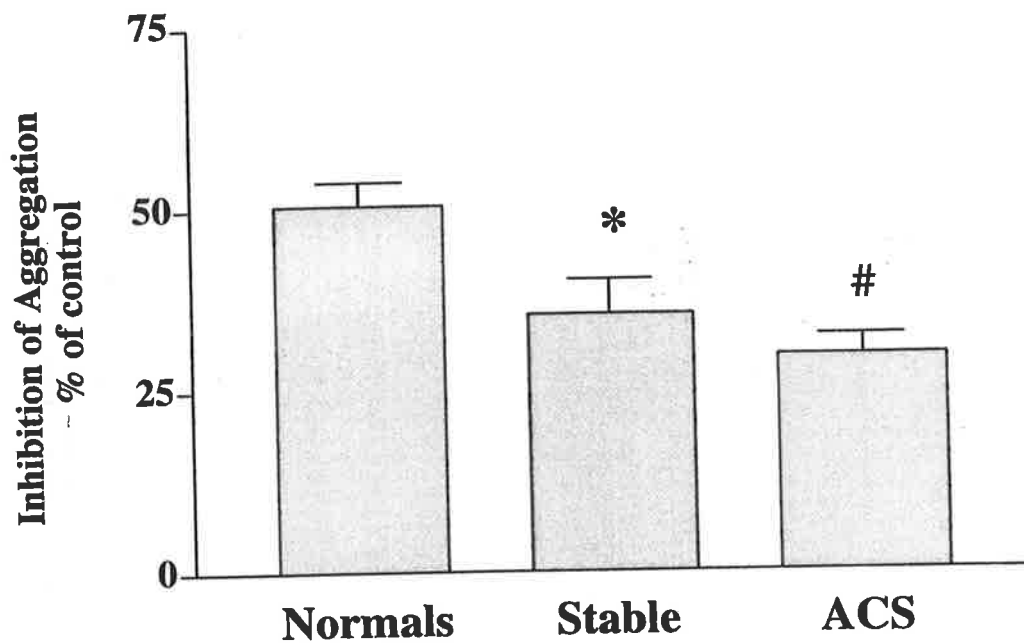


Figure 6.2.

Comparison of baseline SNP (10 μ M) responsiveness between normal subjects, stable angina and acute coronary syndrome (ACS) patients (ANOVA, $F=9.30$, $p=0.0002$). There was a significant decrease in the response towards SNP for stable (* $p<0.05$) and acute coronary syndrome (# $p<0.001$) patients compared with normal subjects.

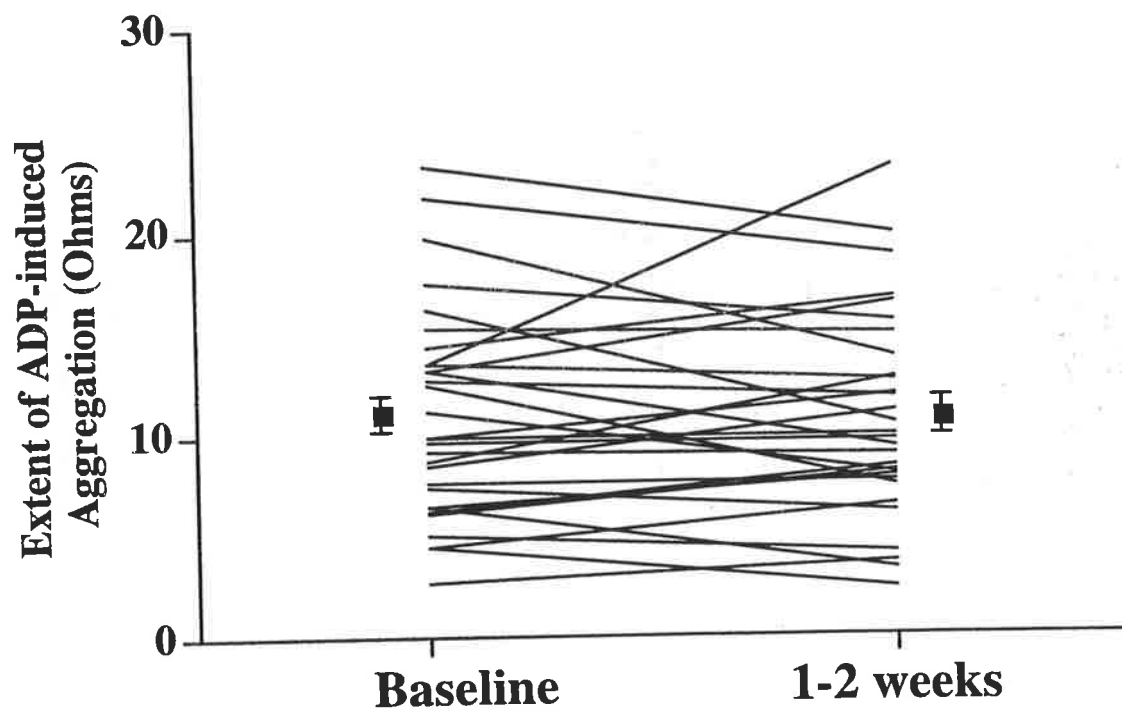


Figure 6.3.

Effect of short-term perhexiline therapy on extent of ADP-induced aggregation from stable angina patients (n=30). There was no significant decrease in the extent of aggregation by perhexiline ($p=0.70$).

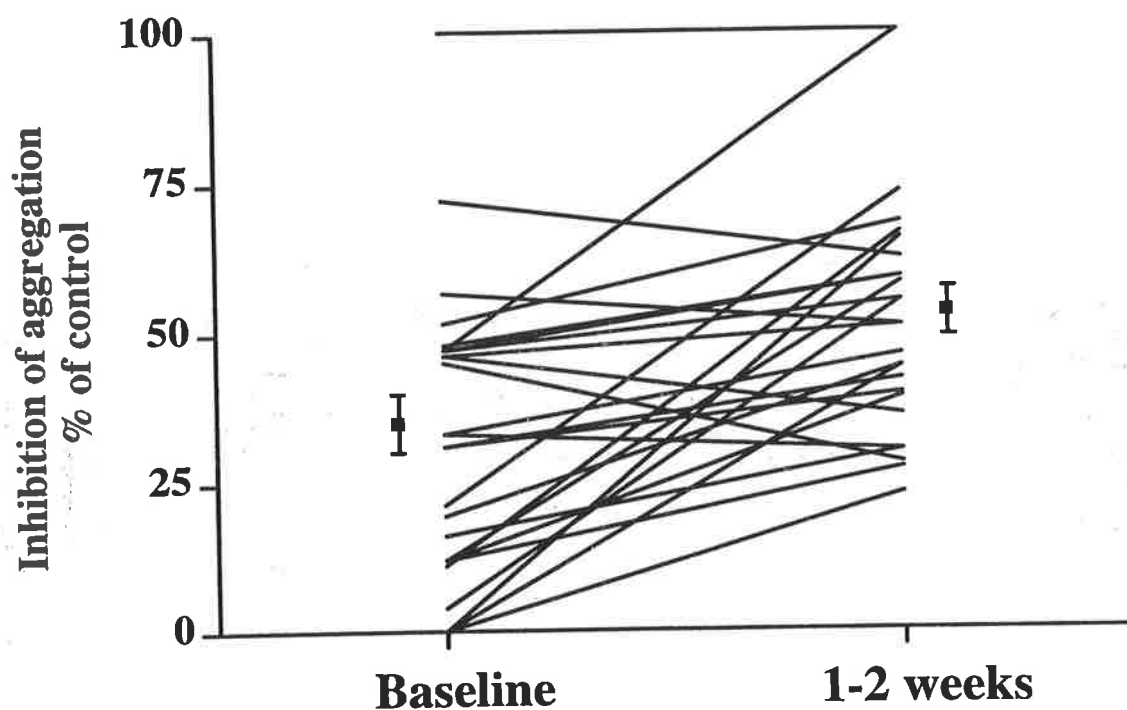


Figure 6.4.

Effect of short-term perhexiline therapy on SNP (10 μ M) responsiveness in blood samples from stable angina patients (n=30). There was a significant increase in SNP responsiveness following short-term perhexiline therapy ($p<0.001$).

After short-term perhexiline therapy the SNP response of stable angina patients was similar to that of normal subjects.

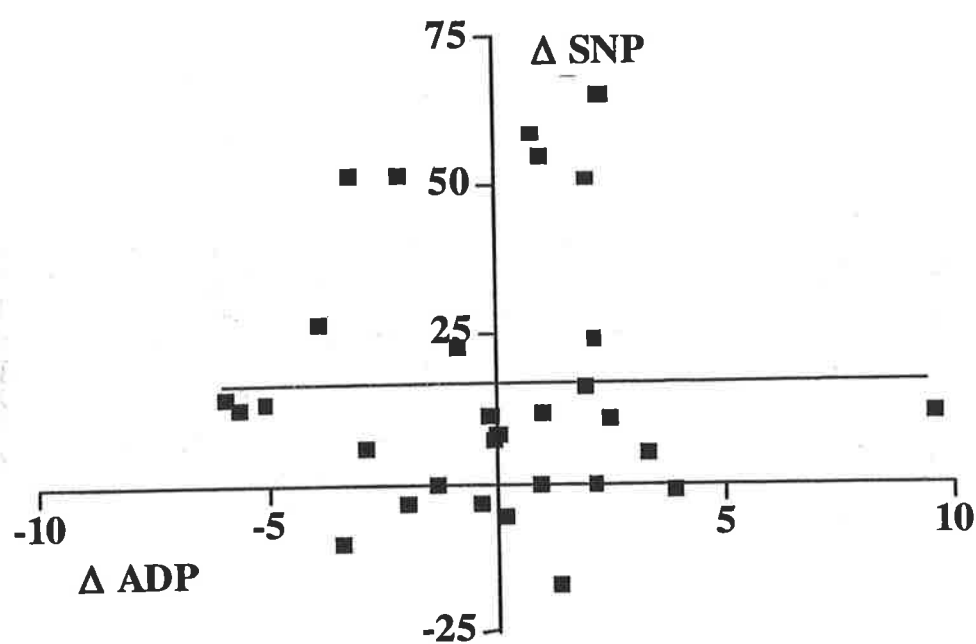


Figure 6.5.

Correlation between change in extent of aggregation (Δ ADP) with change in SNP response (Δ SNP). No significant correlation was observed ($r^2=0.001$, $p=0.98$).

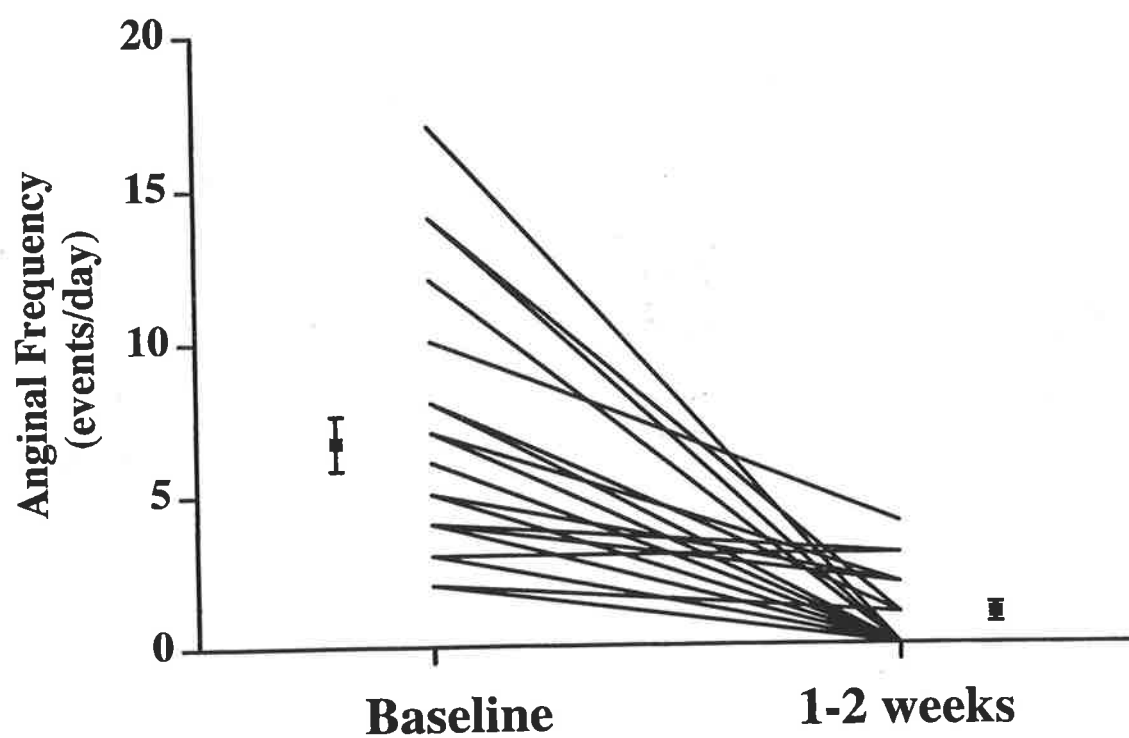


Figure 6.6.

Effect of short-term perhexiline therapy on anginal frequency (n=22). Short-term perhexiline therapy significantly decreased the number of reported anginal episodes per day ($p<0.001$).

