Studies in Myocardial Ischaemia and Infarction: Effects of N-Acetylcysteine on Oxidative Stress and Myocardial Salvage.

Margaret Anne Arstall, MBBS

A thesis submitted for the degree of

Doctor of Philosophy

Cardiology Unit, The Queen Elizabeth Hospital

Department of Medicine

Faculty of Medicine

The University of Adelaide

July 1995

TABLE OF CONTENTS

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 previously published or written by any other person, except where due reference has been made in the text.

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

ACKNOWLEDGMENTS

During the work of this thesis I was funded as a Postgraduate Medical Research Scholar by The National Heart Foundation of Australia.

I wish to acknowledge the inspiration, guidance and encouragement of my supervisors Prof John D Horowitz and Dr W Henry Betts.

The work of Dr Jeifu Yang and Ms Irene Stafford of the Cardiology Research Laboratory, TQEH in the development and utilisation of the sulphydryl assay (Chapter 2.4) was invaluable to this thesis. The inspiration and encouragement of Prof Ivan del la Lande, Senior Research Fellow in the Cardiology Department of TQEH, as regards the development of the Langendorff-perfused rat heart model was much appreciated. Also, the practical assistance in the development of the malondialdehyde assay and preparation of neutrophils by Ms Geraldine Murphy and Ms Mary Rokasinski of the Rheumatology Research Laboratory, TQEH was greatly appreciated. The advice of Dr Giuseppe Lazzarino of the University of Rome and Dr Bruno Giardina of the Catholic University of Rome concerning the development of the malondialdehyde assay was essential to the final methodology and gratefully acknowledged. The advice of Dr Harvey White of Greenlane Hospital, Auckland, New Zealand concerning the study in Chapter 5 was very helpful and gratefully accepted. Lastly, but by no means leastly, I wish to thank the staff of The Queen Elizabeth Hospital Cardiac Catheterisation Laboratory and Coronary Care Unit for their professional attitude, co-operation, support and patience.

Many thanks to the people who consented to be involved in the various human studies. Their volunteered help was pivotal to this thesis.

Lastly, I wish to sincerely thank my father for his unfailing support during good times and bad.

PUBLICATIONS

MA Arstall, J Yang, I Stafford, WH Betts, JD Horowitz. N-acetylcysteine in combination with nitroglycerin and streptokinase for the treatment of evolving acute myocardial infarction: safety and biochemical effects. Circulation (in press)

MA Arstall, S Stewart, MA Haste, JD Horowitz. Streptokinase-induced aggravation of myocardial ischaemia. International Journal of Cardiology (in press)

SUMMARY

Prolonged myocardial ischaemia results in depletion of endogenous tissue anti-oxidant mechanisms. Where such ischaemia is followed by sudden pharmacologically or mechanically induced reperfusion of the previously ischaemic area is associated with a rapid, but sometimes prolonged phase of release of oxygen-derived radical species and oxidants. This may result in exacerbation of cellular injury, and are believed to be primarily responsible for the phenomenon of myocardial "stunning", reperfusion arrhythmias and possibly further necrosis after reperfusion. Strategies aimed at limiting oxidative stress in humans have met with little success to date. However, thiol-containing drugs have shown some promise, perhaps based upon extensive tissue penetration, multiple pharmacological effects and minimal toxicity. Of such agents, N-acetylcysteine (NAC) is already in clinical use as adjunct to glyceryl trinitrate (GTN) therapy in patients with ischaemic heart disease, and has been shown *in-vitro* both to limit free radical release from neutrophils and to increase clearance of ("scavenge") a variety of free radicals and oxidants.

Plasma biochemical markers of such effects are required to assess the extent of such changes. Two such markers of oxidative stress utilised in this series of studies were changes in the global or regional redox state of glutathione and release of the lipid peroxidation product, malondialdehyde (MDA). A sensitive and specific assay for malondialdehyde in plasma was developed.

Three models of myocardial ischaemia and reperfusion were developed and utilised to further assess the mechanism of action and potential utility of NAC:

1. An isolated Krebs-perfused rat heart, measuring cardiac haemodynamics and MDA concentrations in the coronary effluent,

2. An *in vivo* human model of pacing-induced myocardial ischaemia in patients with stable angina

pectoris and significant coronary stenoses in the left coronary system, measuring cardiac and coronary haemodynamics and the trans-coronary gradients of lactate, MDA utilising coronary sinus catheterisation,

3. An *in vivo* human model of patients receiving treatment with intravenous streptokinase for evolving acute myocardial infarction.

In all models the effect of the use of NAC either alone or in combination with GTN were assessed.

In the model of isolated Krebs-perfused rat hearts, total global ischaemia for 30 minutes followed by reperfusion was not associated with a marked or sustained release of myocardial MDA into the coronary effluent during reperfusion, but marked LV dysfunction after 30 minutes reperfusion. Only if a metabolic or oxidative stress was used in combination with ischaemia, such as depletion of glucose prior to ischaemia or the infusion of activated human neutrophils before and after ischaemia, was there a small, non-sustained but statistically significantly increased myocardial MDA release in the early reperfusion period. This was accompanied by significantly worse LV dysfunction as compared to ischaemia alone. Perfusion of the rat heart with 200 μmol/L NAC either throughout the experimental period or at reperfusion alone, failed to protect the heart from ischaemia/reperfusion induced impairment of left ventricular systolic and diastolic dysfunction or myocardial MDA release into the coronary effluent.

Right ventricular pacing in patients with (n = 12) or without (n = 2) significant left coronary artery disease at 140 bpm for 2 to 3 minutes was associated with a significant myocardial release of MDA indicative of oxidative stress during ventricular tachycardia. In contrast, there was no significant release of MDA from the non-ischaemic femoral vascular bed (n = 2) after mild exercise or during rapid atrial pacing in patients (n = 5) with significant left coronary artery disease. Myocardial lactate release measured by blood sampling from the coronary sinus through a 90 cm

catheter is artefactually decreased due to extraction of the lactate by the catheter. This resulted in an underestimation of the metabolic extent of myocardial ischaemia during rapid pacing. Intravenous NAC infusion (5g over 10 minutes prior to onset of ischaemia, n = 7) decreased myocardial lactate flux but there was no significant decrease of myocardial MDA flux in this small study.

Prior to reperfusion of an occluded coronary artery resulting in evolving acute myocardial infarction significant aggravation of myocardial ischaemia, manifested both by episodic increases in S-T segment elevation on the electrocardiogram (ECG) occurred within the first 20 minutes after initiation of intravenous streptokinase infusion (n = 20). This was temporally, but not quantitatively associated with transient hypotension in most patients. Therefore, is likely that streptokinase aggravates ischaemia prior to reperfusion, although probably not via the induction of hypotension. This data suggests that better protection of the ischaemia myocardium prior to reperfusion may improve overall myocardial salvage. It is also possible that this effect contributes to the "early hazard" of thrombolytic therapy.

In a study of patients treated with streptokinase for evolving acute myocardial infarction, the intravenous infusion of NAC in combination with GTN and streptokinase (n = 20) was associated with a statistically significant reduction in oxidative stress as compared to patients treated with GTN and streptokinase alone (n = 7). Plasma MDA concentration was lower over the first 4 hours and plasma GSH:GSSG ratio higher at 4 and 24 hours in the NAC-treated patients. Plasma concentration of GSH was directly proportional to the plasma concentration of NAC. There was also a non-significant trend towards more rapid reperfusion of the occluded infarct related artery and better cardiac haemodynamics in the early post-infarction period.

Therefore, NAC appeared to decrease oxidative stress in human models of myocardial ischaemia and reperfusion with no significant adverse effects in humans. In combination with GTN, it may improve the speed of reperfusion and increase myocardial salvage in evolving acute myocardial infarction. These compelling results require further investigation in larger clinical trials to assess the clinical effect of the reduction of oxidative stress during myocardial ischaemia and infarction. Furthermore, the exact mechanism of the interactive role of GTN and NAC on myocardial ischaemia, their possible synergistic effect with streptokinase, and cytoprotection during reperfusion requires further investigation.

Chapter 1:

Introduction

1.1 Normal coronary arterial structure and function, and relationship with myocardial metabolism

In order to understand the structural and pathophysiological changes within the heart that result from myocardial ischaemia and reperfusion it is first necessary to understand the relevant cardiac anatomy and physiology. Comparisons between the normal and pathological situation can then be made.

1.1.1 Structure of the coronary arteries

The epicardial coronary arteries are large conduit vessels on the myocardial surface, that branch into smaller vessels that perpendicularly penetrate the myocardium. These arterioles supply a dense capillary network, with flow being regulated by precapillary sphincters. The normal human heart has a variable density of collateral vessels (anastomotic connections without an intervening capillary bed between portions of the same or other coronary arteries). These are usually less than 200 μm in diameter and not visible on coronary angiography of the normal heart.

The normal artery consists of three layers. The inner most intimal layer is lined by endothelium on the luminal aspect and an internal elastic lamina on the outer aspect. The next layer, the media, contains varying amounts of smooth muscle and is surrounded externally by an external elastic lamina. The outer layer is the adventitia.

The intima, consisting largely of endothelial cells, is relatively thin. It partly acts as a semi-permeable membrane. Importantly, it is also an endocrine and paracrine organ producing and metabolising a variety of vasoactive molecules, cytokines and growth factors which regulate blood flow and function. Furthermore, it prevents thombogenesis and is involved in connective tissue

formation within the intima. The endothelial cells are aligned with their long axes in the direction of blood flow (Davies et al. 1988). The endothelial cell borders are irregular and interdigitate, attached to each other by tight junctions and gap junctions, permitting transport of material to and from the lumen via transcytosis. The endothelium rests on a connective tissue matrix consisting of a basement membrane intermixed with collagen fibrils and occasional solitary smooth muscle cells. With age there is an increase in the amount of connective tissue and the number of intimal smooth muscle cells (Ross, 1992).