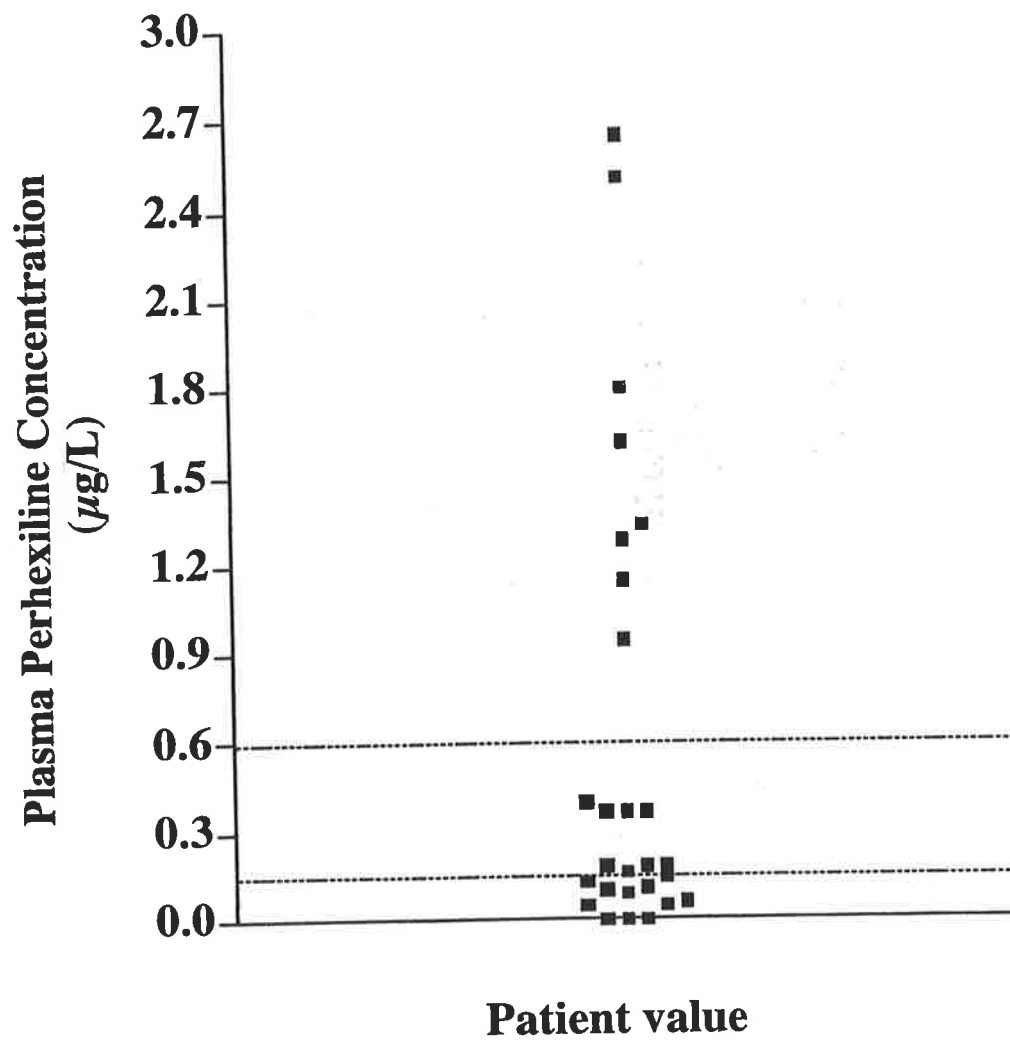


Figure 6.7

Plasma perhexiline concentrations after short-term therapy. The therapeutic range (0.15 to 0.60 $\mu\text{g/L}$) is indicated.

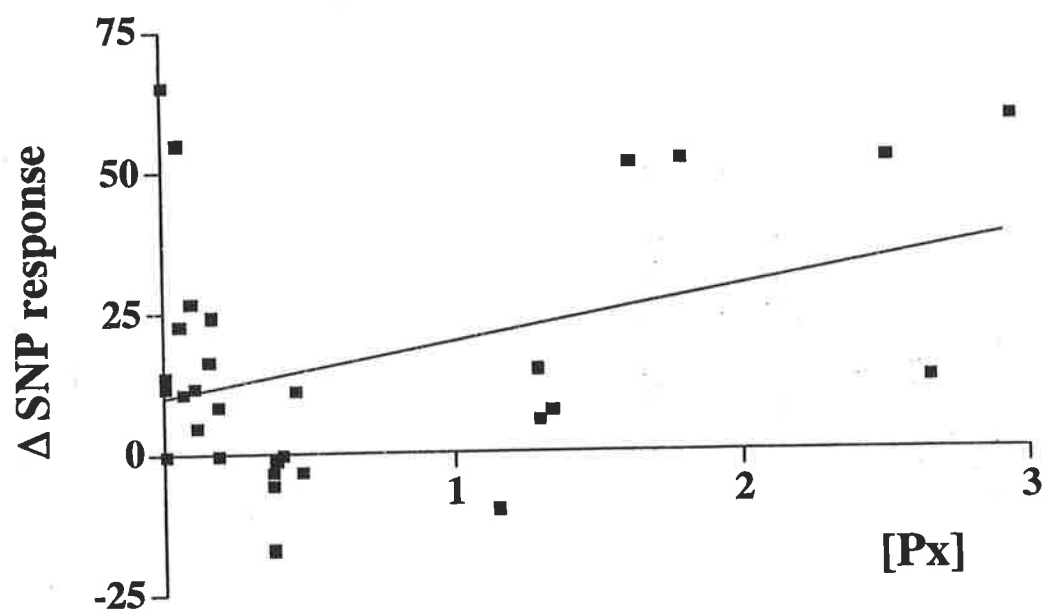


Figure 6.8.

Correlation between change in SNP responsiveness and plasma perhexiline concentration ($r^2=0.13$, $p=0.04$).

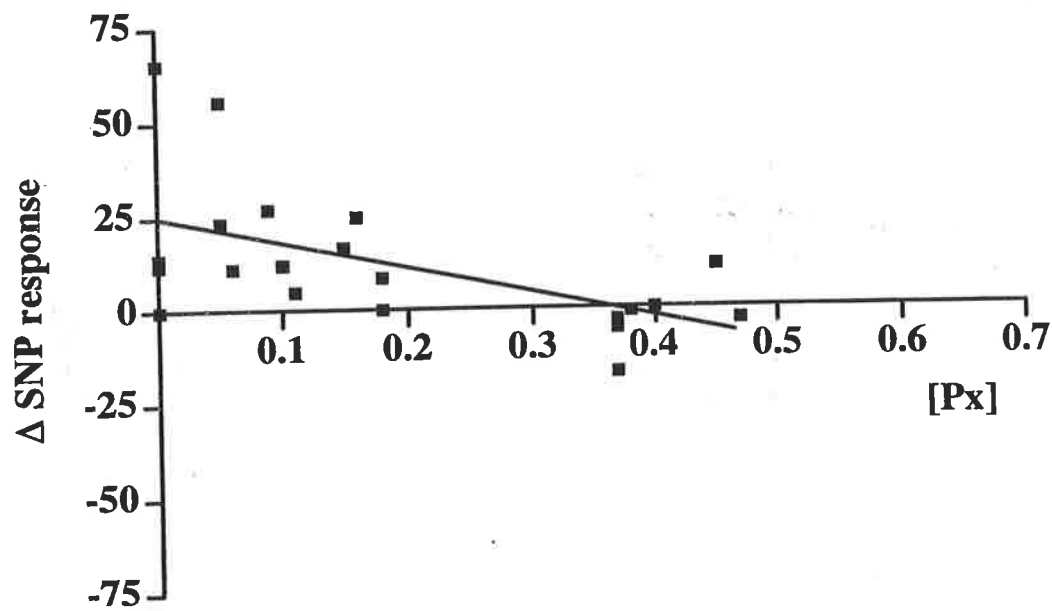


Figure 6.9

Correlation between change in SNP responsiveness and plasma perhexiline concentration below the upper limit (0.60 $\mu\text{g/L}$) of the therapeutic range ($r^2=0.33$, $p=0.01$).

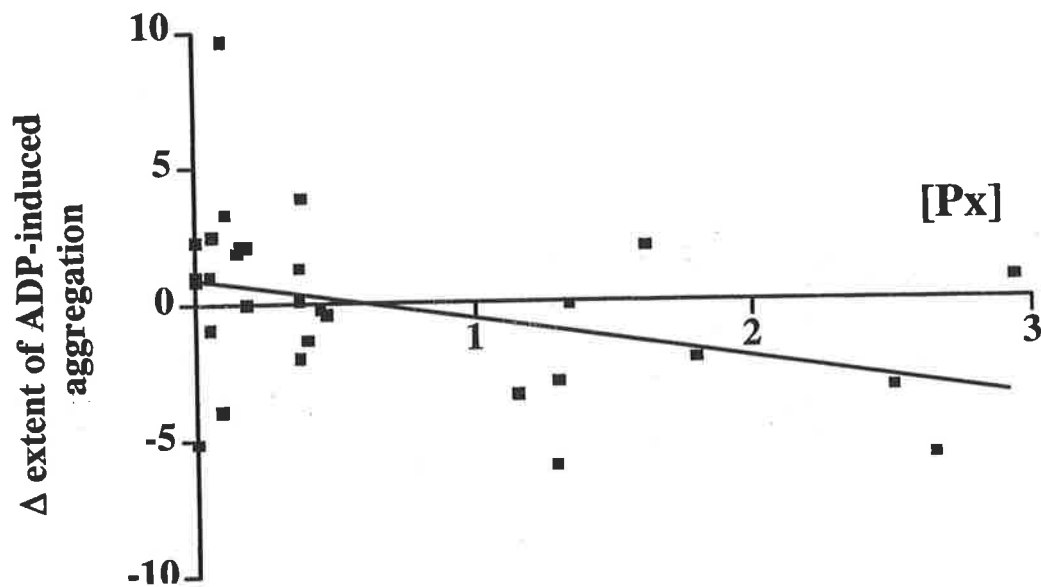


Figure 6.10.

Correlation between change in extent of aggregation and plasma perhexiline concentration ($r^2=0.40$, $p=0.02$).

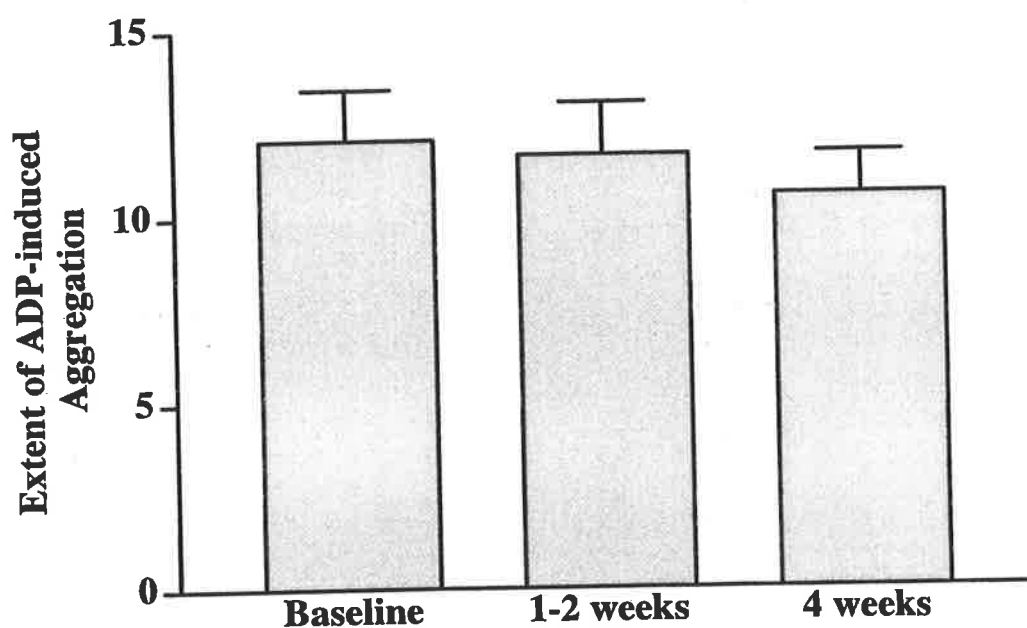


Figure 6.11.

Effect of 4 weeks perhexiline therapy on the extent of ADP-induced aggregation (n=14). No significant difference was observed between the extents of aggregation at each time point (ANOVA, $F=0.34$, $p=0.7$).

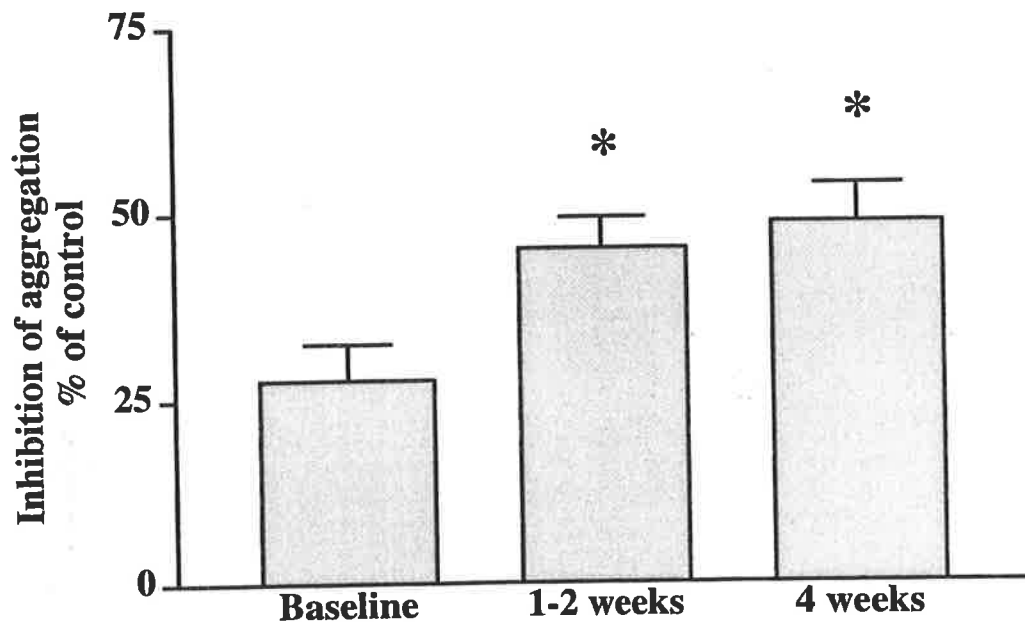


Figure 6.12.

Effect of 4 weeks perhexiline therapy on SNP (10 μ M) responsiveness (n=14) in patients with stable angina pectoris. There was a significant increase in SNP responsiveness after 4 weeks of perhexiline therapy when compared to baseline values. However, this increase was not significantly different from the increase observed at 1-2 weeks of therapy.