The media, containing most of the artery's smooth muscle, is bounded by the internal and external elastic laminae. The elastic laminae are fenestrated layers of elastic fibres which permit molecules and cells to pass in either direction. There are multiple lamellae of connecting smooth muscle cells arranged in spiralling layers, with each cell surrounded by a discontinuous basement membrane and interspersed by collagen and proteoglycan. Each lamella is bounded by elastic laminar on the inner and outer aspects. When there are more than 29 lamellae, vasa vasorum from the adventitiae are necessary for adequate nourishment of the outer lamellae (Ross, 1992).

The adventitia consists of a dense structure of collagen bundles, elastic fibres, fibroblasts and some smooth muscle cells. It carries the vasa vasorum, lymphatic channels and innervation (Ross, 1992).

1.1.2 Endocrine and paracrine aspects of endothelial function

The endothelium has multiple physiological functions. It acts as a semi-permeable membrane and maintains the integrity of the underlying basement membrane. One of its vital roles is in the maintenance of vascular tone. Various vasoactive substances are released. Endothelial derived relaxing factor, which is either nitric oxide (NO) or a NO-like compound (Welch and Loscalzo,

1994; Myers et al. 1990), is a potent vasodilator, continually released, contributing markedly towards a "dilator" component of resting vasomotor state (Vallance et al. 1989). It is produced by a calcium and calmodulin dependent, constitutive NO synthase from L-arginine (see also 1.4.3.3). NO acts on its target cell via a reaction with the ferrous ion in the haem prosthetic group in soluble guanylate cyclase, activating this enzyme, and thus generating cyclic guanosine monophosphate (cGMP) (Moncada and Higgs, 1993). This has a variety of effects, including relaxation in vascular smooth muscle cells, inhibition of platelet adhesion and aggregation, inhibition of leukocyte adhesion and activation and inhibition of smooth muscle cell proliferation. (Vane et al. 1990; Moncada and Higgs, 1993; Welch and Loscalzo, 1994; Kubes et al. 1991). NO is a radical species with a short half life (between 6 and 30 seconds) (Butler et al. 1995) in the circulation where it is inactivated by reacting with the ferrous ion in haemoglobin (Moncada and Higgs, 1993). Because of this, NO acts largely locally. However, there is evidence that NO may bind to the sulphydryls of carrier molecules such as albumin and other sulphydryl groups in plasma proteins in a reversible nitrosation reaction, thus acting as a reservoir of NO, enhancing its stability and preserving its biological action (Stamler et al. 1992a; Welch and Loscalzo, 1994; Stamler et al. 1992b; Keaney, Jr. et al. 1993; Scharfstein et al. 1994).

NO acts in synergy with prostacyclin (PGI_2), another endothelial derived vasodilator. PGI_2, a product of arachidonic acid metabolism, is released in response to pulsatile pressure and a variety of endogenous stimulants such as bradykinin and serotonin. Production is inhibited by glucocorticoids and inhibitors of guanylate cyclase. PGI_2 activates adenylate cyclase in the target cell leading to formation of cyclic adenosine monophosphate (cAMP). Like NO, PGI_2 relaxes vascular smooth muscle and inhibits platelet aggregation. It also increases the activity of enzymes that metabolise cholesterol esters in smooth muscle cells and suppresses their accumulation in

macrophages. PGI_2 inhibits the release of several growth factors from smooth muscle cells. Its half-life is less than one circulation time, being non-enzymatically hydrolysed in plasma. Therefore, like NO, it is predominantly a paracrine agent (Vane et al. 1990).

In contrast, endothelin-1 (ET-1) is released from endothelial cells and acts predominantly as a vasoconstrictor. It is produced from two enzymatic proteolytic cleavages of an approximately 200 amino acid preproendothelin to form a 38-39 amino acid "big" endothelin and subsequently the 21 amino acid ET-1 (Simonson and Dunn, 1990). ET-1 appears to be slowly synthesised in response to many substances including thrombin and adrenaline. Some "big" endothelin has also been shown to be present in plasma, but has only approximately 10% of the activity of ET-1 (Simonson and Dunn, 1990; Luscher, 1994). ET-1 is the most potent vasoconstrictor so far discovered, causing sustained vasoconstriction, although it is eliminated from the circulation within minutes. It binds to specific endothelin-A receptors on the smooth muscle cell surface which initiates a complex intracellular response involving the activation of phospholipase C and protein kinase C causing an elevation of intracellular calcium concentration (Simonson and Dunn, 1990; Luscher, 1994; White et al. 1993). Stimulation by ET-1 of endothelin-B receptors leads to release of NO and PGI_2, which limit its net vasoconstrictor effects (Seo et al. 1994).

The endothelial cell surface also contains angiotensin-converting enzyme, thereby interacting in angiotensin and bradykinin metabolism (Vane et al. 1990). Also a variety of adhesion molecules, growth factors and cytokines may be secreted by the endothelium in response to injury (Ross, 1993; Vane et al. 1990).

The endothelium provides a nonthrombogenic surface. Inclusive to the antiplatelet effects of nitric

oxide and prostacyclin (Kubes et al. 1991), tissue plasminogen activator, urokinase and heparan sulphate are released by the endothelium and have anti-thrombotic properties. Von Willebrand factor also adheres to the endothelial surface (Ross, 1993).

1.1.3 Coronary flow

Under physiological conditions coronary blood flow varies greatly (Lombardo et al. 1953). Because the heart is an aerobic organ, increased requirement for oxidative metabolism induced by increased cardiac work are met by increased coronary blood flow, rather than increased oxygen extraction (Lombardo et al. 1953; Rubio and Berne, 1975; Camici et al. 1989). Oxidative metabolism can be accurately estimated from the measurement of the rate of myocardial oxygen consumption (MVO_2) (Lombardo et al. 1953; Ardehali and Ports, 1990; Ando et al. 1989). There are several determinants of myocardial oxygen consumption (Table1.1). Firstly, the basal metabolic state for electrical depolarisation, repolarisation and physiological processes not directly associated with contraction are a small proportion of the total myocardial oxygen demand (Ando et al. 1989). A large proportion of MVO_2 occurs in relation to myocardial wall tension and contractility, as reflected by the left ventricular systolic pressure volume area (Suga et al. 1984). This pressure-volume area is influenced both by external mechanical work applied to the heart (area of the pressure volume loop), and the end-systolic elastic potential energy in the ventricular wall (Figure 1.1). This linearly correlates with MVO_2 regardless of loading conditions. Furthermore, in the intact heart, as contractility increases (for example in response to increased catecholamines), heart size and therefore wall tension decreases. However in the normal heart, heart size cannot change greatly, so that an increase in contractility usually leads to an increase in MVO_2 (Teplick et al. 1986; Suga et al. 1983). Also, heart rate is a direct determinant of MVO_2 (Rooke and Feigl, 1982). MVO_2 is also influenced by the substrate utilised by the myocardium

for energy metabolism. In particular MVO_2 is directly proportional to the fraction of energy derived from fatty acid metabolism, which varies directly with arterial fatty acid concentration and inversely with arterial glucose and insulin concentration (Vik Mo and Mjos, 1981).

Under normal physiological circumstances, coronary flow is predominantly controlled within the resistance vessels that penetrate at right angles from the epicardial surface to the endocardium. The variable pressure gradient across the coronary vascular bed is the difference of the pressure at the origin of the epicardial coronary arteries and the potentially occlusive pressure in the left ventricle in diastole. Flow is therefore a function of this pressure gradient and the resistance offered by the vascular bed (Rubio and Berne, 1975). Autoregulation of the coronary flow occurs to maintain myocardial perfusion within a relatively narrow range despite changes in perfusion pressure. This complex, but important phenomenon of alteration in coronary vasomotor tone is more prominent in the subepicardium as compared the subendocardium of the left ventricle. Essentially autoregulation is achieved via regional or global coronary vasodilation at the microvascular level in the face of factors which would otherwise result in decreased flow (Marcus et al. 1990).

Total coronary vascular resistance is influenced by both factors extrinsic and intrinsic to the vascular bed. An important extrinsic factor is intramyocardial wall tension. As this is much higher during systole and the resistance vessels are "throttled" leading to most coronary flow occurring during diastole. Furthermore, these extrinsic compressive forces are probably greater in the endocardial third of the left ventricle than the epicardial third (Stein et al. 1980). Therefore, when the perfusion gradient drops, either due to decreased driving pressure (secondary to a decreased mean arterial pressure or significant stenosis in an epicardial vessel), or rise in ventricular diastolic

TABLE 1.1 Determinants of myocardial oxygen consumption

Category	Influential factors	reference
Basal	maintenance of cell viability	Ando et al. 1989
	electrical depolarisation and repolarisation	Klocke et al. 1966
	maintenance of the active state	Ando et al. 1989
	proportion of V_1:V_3 myosin isoforms	Tubau et al. 1987
Work of contraction	muscle shortening	Covell et al. 1967
	LV wall tension	Ardehali and Ports, 1990 Rooke and Feigl, 1982
	LV contractility	Ardehali and Ports, 1990 Suga et al. 1983 Suga et al. 1984
	heart rate	Rooke and Feigl, 1982
External effects	external mechanical work	Suga et al. 1984
	metabolism of catecholamines	Suga et al. 1983 Teplick et al. 1986
	stimuli for variable fatty acid uptake	Vik Mo and Mjos, 1981