*** $p < 0.05$ versus baseline values**

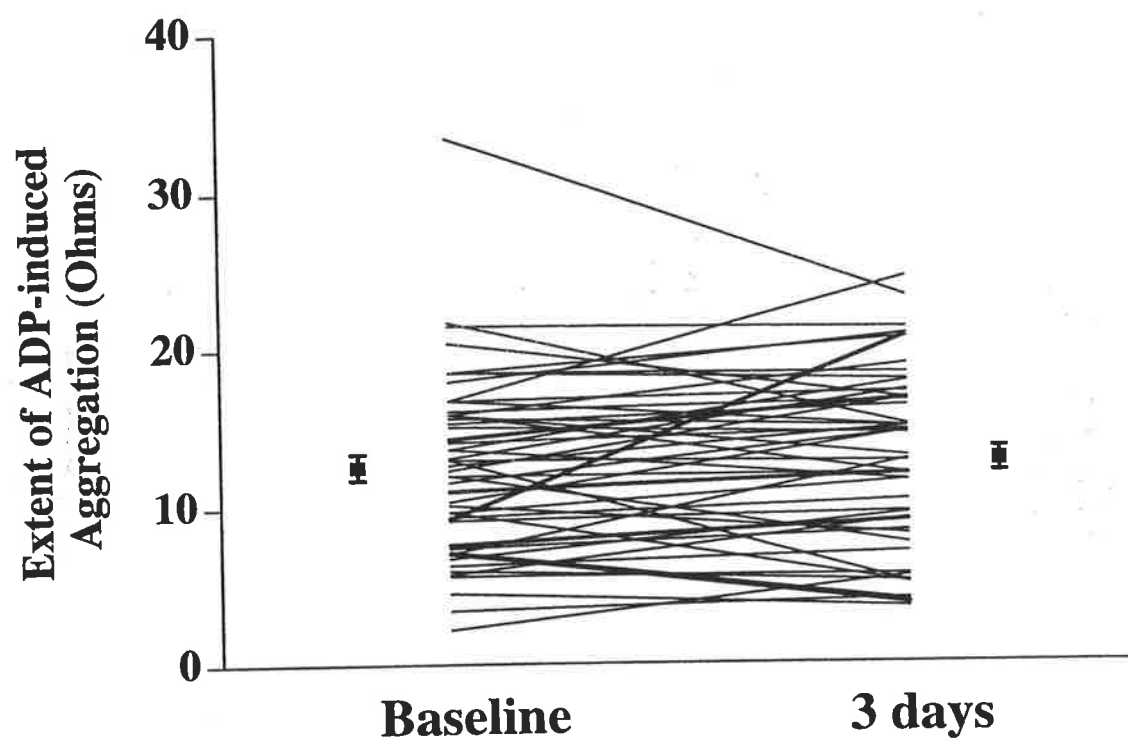


Figure 6.13.

Effect of 3 days perhexiline therapy on the extent of ADP-induced aggregation in blood samples from acute coronary syndrome patients (n=50). Perhexiline therapy did not significantly decrease the extent of aggregation ($p=0.8$).

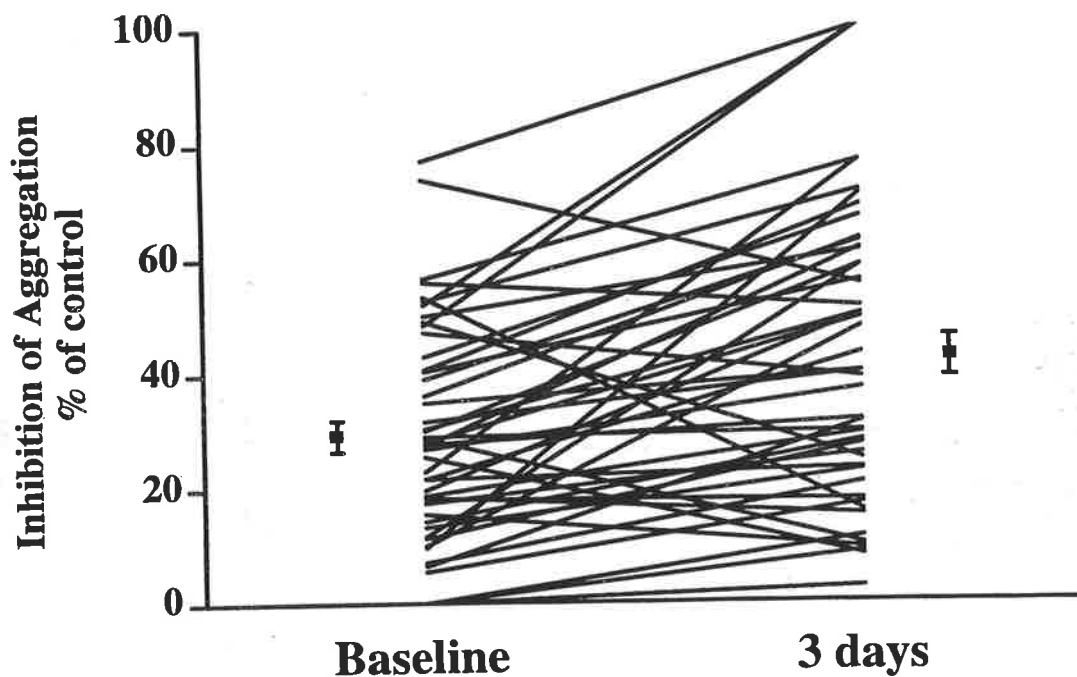


Figure 6.14.

Effect of 3 days perhexiline therapy on SNP (10 μ M) responsiveness in whole blood samples from acute coronary syndrome patients (n=50). There was a significant increase in SNP responsiveness following 3 days of perhexiline therapy ($p<0.001$).

note: The SNP response has been restored to approximately the value observed in normal subjects.

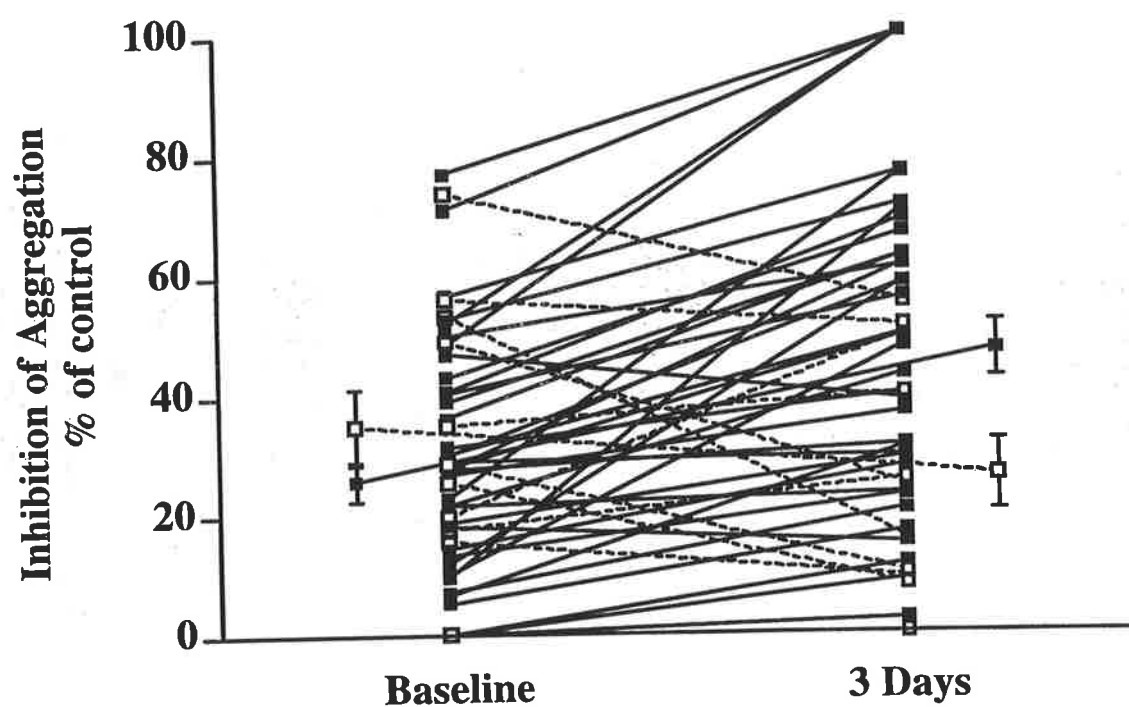


Figure 6.15.

Group and mean data: Effect of symptomatic status on SNP (10 μ M) responsiveness in acute coronary syndrome patients. Resolution of ischaemic symptoms within 3 days of perhexiline (closed symbols, n=29) was associated with significantly greater ($p<0.01$) increase in platelet response to SNP than non-resolution (open symbols, n=11).

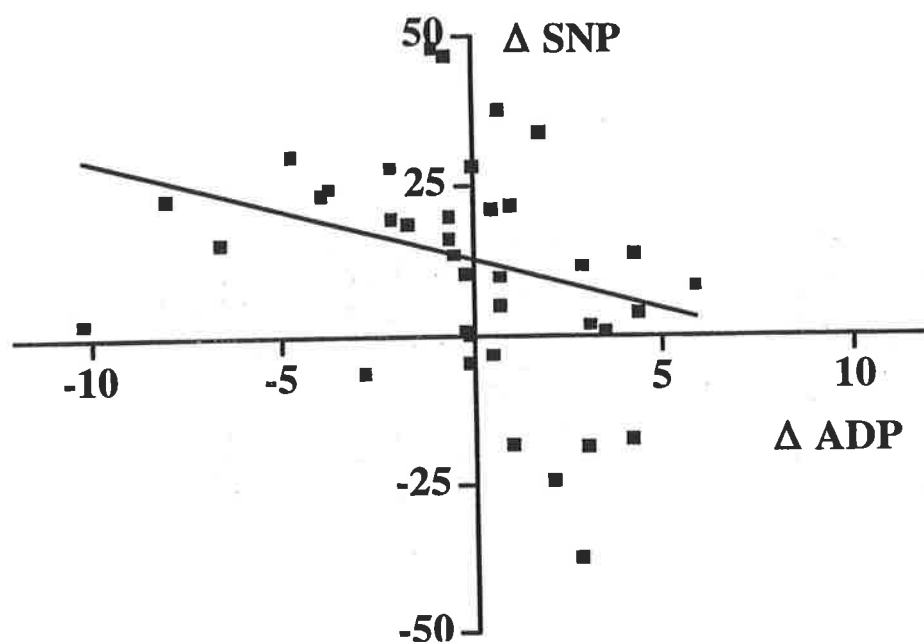


Figure 6.16.

Correlation between the change in extent of aggregation and change in SNP responsiveness following 3 days perhexiline therapy. No significant correlation was observed ($r^2=0.01$, $p=0.6$).

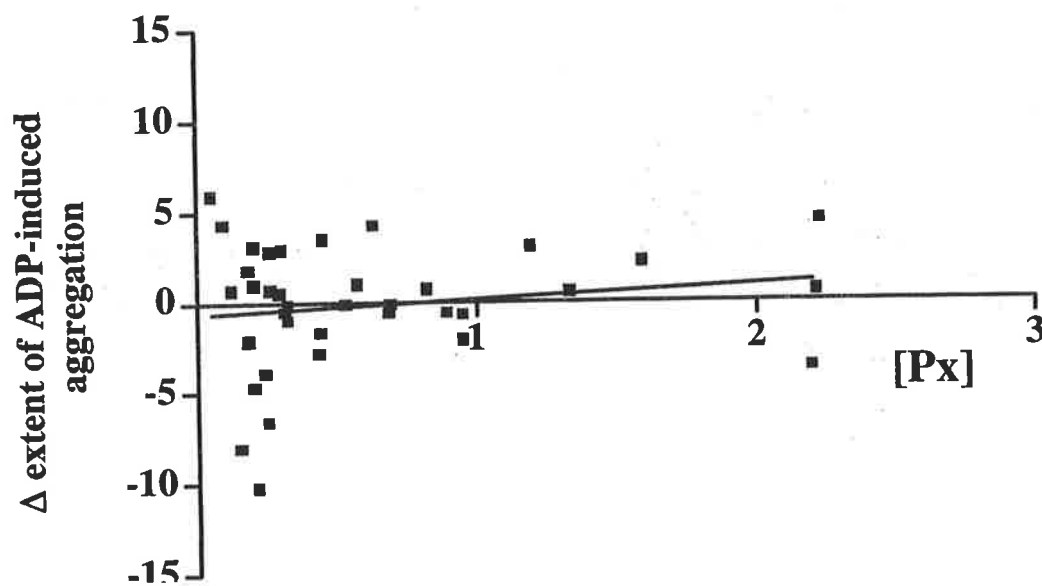


Figure 6.17.

Correlation between the change in extent of ADP-induced aggregation and plasma perhexiline concentration (Px). No significant correlation was observed ($r^2=0.02$, $p=0.4$).

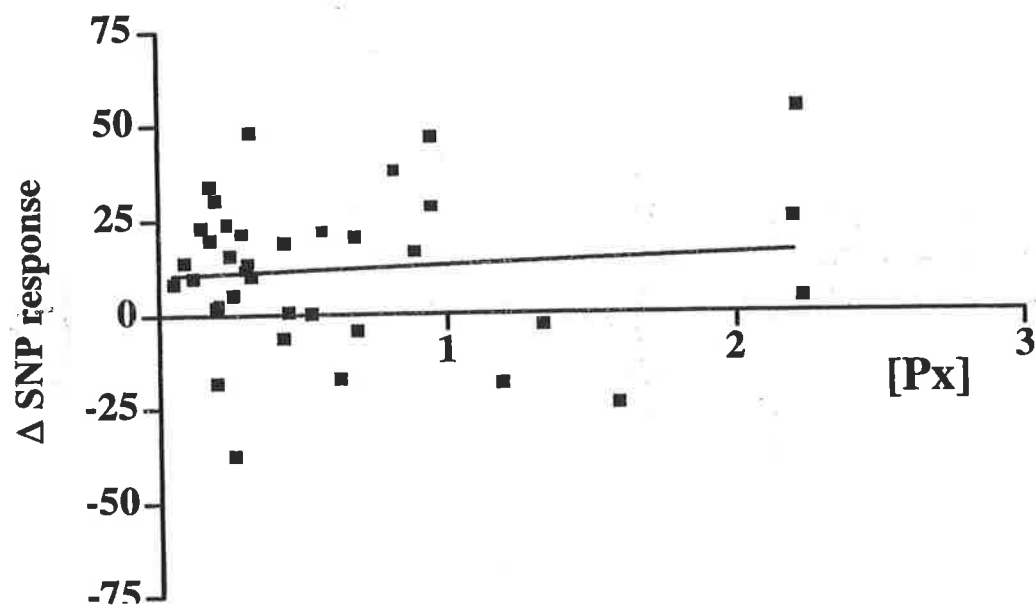


Figure 6.18.

Correlation between the change in SNP responsiveness and plasma perhexiline concentration (Px). No significant correlation was observed ($r^2=0.07$, $p=0.1$).

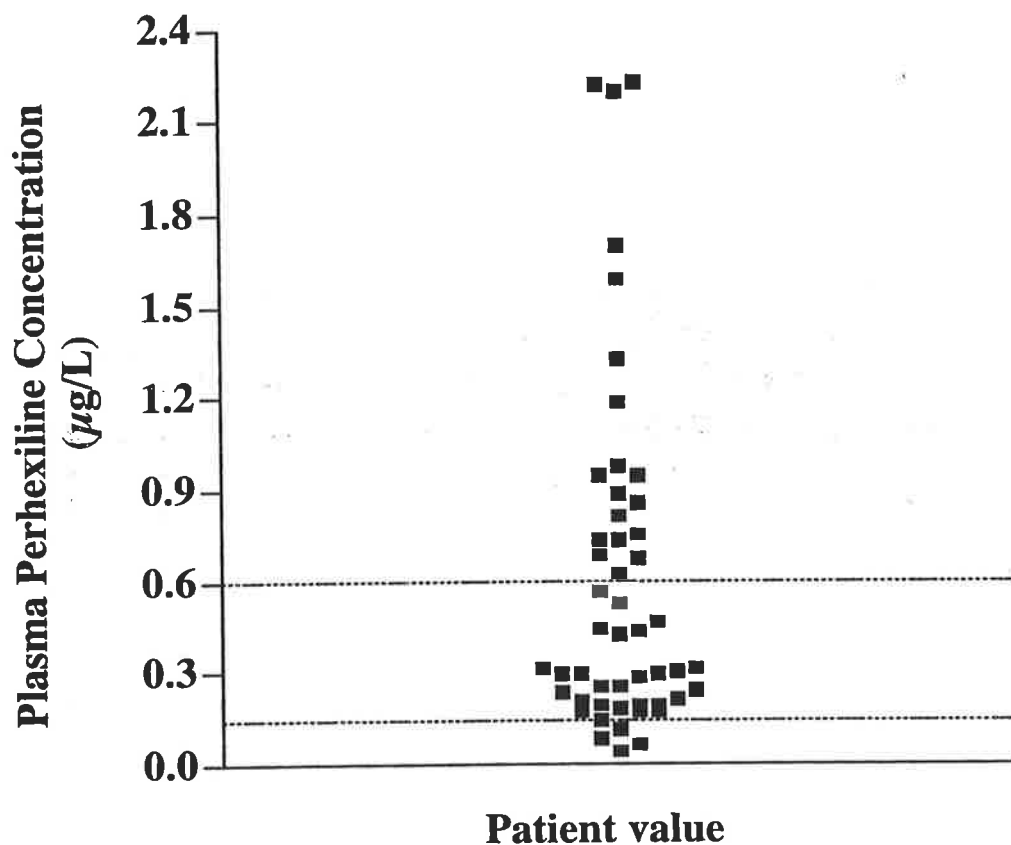


Figure 6.19.

Plasma perhexiline concentrations for acute coronary syndrome patients after 3 days of perhexiline therapy. The therapeutic range (0.15 to 0.60 µg/L is indicated). 19 patients had perhexiline levels higher than the upper limit of the therapeutic concentration range.

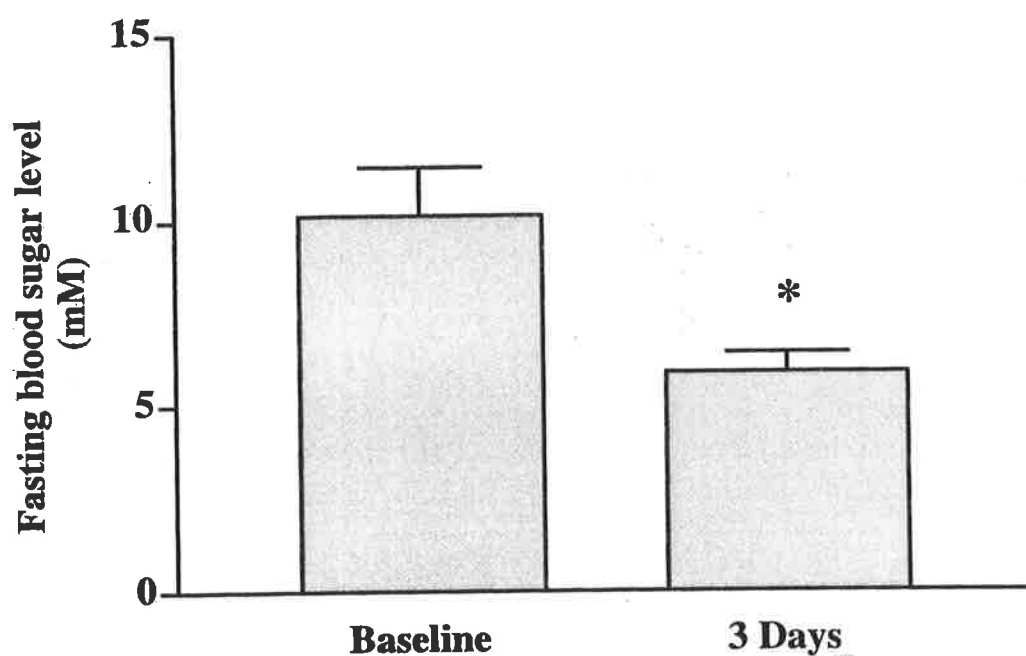


Figure 6.20.

Effect of 3 days perhexiline therapy on fasting blood sugar levels in acute coronary syndrome patients with diabetes mellitus (n=17). A significant reduction in blood sugar levels ($p<0.001$) was evident after 72 hours perhexiline therapy.

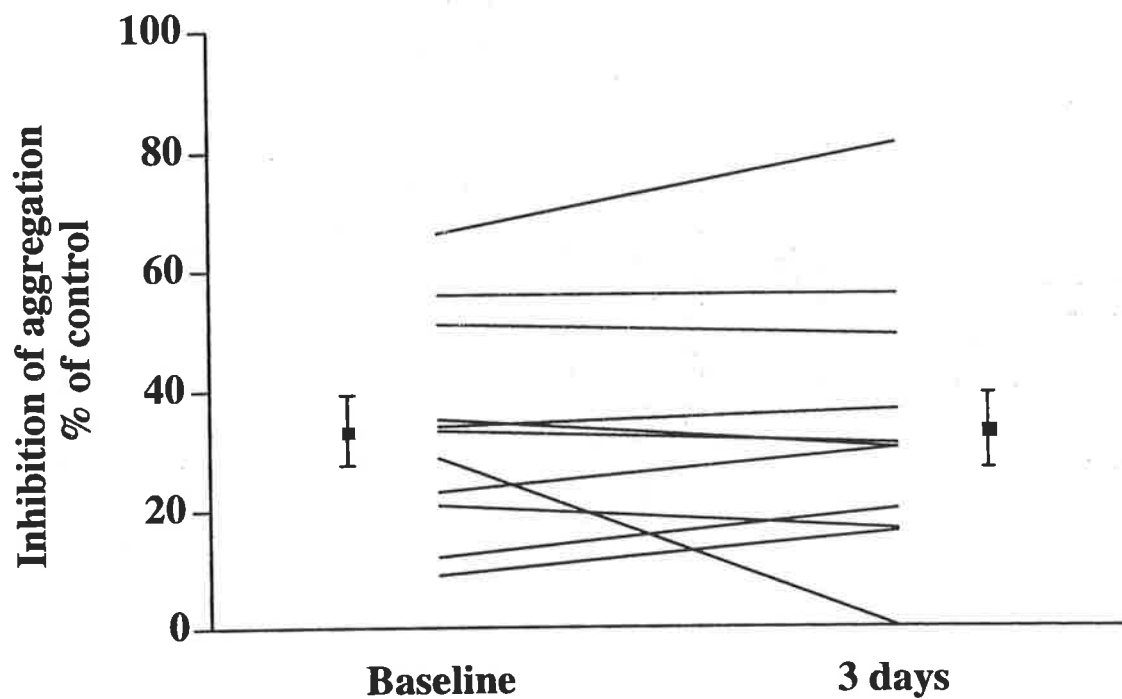


Figure 6.21.

Effect of 3 days conventional therapy (no perhexiline) on SNP (10 μ M) responsiveness. Acute coronary syndrome patients (n=12) not receiving perhexiline demonstrated no significant difference (p=0.26) in SNP responsiveness after 3 days of conventional therapy.

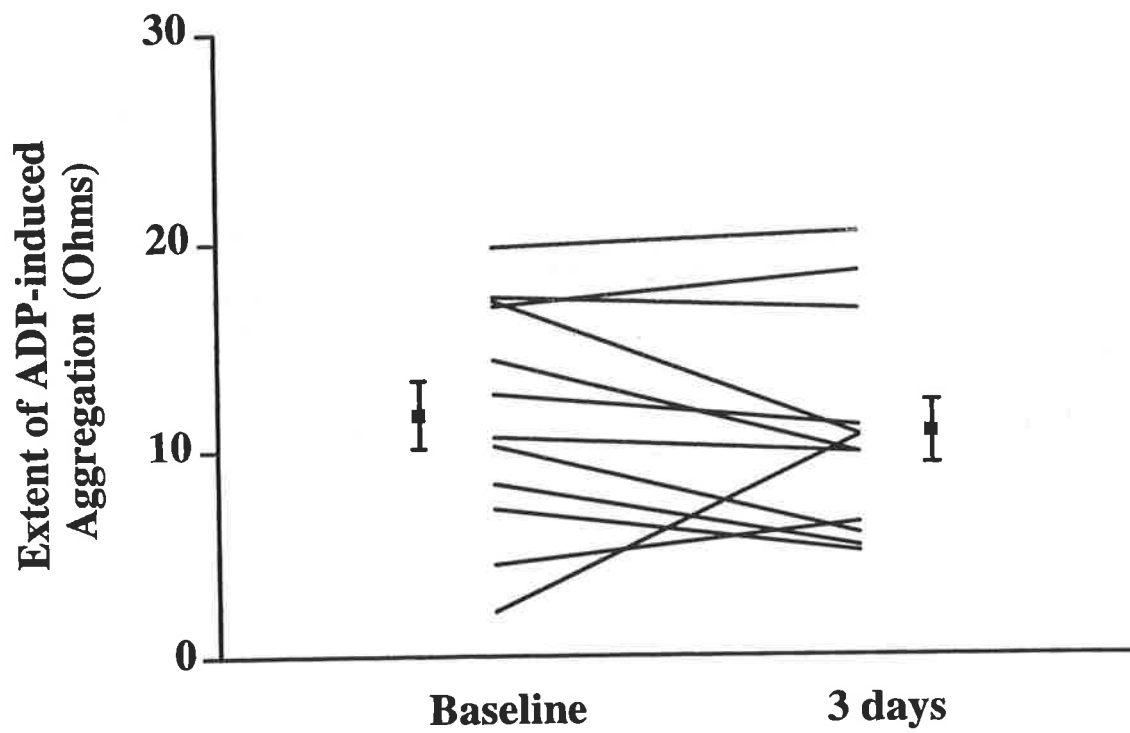


Figure 6.22.

Effects of 3 days conventional therapy (no perhexiline) on extent of ADP-induced aggregation in blood samples from acute coronary syndrome patients (n=12). No significant difference in extent of aggregation was observed ($p=0.57$).

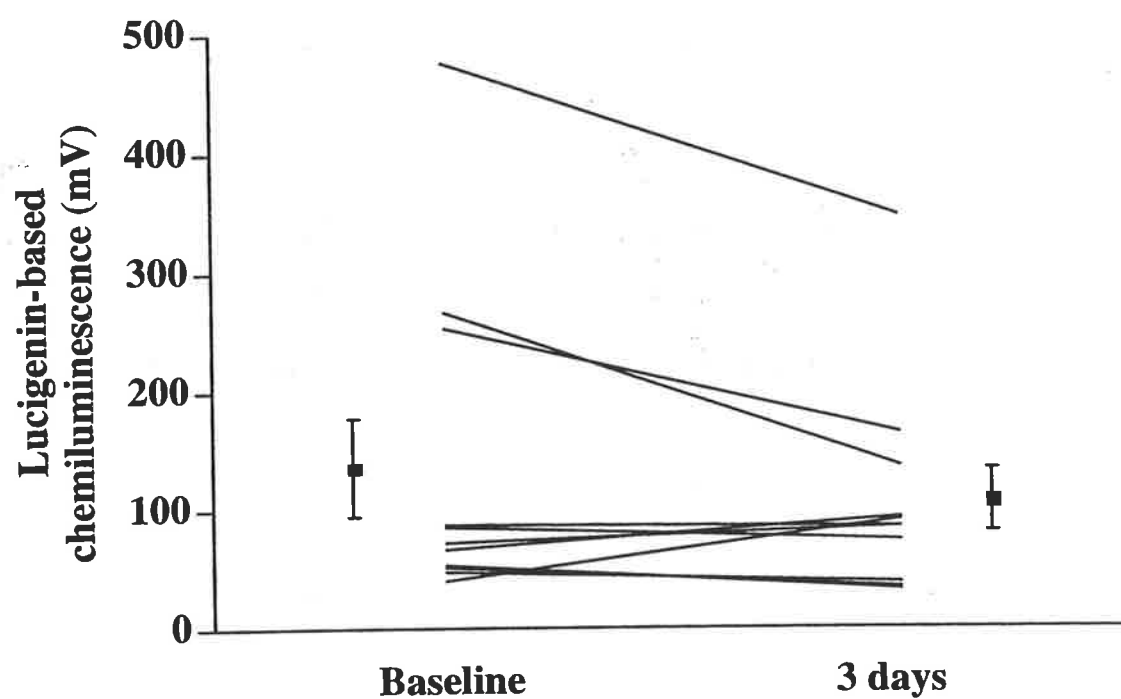


Figure 6.23.