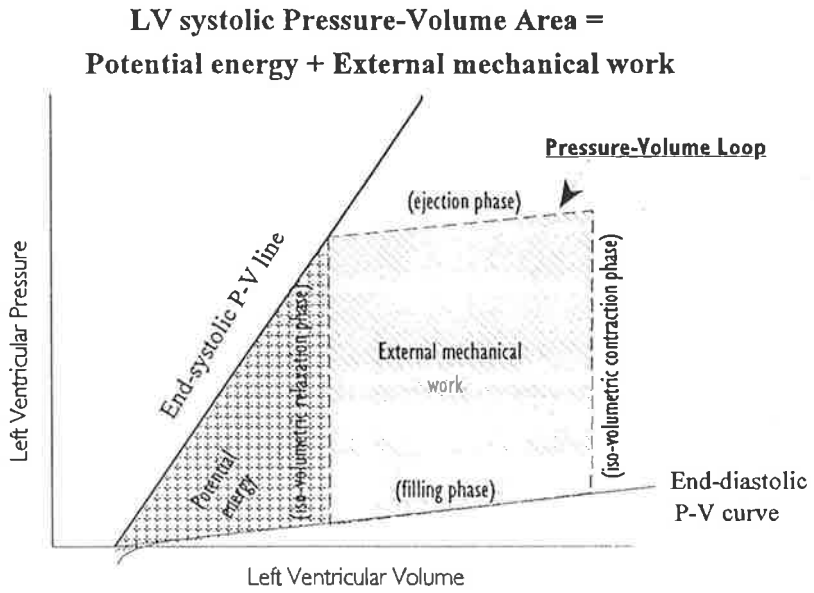


FIGURE1.1 Relationship of the pressure-volume loop to LV systolic pressure-volume area. (Suga et al. 1984)

pressure, the subendocardial flow is the most likely to be compromised (Brazier et al. 1974).

There are many factors intrinsic to the vasculature that influence total coronary vascular resistance. An important control system is the vasoactivity of the endothelium, which is discussed in 1.1.2. Coronary vascular tone is markedly influenced by the autonomic nervous system which richly innervates the vessels. Vasoconstriction is caused by stimulation of both α_1 and α_2 adrenoceptors mediated by an increase in vascular smooth muscle intracellular calcium concentration (Woodman and Vatner, 1987). In contrast, activation of β_1 and β_2 adrenoceptors in both large and small coronary arteries induces vasodilation (Vatner et al. 1986). It appears that adrenergic constrictor tone predominates at rest (Vatner et al. 1970). Cholinergic stimulation from the vagal nerve appears to indirectly mediate small vessel dilatation (Higgins et al. 1973) (see 1.1.2). Baroreceptor activity influences autonomic outflow to the coronary vasculature in a reflex manner (Hackett et al. 1972).

Changes in regional myocardial metabolism and accumulation of metabolites, affect coronary blood flow and therefore influence autoregulation. Metabolic products of hypoperfused regions of myocardium act as vasodilators, lowering vascular resistance and increasing coronary blood flow rather than increasing oxygen extraction from blood. Possible metabolic products include oxygen itself, carbon dioxide, potassium and adenosine. Molecular oxygen appears to be a major determinant of constrictor tone within the precapillary sphincters. Therefore, decreasing oxygen tension allows the sphincters to relax and increase perfusion of the region (Duling, 1972). In grossly ischaemic myocardium, the early increase in extracellular potassium concentration that follows may modify the transmembrane potential of vascular smooth muscle, causing relaxation and coronary vasodilation (Gellai et al. 1973).

Probably one of the most powerful vasodilators of this category is adenosine, which is produced from adenosine monophosphate (AMP) by the enzyme 5'-nucleotidase when myocardial cells are unable to maintain adenosine triphosphate (ATP) resynthesis in balance with ATP utilisation. Adenosine has a paracrine effect, blocking calcium influx into vascular smooth muscle cells via a specific receptor, thus leading to relaxation. The endothelium rapidly metabolises adenosine via the enzyme adenosine deaminase to inosine and hypoxanthine. Furthermore, the resultant vasodilation causes a rise in coronary flow, washing out any remaining adenosine, thereby limiting its response (McKenzie et al. 1982; Collins, 1993).

To maintain adequate coronary flow at times of high MVO_2, the coronary vasculature is capable of significant vasodilation over and above the resting state. This may be manifest by a marked increase in coronary flow, or reactive hyperaemia, after a stimulus such as transient coronary artery occlusion. The difference between basal and maximal flow is called the coronary flow reserve. Methods of estimation of coronary flow reserve in humans include the comparison of coronary flow at rest and after injection of a coronary vasodilator such as dipyridamole or adenosine. However, it is impossible to be certain whether maximum vasodilation has been achieved. Measurement of coronary flow can be carried out via the coronary sinus thermodilution method (Ganz et al. 1971), Doppler flowmeters in the epicardial coronary arteries (Wilson et al. 1985) or positron emission tomography (Bergmann et al. 1989).

1.1.4 Myocardial metabolism

Free fatty acids (FFA) are the major substrates for energy metabolism, via lipid oxidation, in the normal fasting human heart at rest, contributing approximately 80% of the caloric requirements. Furthermore, once FFA is extracted by the myocardium, it is rapidly oxidised (Wisneski et al.

1987) However carbohydrates, namely glucose, pyruvate, lactate, ketones and glutamate are also extracted at rest. Alanine and citrate are released in small amounts (Camici et al. 1991; Camici et al. 1989; Thomassen et al. 1988) (Figure 1.2). Of the glucose extracted, 60 - 70% is stored as glycogen rather than immediately oxidised (Wisneski et al. 1985) In the fed state carbohydrate metabolism predominates with increased rates of glucose and lactate uptake (Camici et al. 1991).

The factors that regulate this variable substrate utilisation are complex and partly depend on substrate availability (Wisneski et al. 1987). For example, lactate and glucose uptake is directly proportional to their respective arterial concentrations and inversely proportional to arterial FFA concentration (Gertz et al. 1980; Kaijser and Berglund, 1992; Wisneski et al. 1985). FFA appears to be extracted by the myocardium via an endothelial membrane fatty acid binding protein, which is saturable by increasing arterial FFA concentration or coronary flow (Vyska et al. 1991). Various hormones also influence substrate extraction. Insulin facilitates glucose uptake and inhibits lipolysis in adipose tissue, decreasing arterial FFA concentration and availability to the myocardium. Catecholamines facilitate FFA uptake and oxidation and decrease glycolysis in myocardial cells (Camici et al. 1991). In the normal heart, as cardiac work increases, the increased oxidative demands are met by an increase in carbohydrate metabolism, both from glycogen stores in the myocardial cell and uptake of glucose and in particular, lactate (Kaijser and Berglund, 1992) (Figure 1.3). This increased carbohydrate oxidation accounts for approximately 60% of the caloric requirements as FFA uptake remains unchanged (Camici et al. 1991). After myocardial stress, cardiac haemodynamics return to the resting state within minutes, but increased carbohydrate uptake continues for at least 30 minutes (Camici et al. 1989).

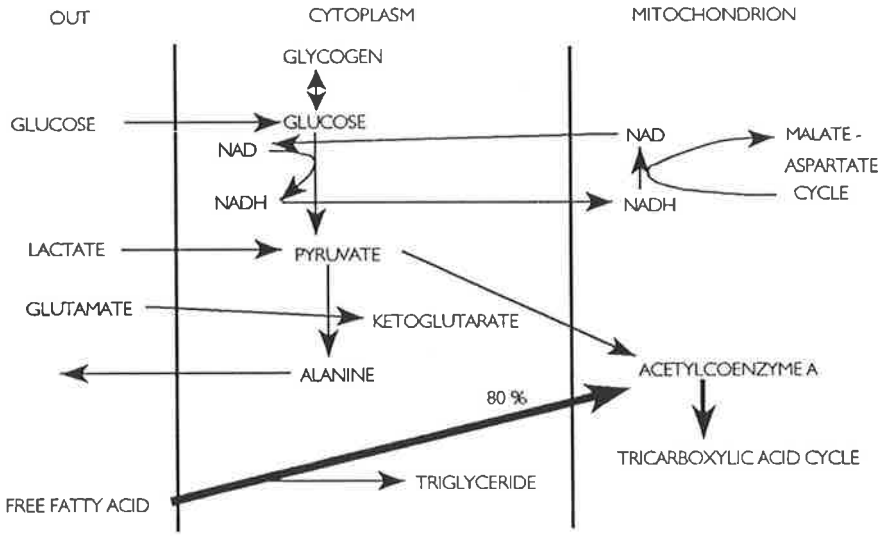


FIGURE 1.2 Myocardial metabolism at rest and fasting. (Camici et al. 1991)

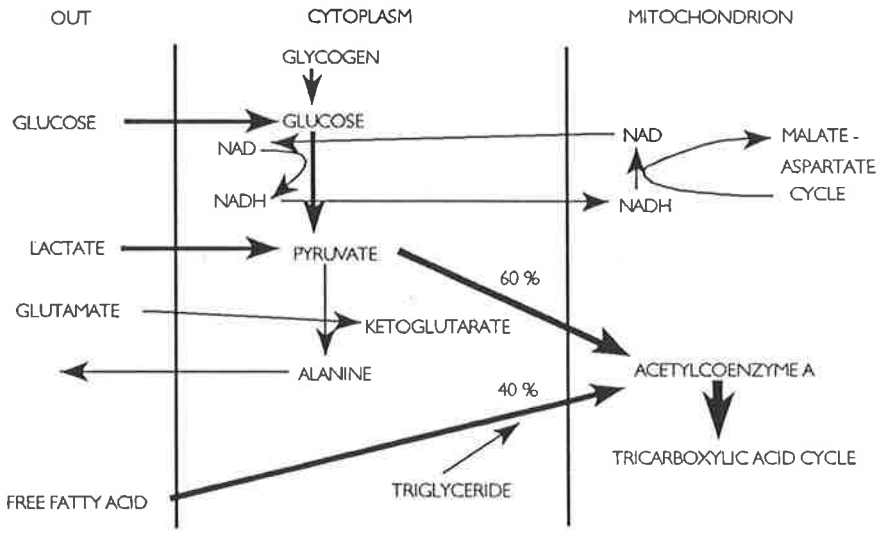


FIGURE 1.3 Myocardial metabolism during stress. (Camici et al 1991)