Effect of perhexiline therapy on superoxide content in blood samples from acute coronary syndrome patients (n=11). No significant changes were observed.

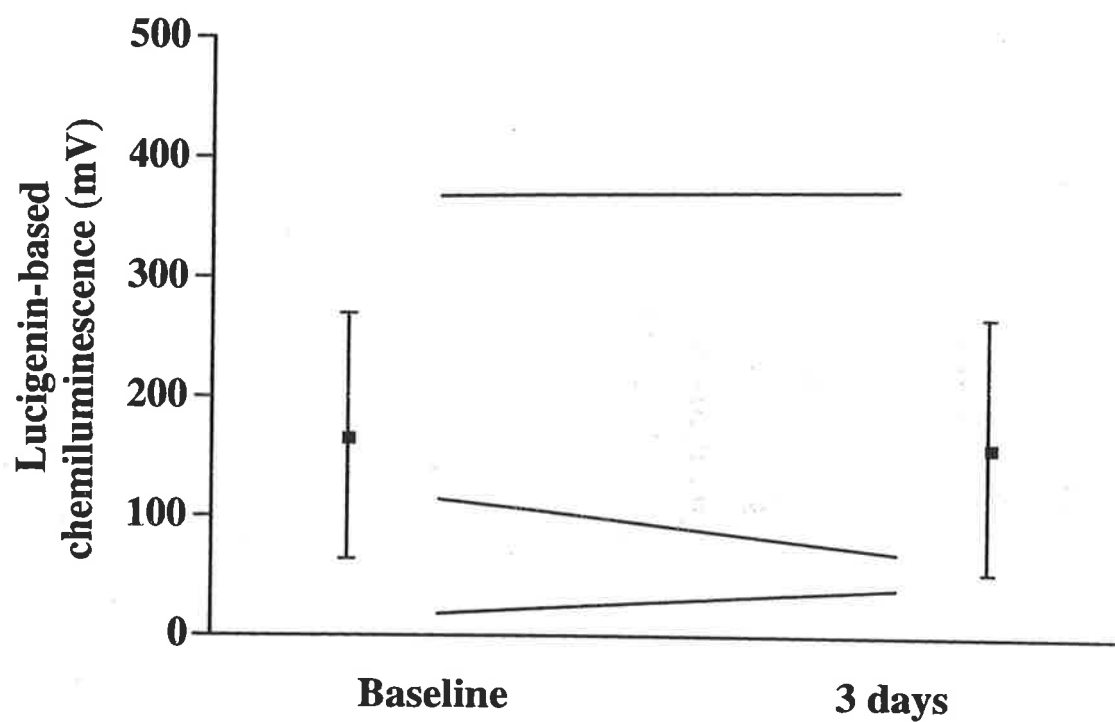


Figure 6.24.

Effects of 3 days conventional therapy on superoxide content in blood samples from acute coronary syndrome patients not receiving perhexiline (n=3). Superoxide content was not altered by the efflux of time or improvement in symptomatic status.

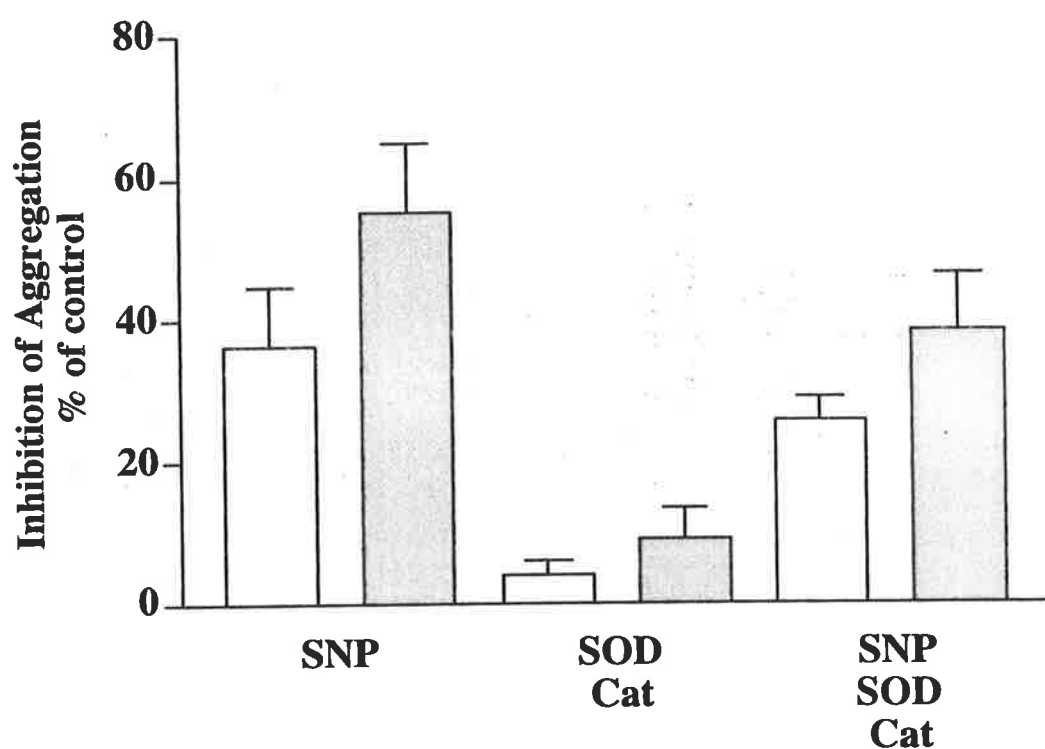


Figure 6.25.

Effect of superoxide dismutase (SOD, 300U/ml) plus catalase (Cat, 300U/ml) on the SNP (10 μ M) response of acute coronary syndrome patients (n=6) at baseline (clear bars) and after (shaded) 3 days perhexiline therapy. Both at baseline and after perhexiline therapy SOD plus catalase did not significantly affect baseline aggregation or inhibition of aggregation by SNP.

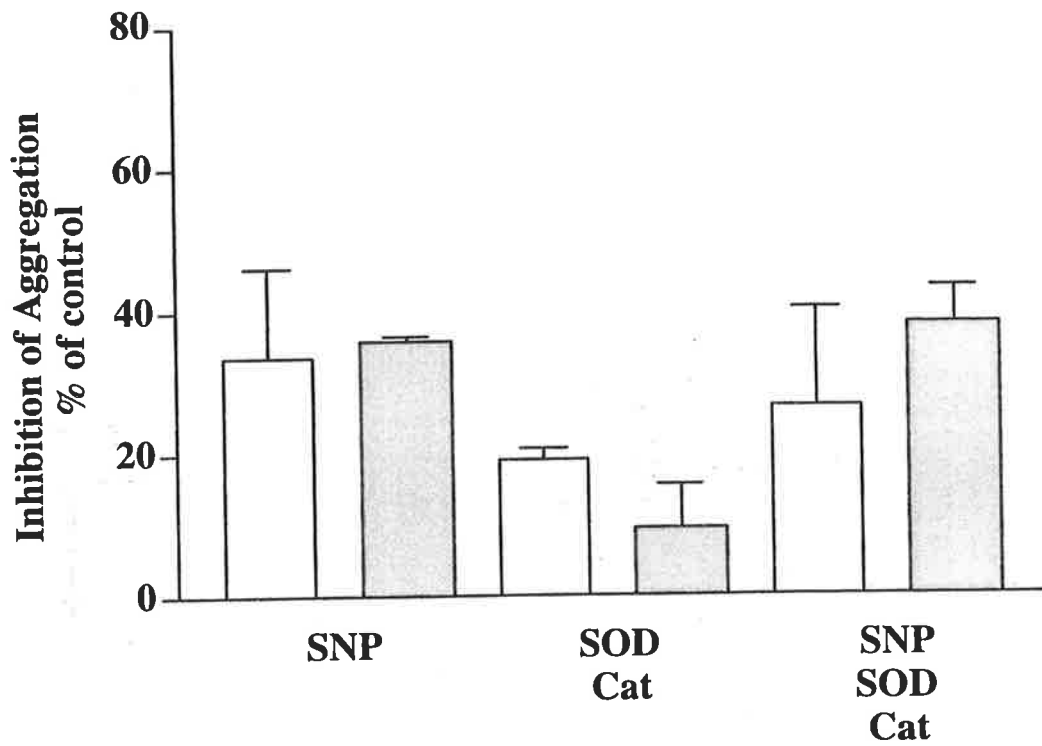


Figure 6.26.

Effect of superoxide dismutase (SOD, 300U/ml) plus catalase (Cat, 300U/ml) on the SNP (10 μ M) response of acute coronary syndrome patients (n=4) at baseline (clear bars) and after (shaded) 3 days conventional therapy. At baseline SOD plus catalase produced a significant inhibition of aggregation but did not affect baseline responsiveness to SNP. After 3 days of conventional therapy SOD and catalase did not affect the SNP response, but did demonstrate a significant increase from baseline in degree of inhibition of aggregation.

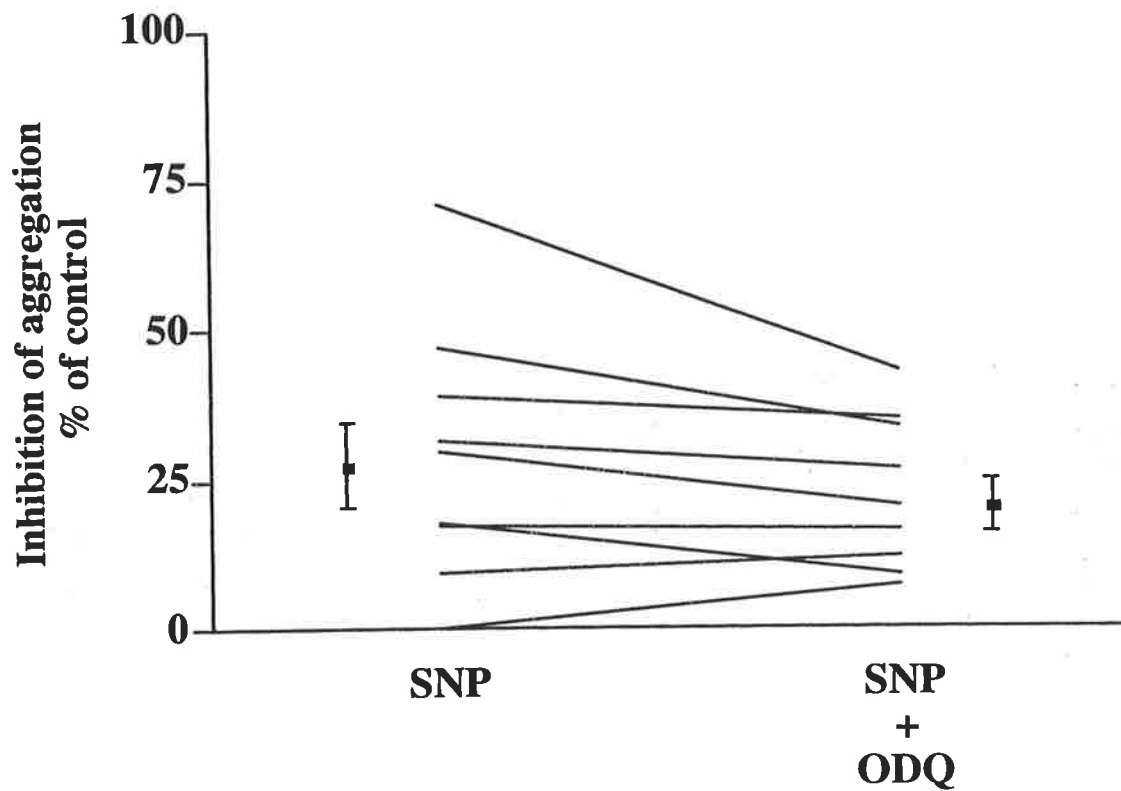


Figure 6.27.

Effect of ODQ (10 μ M) on baseline SNP (10 μ M) responsiveness in blood samples from acute coronary syndrome patients receiving perhexiline (n=10). ODQ significantly decreased baseline SNP responsiveness from 27.5 ± 7.0 to 20.4 ± 4.4 % inhibition of aggregation, $p < 0.05$.

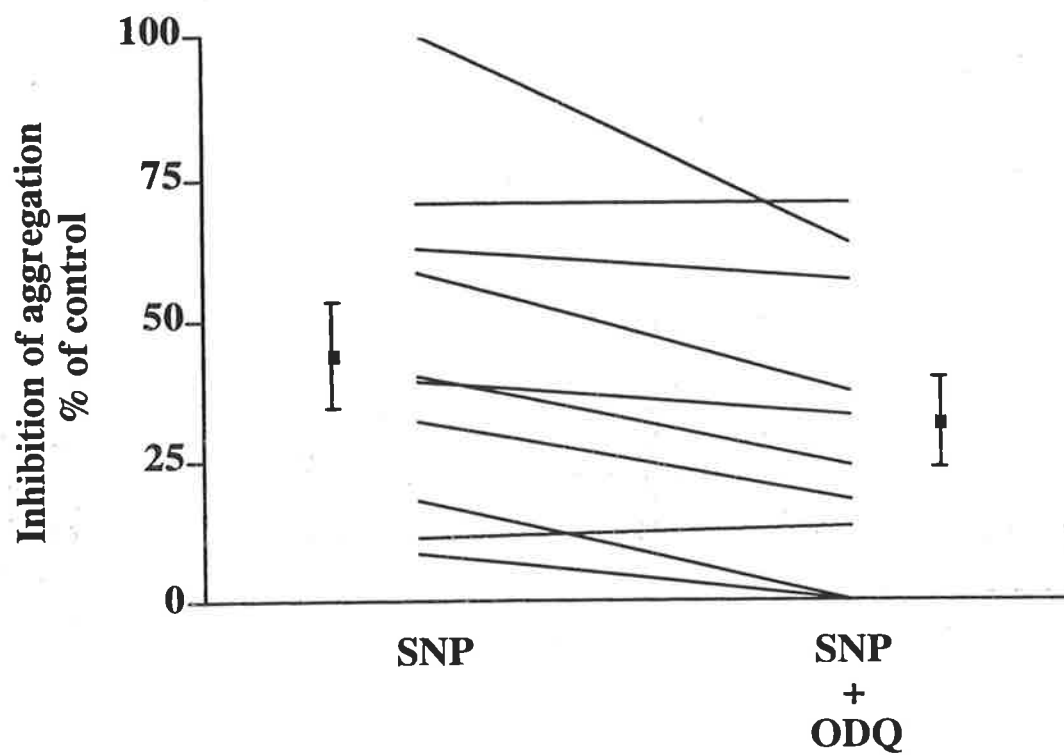


Figure 6.28.