1.2 Acute myocardial ischaemia and infarction

1.2.1 Epidemiology of ischaemic heart disease

Ischaemic heart disease remains the single most common cause of premature death and death in all ages and sexes in most Western countries. Table 1.2 outlines the cause of death in all ages in Australia in 1993. In 1993, 43.8% of all deaths were due to cardiovascular disease. Of these deaths, 55.8% were caused by acute myocardial infarction. In general, males have a higher death rate from coronary artery disease in all age groups, with the incidence directly proportional to age. This dominance of cardiovascular deaths has occurred for more than 40 years. However, the overall incidence of death from cardiovascular disease, including coronary artery disease progressively declined from the latter half of the 1960s. Mortality rates of age-matched individuals from coronary heart disease decreased 66% in men and 67% in women between 1967 and 1992. The extent of the contribution from a decrease in the prevalence of hypercholesterolaemia, smoking and uncontrolled hypertension to this decline was estimated to be up to 75% in women and 50% in men. Changes in health service access and management appeared to have contributed to approximately 40% of the decline (National Heart Foundation of Australia, 1992). Death from acute myocardial infarction is more frequent within the first day of infarction, and usually within the first few hours after the onset of chest pain. After this time, the chance of survival steadily increases (National Heart Foundation of Australia, 1992; Senes-Ferrari, 1994).

1.2.2 Pathogenesis of atherosclerosis

Atherosclerosis is the usual underlying pathological process that predisposes to the onset of myocardial ischaemia and infarction (Muller et al. 1989). Other causes such as coronary artery dissection and coronary artery spasm in the absence of atherosclerosis are very rare and will not be considered here further.

TABLE 1.2 Total Australian deaths 1993 for all ages

Cause of death	Males		Females		Persons	
	No	%	No	%	No	%
Cardiovascular disease:						
Coronary heart disease	16335	25.1	13424	23.8	29759	24.5
Stroke	4818	7.4	7319	13.0	12137	10.0
Other cardiovascular disease	5216	8.0	6124	10.8	11340	9.3
All cardiovascular disease	26369	40.5	26867	47.5	53236	43.8
Cancers:						
Lung cancer	4737	7.3	1859	3.3	6596	5.4
Breast cancer	16	0.0	2641	4.7	2657	2.2
All cancers	18479	28.4	14212	25.1	32691	26.9
Traffic accidents	1384	2.1	572	1.0	1956	1.6
AIDS	689	1.1	29	0.1	718	0.6
All other	18164	27.9	14829	26.2	32993	27.1
All causes	65085		56509		121594	

Legend: No, number.

1.2.2.1 Prevalence of atherosclerosis

Several autopsy studies have been performed to determine the prevalence of systemic and coronary atherosclerotic disease. Fatty streaks are present in the aortae of most children aged greater than 3 years, with severity increasing rapidly in adolescence (Holman et al. 1958). However, an autopsy study of Americans aged 1 to 69 years showed no correlation of the prevalence of atherosclerosis with age in adults. The highest prevalence and greatest extent of lesions was found in white men aged less than 40 years (Strong and McGill, 1961). This supports the hypothesis that atherosclerotic coronary disease appears early in life, but does not manifest itself as ischaemic heart disease for many years. A recent autopsy study to assess whether the prevalence of coronary atherosclerosis had decreased in a society where the death rate from coronary heart disease had decreased was carried out by Joseph *et al* (Joseph et al. 1993). In a group aged 14 to 35 years, 86% male and 86% white dying from non-cardiac causes, the prevalence of early or progressive atherosclerotic coronary lesions was 78%, with greater than 50% stenoses seen in 21%. The prevalence in males was 76%, which was similar to that found in a past autopsy study of Korean war victims, where the prevalence was 77% (Enos et al. 1955).

1.2.2.2 Coronary risk factors

With the recognition that atherosclerosis was the underlying disease process that leads to ischaemic heart disease, and the use of epidemiological data to determine groups at risk of developing ischaemic heart disease, several "coronary risk factors" have been identified, although not all of these risk factors are necessarily directly causative. However, all coronary risk factors have been shown by epidemiological or other studies to be associated with progression of coronary atherosclerosis and/or development of symptomatic myocardial ischaemia. Age, male sex, hyperlipidaemia, in particular hypercholesterolaemia, smoking, systolic and diastolic

hypertension, diabetes mellitus, a family history of premature ischaemic heart disease, obesity, low plasma concentration of vitamin E, socioeconomic status, occupation, perceived psychological stress and sedentary lifestyle are all recognised as coronary risk factors.