Effect of ODQ (10 μ M) on SNP (10 μ M) responsiveness after perhexiline therapy.

ODQ significantly decreased platelet SNP responsiveness from 43.9 ± 9.2 to 31.7 ± 8.0

% inhibition of aggregation, $p < 0.01$.

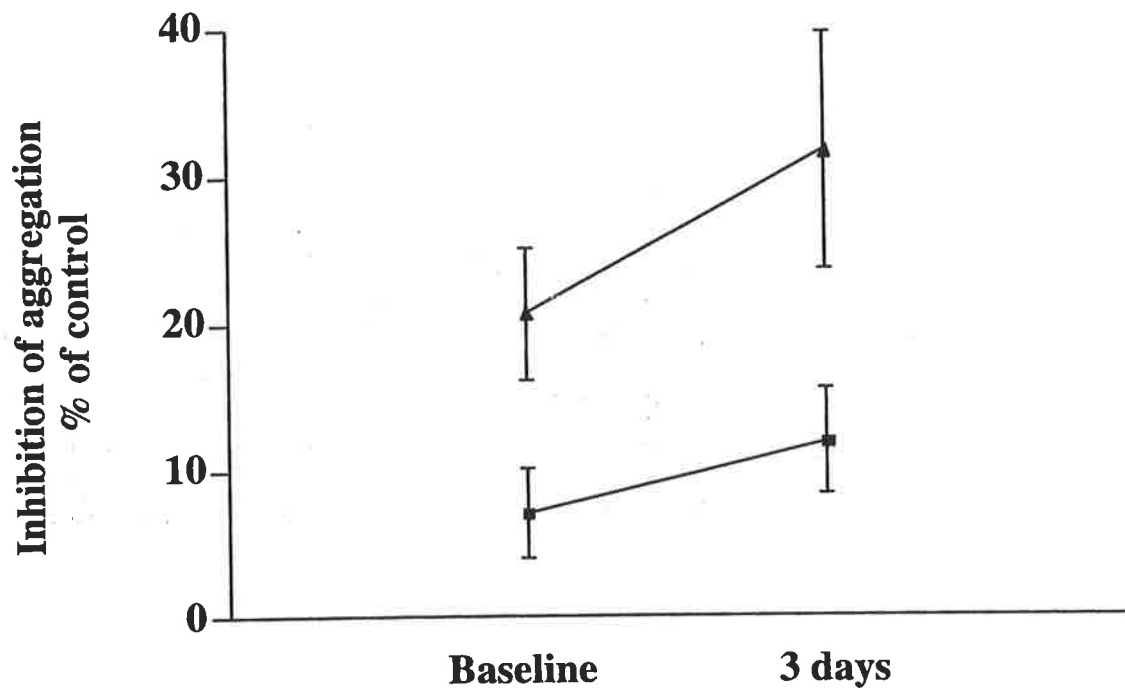


Figure 6.29.

Individual components (▲-guanylate cyclase-independent and ■-guanylate cyclase-dependent) of SNP mediated inhibition of aggregation. After perhexiline therapy the guanylate cyclase-independent component of inhibition increased from 20.7 ± 4.4 to 31.7 ± 8.0 % inhibition of aggregation ($p=0.13$), while the guanylate cyclase-dependent component increased from 7.1 ± 3.1 to 12.0 ± 3.6 % inhibition of aggregation ($p=0.06$).

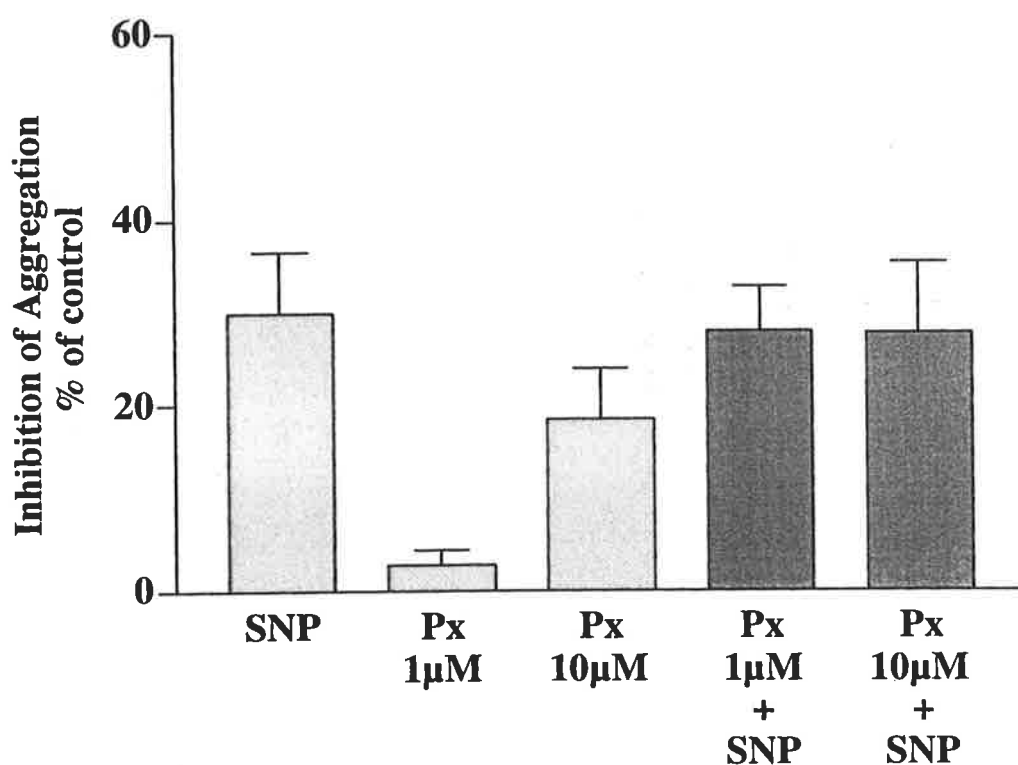


Figure 6.30.

Acute effect of perhexiline on SNP responsiveness in vitro. SNP (10 μ M) produced 29 ± 6.7 % inhibition of ADP-induced aggregation. Perhexiline 1 and 10 μ M produced 2.9 ± 1.6 and 18.5 ± 5.3 % inhibition of aggregation respectively. When perhexiline was pre-incubated with SNP the resultant inhibition of aggregation was less than the sum of the individual extents of inhibition for perhexiline and SNP.

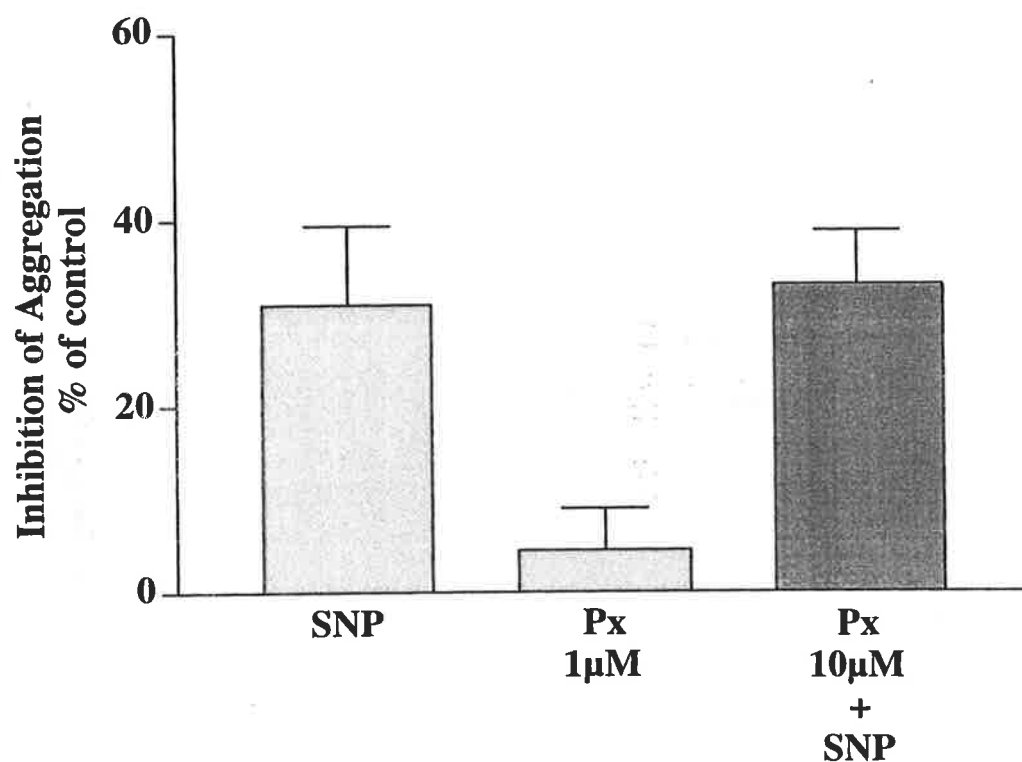


Figure 6.31.

Acute effect of perhexiline on SNP responsiveness in vitro. SNP (10μM) produced 31±8.3 % inhibition of multiple agonist-induced aggregation. Perhexiline (1μM) produced 4.5±4.5 % inhibition of aggregation. When perhexiline was pre-incubated with SNP, the resultant inhibition of aggregation was 33±5.7 %.

Chapter 7

PERHEXILINE INHIBITS SHEEP PLATELET AGGREGATION.

7 Chapter 7: Perhexiline Inhibits Sheep Platelet Aggregation

7.1 Summary

The objective of this study was to examine the effects of acute and chronic perhexiline monotherapy on ADP-induced platelet aggregation and platelet nitric oxide responsiveness. Adult female sheep (25-30 kg) were infused with either perhexiline (3-5 mg/kg, n=6), etomoxir (10 mg/kg, n=3) and vehicle (5% dextrose, n=6) for 60 min. Blood samples were collected before and after the infusion and examined for ADP and nitric oxide responsiveness. ADP responsiveness was not altered by either perhexiline or etomoxir. Sheep platelets were totally unresponsive to the anti-aggregatory effect of the nitric oxide donor sodium nitroprusside..

Two sheep were examined for the effect of chronic (2-3 days) oral perhexiline (200 and 600 mg bd) treatment. Although no inhibition of aggregation was evident for perhexiline 200 mg bd from mean data, at day 2 of treatment perhexiline at this dosage inhibited ADP (1 μ M)-induced aggregation by 61 ± 10 %. In addition, perhexiline 600 mg bd inhibited ADP-induced aggregation at all concentration examined ($p < 0.001$).

These results suggest that perhexiline has a direct anti-aggregatory effect independent of its interaction with nitric oxide, which might contribute to its beneficial effects in patients with stable angina and acute coronary syndromes.

7.2 Introduction

The results of Chapter 6 demonstrate that perhexiline produced a significant improvement in platelet nitric oxide responsiveness but, no significant decrease in the extent of ADP-induced aggregation. This result is somewhat concerning, but not totally unexpected given the fact that many other anti-platelet agents are administered to both stable angina or acute coronary syndrome patients.

A possible way around this problem would be to investigate the effect of perhexiline monotherapy in patients with severe aortic stenosis. Unger et al have previously demonstrated that perhexiline significantly decreased symptoms in aortic stenosis patients, who were unsuitable for aortic valve replacement (Unger, et al. 1997). However, even within the aortic stenosis population a large number of patients will be using medications known to affect platelet aggregation (eg. aspirin). Thus it would be difficult to accumulate a group of such patients treated purely with perhexiline.

With this in mind a series of experiments were conducted in normal sheep to investigate the effects of perhexiline monotherapy on platelet aggregation.

7.3 Objectives

To examine the effect of acute and chronic perhexiline administration on ADP-induced sheep platelet aggregation. Acute effects were compared with those of the specific

carnitine palmitoyltransferase-1 inhibitor, etomoxir. Further experiments were planned to examine the effects of both agents on nitric oxide responsiveness.

7.4 Methods

7.4.1 Animals

Adult female sheep (n=6, 25-30 kg) were used in all experiments. Animals were kept in wire-bottomed cages in a controlled-temperature room with a 12 hour light / dark cycle. Sheep were fasted for 24 hours prior to the experiment, but water was provided *ad libitum*.

7.4.2 Experimental protocol

7.4.2.1 Intravenous agents

Experiments were started between 3-4 pm. Perhexiline lactate (3-5 mg/kg body wt.) was dissolves in 5% dextrose and administered via an intravenous canula in the jugula vein. Etomoxir (10 mg/kg body wt.) was dissolved in 5% dextrose plus lactate. In control experiments sheep were given the same volume of 5% dextrose and lactate. A 1 week washout period was observed between successive experiments in the same sheep.

As Etomoxir as an irreversible carnitine palmitoyltransferase-1 inhibitor it was administered to sheep 1 week after perhexiline or control infusions.

7.4.2.1.1 Blood sampling

Sheep were anaesthetised with intravenous Nembutal (0.5-1.0 mg/kg) for the course of the experiment (1.5-2.0 hours). 15 minutes after the sheep was anaesthetised and a 10 ml blood sample was withdrawn into tubes containing acid-citrate anticoagulant and examined for platelet responsiveness to ADP (0.1-5 μ M), utilising whole blood impedance aggregometry as described in Chapter 2.

All infusions commenced 30 mins after the sheep was anaesthetised and continued for 60 mins. At the end of this infusion period another blood sample was collected for impedance aggregometry studies. When perhexiline was infused an additional amount of blood was withdrawn to assess plasma perhexiline concentrations.

7.4.2.2 Oral Perhexiline

An additional 2 adult female sheep were investigated for the effect of oral perhexiline on ADP-induced platelet aggregation. One of these sheep received 200 mg bd of perhexiline for 2 days, while the other received 600 mg bd of perhexiline for 3 days. Blood samples were collected immediately prior of the first administration of perhexiline, then daily (at the same time) each days thereafter. Additional blood was withdrawn at each time interval and assessed for plasma perhexiline concentration. Control experiments were performed over 3 days, 1 week prior to the commencement of perhexiline treatment.

7.4.2.3 Nitric oxide responsiveness

At each blood collection (both intravenous and oral) the nitric oxide donor, sodium nitroprusside (SNP, 10 μ M) was examined for its effect on ADP (1 μ M)-induced platelet aggregation.

7.4.3 Statistical analysis

The significance of differences was assessed utilising analysis of variance (ANOVA), and results are expressed as mean $\pm$ SEM.

7.4.4 Chemicals

Perhexiline lactate and oral perhexiline (Pexid) was obtained from Sigma Pharmaceutical Company, Melbourne, Australia. Etomoxir was obtained from Research Biochemicals International, Natick, MA, USA.

7.5 Results

7.5.1 Effects on ADP-induced aggregation

7.5.1.1 Intravenous infusions

Figure 7.1 shows the inhibition of platelet aggregation from baseline values for sheep infused with either perhexiline (n=6), etomoxir (n=3) or vehicle control (5% dextrose;

n=6). No significant difference was observed between either of these groups ($F=0.37$, $p=0.77$). Thus neither perhexiline, etomoxir or vehicle significantly affected aggregation

At the end of the infusion period plasma perhexiline levels were $0.33 \pm 0.05 \mu\text{g/L}$, corresponding to the mid-point of the human therapeutic range.

7.5.1.2 Oral Perhexiline

To investigate if chronic perhexiline might produce an anti-aggregatory effects two sheep were administered oral perhexiline for 2-3 days.

Figure 7.2. shows both the sham (3 days without oral perhexiline) period and perhexiline treatment (200 mg bd). Perhexiline at this dosage produced equivocal inhibition of ADP-induced sheep platelet aggregation (see also Figure 7.4). Although blood samples were collected at the end of the infusion period for determination of plasma perhexiline levels, no detectable levels were observed; thus levels in the systemic circulation were less than $0.05 \mu\text{g/L}$.

Figure 7.3. shows the control period and the effect of perhexiline treatment (600 mg bd) on ADP-induced sheep platelet aggregation. In contrast to the lower dose of perhexiline, 600 mg bd produced significant inhibition of aggregation at all times tested ($p < 0.001$; see also Figure 7.4). However, plasma perhexiline levels were again undetectable.

7.5.2 Effects on nitric oxide responsiveness

Platelet responsiveness to the nitric oxide donor, SNP was examined in every blood for sheep treated with both intravenous and oral perhexiline. Sheep platelets were totally unresponsive to the anti-aggregatory effects of the nitric oxide donor sodium nitroprusside. This lack of response was not altered by either perhexiline or etomoxir.

7.6 Discussion

Although the current series of experiments was small, the results provide some additional insights as to the effects of perhexiline on platelet aggregation *in vivo*.

In experiments where perhexiline was infused, although plasma perhexiline levels were “therapeutic”, no effect on ADP-induced platelet aggregation was observed. This lack of effect may be a reflection of the observations by Kennedy and Unger (unpublished) that the onset of metabolic effects of perhexiline in intact cells is relatively slow (days). Thus the period of the infusion might not be sufficient for perhexiline to manifest a response. This putative slow onset of effect may also be relevant to the data described in Chapter 6.

From a pharmacological point of view, it is quite possible that uptake of perhexiline into target tissues may be slow, as has previously been demonstrated for washout (Barry, et al. 1985). Perhaps additionally, the previously described concentration gradient of

perhexiline in mitochondria, leading to rapid accumulation (Deschamps, et al. 1994) may not apply to intact cells.

Experiments in which perhexiline was orally administered to sheep demonstrated definite effects on aggregation. With perhexiline 200 mg bd for 2 days there was no definite effect on platelet aggregation, although possible inhibition was noted at day 2 (Figure 7.4). Although 200 mg bd of perhexiline is the same loading regimen used for cardiac patients (see Chapter 6), plasma perhexiline levels were not detected. However, when 600 mg bd of perhexiline was administered for 3 days there was a marked decrease in the extent of ADP-induced aggregation; plasma levels were again undetectable. The lack of plasma perhexiline is likely to reflect accelerated perhexiline clearance when compared to humans. It is likely that perhexiline is well absorbed, as it has been shown to be in rats (Sewell, et al. 1989), but that there is extensive presystemic clearance in the liver. Thus inhibition of platelet aggregation in these experiments probably reflects interaction between perhexiline and platelets in the portal circulation.

7.7 Conclusion

Overall, this observation suggests that perhexiline has a biologically relevant an anti-aggregatory effect independent of its interaction with nitric oxide, which might contribute to its beneficial effects in patients with refractory angina and therefore should be a focus of future experimental investigation.

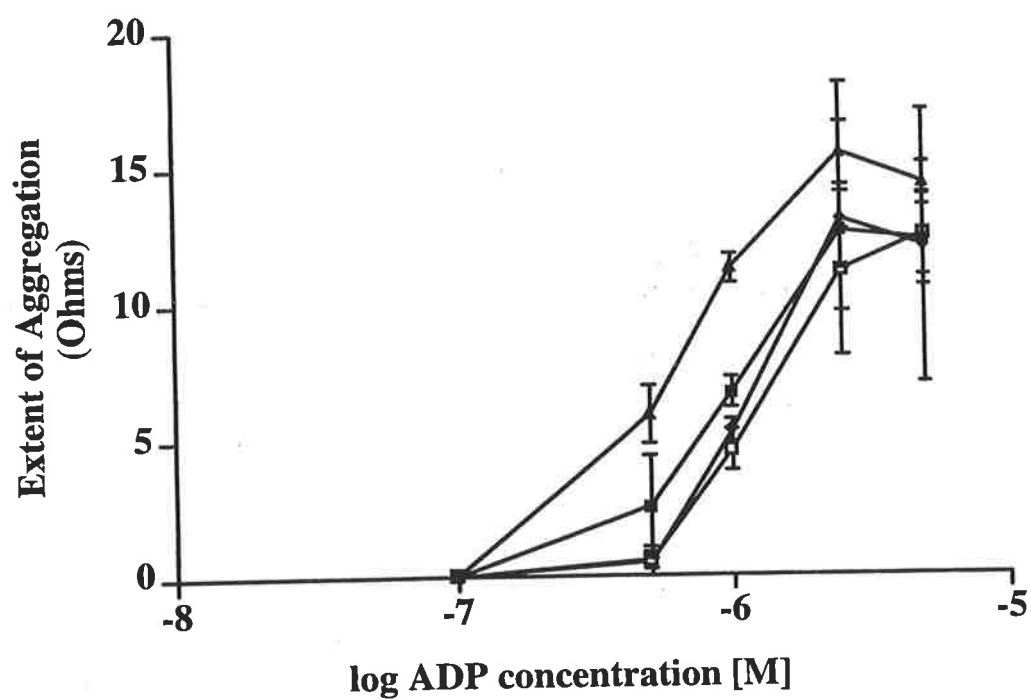


Figure 7.1.

Effect of 60 min infusion of either vehicle (n=6, ■), perhexiline (n=6, □) and etomoxir (n=3, ◆) on extent of ADP-induced sheep platelet aggregation (baseline, ▲). No significant difference was observed between any group and vehicle ($F=0.37$, $p=0.77$).

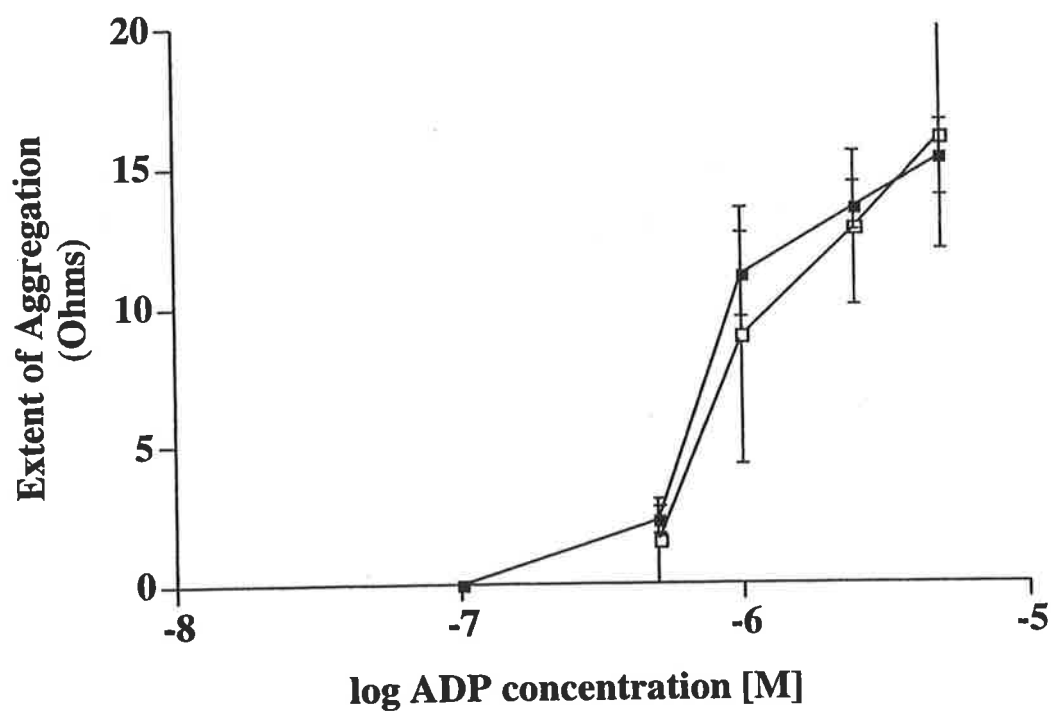


Figure 7.2.

Effect of 3 days oral perhexiline (200 mg bd, □) on ADP-induced sheep platelet aggregation. Perhexiline treatment did not affect control (■) platelet responsiveness.

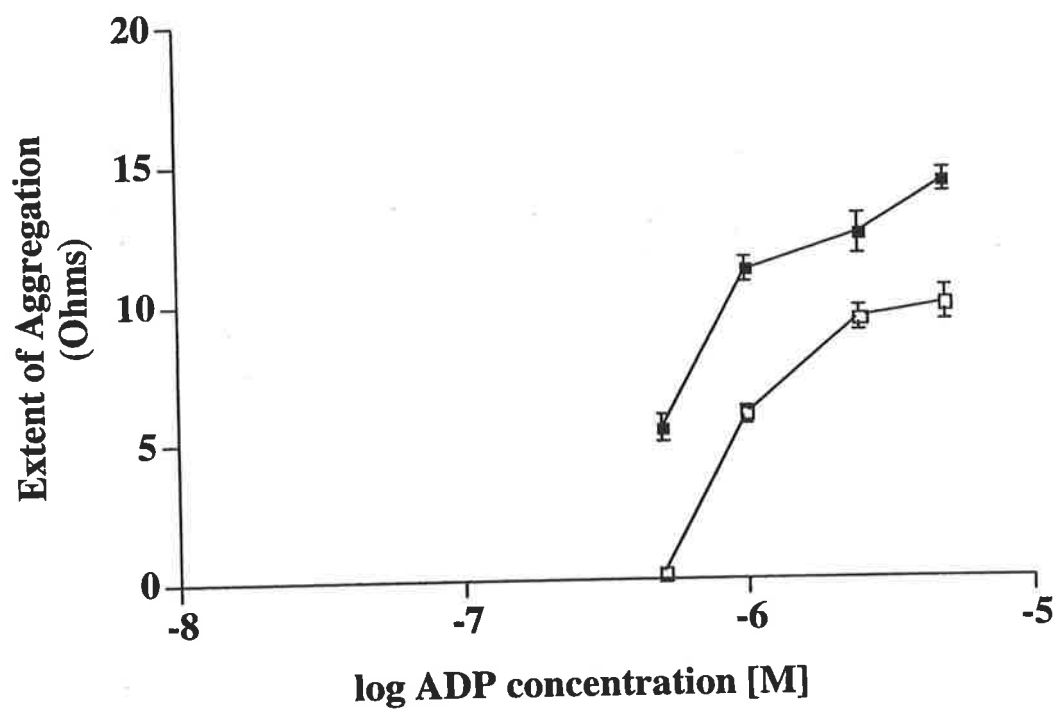


Figure 7.3.

Effect of 3 days oral perhexiline (600 mg bd, □) on ADP-induced sheep platelet responsiveness. Perhexiline treatment significantly reduced extent of ADP-induced aggregation when compared control (■) platelet responsiveness.

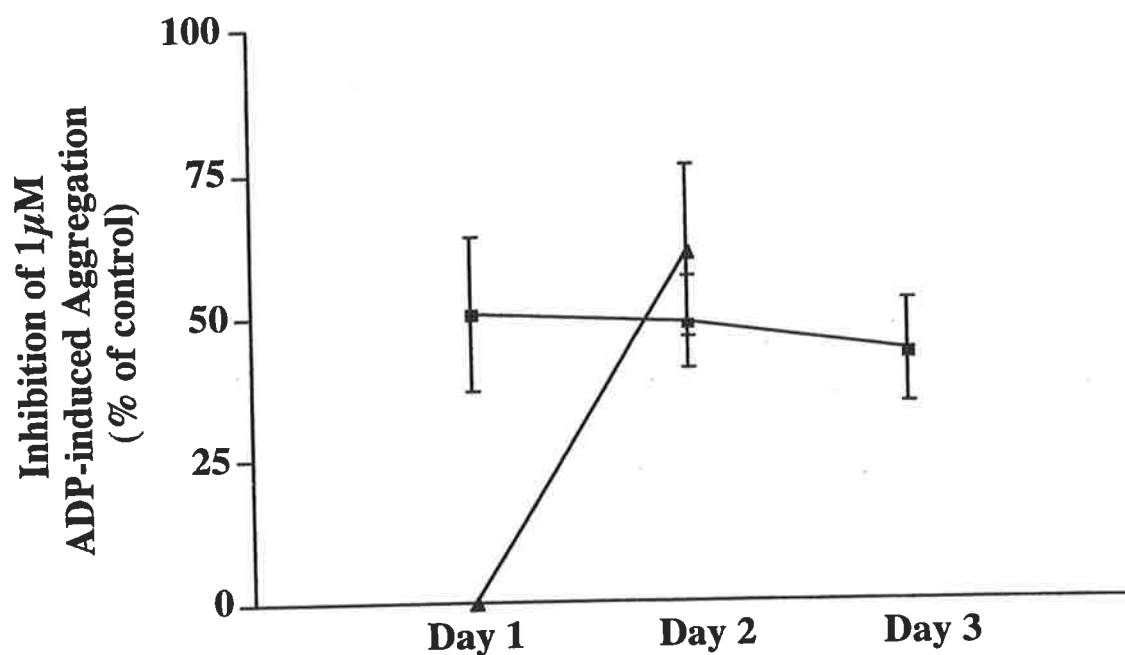


Figure 7.4.

Effect of 2-3 days of oral perhexiline, 200 mg bd (▲) and 600 mg bd (■), on ADP (1 μ M)-induced sheep platelet aggregation. Perhexiline 600 mg bd inhibited aggregation from day 1, while perhexiline 200 mg bd inhibited aggregation at day 2.

Chapter 8

GENERAL DISCUSSION AND FUTURE DIRECTIONS

8 Chapter 8: General Discussion and future directions

The major results of the experiments described in this thesis can be summarised as follows:-

(1) Perhexiline inhibits platelet aggregation in vitro in human blood (Chapter 4), and ex vivo in the sheep (Chapter 7). No definite ex vivo anti-aggregatory effect of perhexiline has been detected in patients receiving aspirin and other anti-anginal agents (Chapter 6). The mechanism of the anti-aggregatory effect is not single (Chapter 4) and may partially involve elevation of cGMP levels in platelets (Chapter 4). It is unlikely to involve the major “metabolic” effect of perhexiline, carnitine palmitoyltransferase-1 inhibition (Chapter 5).

(2) Perhexiline sensitises platelets to nitric oxide in patients with stable angina or acute coronary syndromes (Chapter 6), probably by both the guanylate cyclase -dependent and -independent components of the nitric oxide effect. This effectively reverses the phenomenon of nitric oxide resistance in these patients. The mechanism(s) of reversal do not involve changes in superoxide or hydrogen peroxide levels, but may involve the metabolic effects of perhexiline.

(3) Multiple agonist-induced aggregation is a convenient way of mimicking in vivo aggregation in vitro.

There are some implications from these results, especially (1) and (2) above. It might (and has) been argued that both could contribute to the anti-ischaemic effects of perhexiline, and could tend to prevent acute myocardial infarction. However, it is virtually certain that results (1) and (2) have different mechanisms by virtue of:-

- a) the slow onset of result 2) but not 1)
- b) the demonstration of result 2) in the sheep, which is impervious to result (2).

Among the many limitations of the state of knowledge on the pharmacology of perhexiline and its relationship with platelet aggregation which remain after these experiments, the following stand out:-

- 1) Does perhexiline sensitise platelets to nitric oxide in patients with coronary risk factors but no active ischaemia (who probably show nitric oxide resistance) or even normal subjects?
- 2) What is the mechanism of this sensitisation? Is it common to all agents which divert metabolism from long-chain fatty acids towards carbohydrates?
- 3) Why is there a delay in onset of this effect? How does the effect and the delay correlate with:-
 - a) perhexiline levels? (plasma and tissue)

b) perhexiline-induced changes in metabolism?

Is it possible that very low (ie “safe”) doses of perhexiline might exert this effect.

4) Is this effect also relevant to vascular and myocardial actions of nitric oxide? For example, does it restore “normal” endothelial function in patients with coronary syndromes, a phenomena previously attributed in part to impaired endogenous nitric oxide responses in vasculature? (Bell, et al. 1998; Cannon. 1998). Does it improve the ventricular relaxant effects of nitric oxide, probably critical to beneficial effects of nitric oxide in the left ventricular hypertrophy of aortic stenosis (Matter, et al. 1999), and does this contribute to the beneficial effects of perhexiline in aortic stenosis?

5) What are the mechanisms of result 1)? Given that this is largely nitric oxide- -independent (from the sheep experiments and partially from Chapter 4 results), is it duplicated by other anti-aggregatory agents?

As regards study limitations, there is essentially one major problem, which is that virtually all the results have been obtained using ADP-induced aggregation, largely in whole blood. Of course, this technique is a “mainstream” one which yields quantitation more readily than other agonist-induced aggregation techniques (Puri and Colman. 1997), but it may conceivably produce illusive results as not all physiological / pathological aggregation is ADP-induced. There is therefore considerable need to

validate at least the key findings of this study with other aggregants, as well as with other techniques, such as mobilisation of intraplatelet calcium, platelet surface expression of activated GPIIb/IIIa or P-selectin or thromboxane A₂ formation; such studies are indeed planned.

Thus there are several priorities for future experiments. These can be summarised as follows:-

- 1) Technical validation: (ie same hypothesis, different techniques) - see above.
- 2) Mechanistic evaluation: relevant for both categories of perhexiline effect. Category (1) will require the use of specific antagonists (eg to adenosine, prostanoic acid etc effects). Category (2) will focus on parallel metabolic studies and use of other “metabolic” anti-ischaemic drugs.
- 3) Evaluation of the perhexiline-nitric oxide interaction in other tissues (eg vasculature, endothelial cells, myocardium).

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9 Bibliography

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Appendix

PUBLICATIONS

Willoughby, S.R., Chirkova, L.P., Horowitz, J.D., and Chirkov, Y.Y., (1996) Multiple agonist induction of aggregation: an approach to examine anti-aggregating effects *in vitro*.
Platelets, v. 7 (5-6), pp. 329-333.

NOTE:

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<http://dx.doi.org/10.3109/09537109609023596>

Willoughby, S.R., Chirkov, Y.Y., Kennedy, J.A., Murphy, G.A., Chirkova, L.P., and Horowitz, J.D., (1998) Inhibition of long-chain fatty acid metabolism does not affect platelet aggregation responses.

European Journal of Pharmacology, v. 356 (2-3), pp. 207-213, September 1998

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

[http://dx.doi.org/10.1016/S0014-2999\(98\)00527-5](http://dx.doi.org/10.1016/S0014-2999(98)00527-5)

Thesis Corrections:

Referee 1.

XIV, para 4, line 1:	"of" not "off"
Ch 1, p 29, para 2, line 1:	should read cGMP and cAMP
Ch 1, p 57, line 1:	should read "inhibit"
Ch 1, p 58, line 10:	"no" effect
Ch 1, p 60, line 4:	"too small to be masked"
Ch 2, p 71, 2.12, line 4:	"so as to prevent"
Ch 2, p 73, line 2:	"as described in sections 2.2.5 and 2.2.8, respectively."
Ch 5, p 165, last line:	delete "of"
Ch 6, p 205, para 2, line 1:	"reproduced" should read "reproduce"
Ch 7, p 256, para 3:	"were" should read "where"

Ch 3, p 104, para 3: Serotonin (1-10 μM , $n=2$) added to ADP (0.05 - 5.0 μM) did not produce a significant increase from the addition of the individual extents of aggregation for each agonist ($p=0.23$). However, adrenaline (0.1-1.0 μM , $n=2$) added to ADP (0.05-5.0 μM) produced a significant increase in extent of aggregation compared to the addition of the individual extents of aggregation ($p<0.001$). It is acknowledged that these experiments (hence the small numbers involved) were only utilised to confirm potentiation of aggregation between pairs of agonists (a previously published observation) in our aggregation system, remembering the main focus of the chapter was the development of a multiple (in this case four) agonist method of induction of aggregation, a previously unpublished observation.

Ch 4: The therapeutic concentration range for perhexiline should be 0.15-0.60 " $\mu\text{g}/\text{ml}$ " not " $\mu\text{g}/\text{L}$ ", which to 0.5-2.0 μM . In vitro anti-aggregatory effects of perhexiline were observed at concentrations as low as 0.1 μM , when aggregation was induced by ADP-alone and as low as 1nM when induced by the multiple agonist model. Thus perhexiline inhibits whole blood aggregation at or below its therapeutic concentration range.

Referee 2.

Ch 1, p 47, para 3, line 4:	insert after 0.15-0.6 $\mu\text{g}/\text{ml}$ "which corresponds to concentrations between 0.5-2.0 μM "
Ch 4, p 104, line 1:	" $\mu\text{g}/\text{L}$ " should read " $\mu\text{g}/\text{ml}$ "
Ch 6, p 179, para 2:	" $\mu\text{g}/\text{L}$ " should read " $\mu\text{g}/\text{ml}$ "
Ch 6, p 183, para 1:	" $\mu\text{g}/\text{L}$ " should read " $\mu\text{g}/\text{ml}$ "
Ch 6, p 198, para 4:	" $\mu\text{g}/\text{L}$ " should read " $\mu\text{g}/\text{ml}$ "
Ch 6, p 224, Figure 6.7:	" $\mu\text{g}/\text{L}$ " should read " $\mu\text{g}/\text{ml}$ "
Ch 6, p 225, Figure 6.6:	" $\mu\text{g}/\text{L}$ " should read " $\mu\text{g}/\text{ml}$ "
Ch 6, p 226, Figure 6.8:	" $\mu\text{g}/\text{L}$ " should read " $\mu\text{g}/\text{ml}$ "
Ch 6, p 227, Figure 6.9:	" $\mu\text{g}/\text{L}$ " should read " $\mu\text{g}/\text{ml}$ "
Ch 6, p 234, Figure 6.17:	" $\mu\text{g}/\text{L}$ " should read " $\mu\text{g}/\text{ml}$ "
Ch 6, p 235, Figure 6.18:	" $\mu\text{g}/\text{L}$ " should read " $\mu\text{g}/\text{ml}$ "
Ch 6, p 236, Figure 6.19:	" $\mu\text{g}/\text{L}$ " should read " $\mu\text{g}/\text{ml}$ "
Ch 7, P 255, para 3:	" $\mu\text{g}/\text{L}$ " should read " $\mu\text{g}/\text{ml}$ "

Ch 5-7: Although the developed multiple agonist approach to induction of aggregation is more physiologically relevant than ADP as a single agonist, it was not utilised in Chapters 5 through 7. The reasons for this choice are as follows: 1) in Chapter 5 perhexiline was compared with other agents of similar metabolic action, as the multiple agonist approach did not produced a shift in the perhexiline concentration response curve when compared to ADP-alone (Ch 4), it was determined that the multiple

agonist approach would not yield any additional information regarding this class of agents, thus ADP-alone was utilised as the method of choice; 2) The observed local maximum peak with the multiple agonist approach occurs at concentrations which are below the therapeutic plasma perhexiline concentration. It is recognised that the multiple agonist approach may prove useful when investigating the anti-aggregatory effects of other agents, for example nitroglycerine, in Chapter 3 it was shown that the multiple agonist approach shifted the in vitro concentration response curve for nitroglycerine to the left by approximately a log unit; and 3) The multiple agonist approach is very time intensive and has limited application when routine blood samples are required to be processed in rapid succession as in Chapters 6 and 7.

Ch 6: The cohort of acute coronary syndrome (ACS) patients not receiving perhexiline, so called "controls", were not matched to those ACS patients receiving perhexiline for severity of ischaemic symptoms, and thus represented a group of "less-at risk patients". It would have been desirable to withhold perhexiline therapy from severe ACS patients, which would have enabled a double blind placebo controlled study design. However, as perhexiline is utilised as routine therapy for severe ACS at The Queen Elizabeth Hospital, ethical approval from the local Hospital Ethic's Committee for such a protocol was refused. Nevertheless ACS patients not receiving perhexiline did not demonstrate the increased platelet responsiveness to nitric oxide which was associated with perhexiline therapy. Thus a novel "indirect" antiplatelet effect of perhexiline was discovered.

Given the encouraging results of Chapter 7 regarding a "direct" antiplatelet effect of perhexiline it would have been desirable to study a subset of normal volunteers receiving perhexiline therapy. However, this was not possible in the current thesis and as stated in Chapter 8 is a priority for future experiments.

Ch 7: This chapter was a "preliminary" investigation into the effect of acute and chronic perhexiline monotherapy on ADP-induced platelet aggregation in the sheep. Perhexiline was administered intravenously for 1 hour (acute) or orally over several days (chronic). There was a discrepancy in the results in the respect that acute perhexiline did not inhibit aggregation when chronic perhexiline did. We proposed in Chapter 7 that this lack of effect was due to slow metabolic onset of action of perhexiline. However, this is not consistent with results from Chapter 4 (in vitro perhexiline inhibits platelet aggregation). An additional explanation of the lack of effect would be that there is the possibility of interference between either perhexiline or platelet aggregation and the anaesthetic (nembutal) utilised. This is possible given that plasma perhexiline levels were in the therapeutic range. This was not investigated in the sheep treated acutely as we eliminated the use of nembutal by moving to a conscious sheep (chronic experiments).

The chronic experiments demonstrated that perhexiline inhibits platelet aggregation after 2-3 days of therapy. Thus inhibition of aggregation may contribute to its perhexiline's anti-ischaemic properties. However, caution must be exercised with this statement especially considering that high doses of perhexiline did not produce detectable plasma perhexiline concentrations in the sheep. To conclusively answer this question it is necessary (as stated previously) to conduct similar experiments in normal volunteers.