Current smoking is well established as a coronary risk factor (Lakier, 1992), although once an individual has stopped smoking the risk progressively declines over 2 to 5 years and approaches that of non-smokers (Dobson et al. 1991). Hyperlipidaemia, in the form of an elevated fasting total cholesterol or triglyceride is also directly correlated to the prevalence of ischaemic heart disease (Levy et al. 1990; Bainton et al. 1992), as is the extent of elevation of resting systolic and/or diastolic blood pressure (Levy et al. 1990; Clausen and Jensen, 1992). Diabetes mellitus (Butler et al. 1985; Balkau et al. 1992) and a family history of premature ischaemic heart disease (Roncaglioni et al. 1992; Brand et al. 1992) are clearly coronary risk factors, but there is conflicting evidence as to whether obesity is an independent coronary risk factor, although several studies suggest it conveys an independent risk (Hubert et al. 1983; Fitzgerald and Jarrett, 1992). The plasma concentration of the dietary antioxidants vitamin E and flavinoids are inversely proportional to the incidence of ischaemic heart disease (Gey et al. 1991; Hertog et al. 1993; Parfitt et al. 1994). Regular physical exercise has been shown to decrease the incidence of symptomatic ischaemic heart disease (Lakka et al. 1994) and decrease the risk of an acute ischaemic event after heavy physical effort (Willich et al. 1993).

Many studies have also suggested that several social and emotional parameters appear to be coronary risk factors. For example, certain occupations have an increased incidence of ischaemic heart disease, even when other risk factors are taken into account (Rosengren et al. 1991; Vena et al. 1986; Dubrow et al. 1988; Sardinas et al. 1986). Similarly, occupational stress and shift work

has an association with ischaemic heart disease (Haan, 1988; Ely and Mostardi, 1986; Knutsson et al. 1986).

1.2.2.3 The lesions of atherosclerosis

From studies of hypercholesterolaemic animals, three processes are known to occur in the progressive formation of the lesions of atherosclerosis:

a) Proliferation of smooth muscle cells, macrophages and lymphocytes.

b) Formation by smooth muscle cells of a connective tissue matrix comprising elastic fibre proteins and proteoglycans.

c) Accumulation of lipid in cells in the form of "foam cells" and surrounding matrix.

Similarly, these changes appear to be similar to those found in the human coronary arteries of hearts removed in transplant operations (Davies et al. 1988).

The earliest recognisable pathological lesion of atherosclerosis is the 'fatty streak' which comprises an aggregation of lipid-rich macrophages and T lymphocytes within the intima and a small number of lipid-filled smooth muscle cells beneath them as the lesion enlarges (Ross, 1993). It appears to the eye as an area of yellow discolouration. Most of the lipid is in the form of cholesterol and cholesterol ester. The overlying endothelium is morphologically normal. They are probably the precursors of the fibrous plaque as their anatomical sites in the arteries of children are the same as those of fibrous plaques in adults (Stary, 1989). Fatty streaks appear to be followed by the development of intermediate lesions which are composed of layers of macrophages and smooth muscle cells (Ross, 1993).

One form of lesion is described as a diffuse intimal thickening, which consists of increased numbers

of smooth muscle cells surrounded by variable amounts of connective tissue. It is unclear whether these lesions progress to the advanced lesions of atherosclerosis or are multi-layered cushions formed because of increased stress on the artery wall, progressing no further (Ross, 1992).

The advanced lesions of atherosclerosis are more complex and occlusive lesions, the fibrous plaques. They are white in appearance, and as they increase in size over time, project into the arterial lumen. The plaque is covered in a dense fibrous cap of connective tissue, embedded in smooth muscle which overlays a region of lipid-laden macrophages, and T-lymphocytes which are frequently activated. Lipid is again usually in the form of cholesterol and cholesterol ester within both the macrophages and smooth muscle cells. The proliferated smooth muscle cells are surrounded by collagen, elastic fibres and proteoglycan. Beneath these cells is a core of necrotic tissue debri, often containing cholesterol crystals and regions of calcification. In their most advanced stages, the plaques contain a large number of capillary and venule-like channels. The proportion of lipid with the fibrous plaque varies between sites and individuals (Ross, 1992). The endothelium overlying atherosclerotic lesions is morphologically abnormal. It is irregularly arranged and varied in size and shape. Leukocytes, usually monocytes, are adhered to the surface or are in transit through the endothelium via gaps between the cells. In more severe lesions, defects are present in the endothelial surface. This denudation of the endothelium may be associated with the presence of adherent platelets (Davies et al. 1988). The distribution and severity of atherosclerotic lesions are not uniform throughout the vascular tree. The proximal portions of the coronary arteries generally show the most intense involvement within the vascular tree, as does the abdominal aorta, whereas the carotid and cerebral vessels are more commonly affected in hypertensive individuals. (Ross, 1993; Ross, 1992).

1.2.2.4 Hypotheses of atherogenesis

There are several proposed hypotheses for the pathogenesis of atherosclerosis. The first and currently predominant hypothesis has been termed "the response to injury hypothesis", and the second, "the monoclonal hypothesis". The monoclonal hypothesis suggests that each atherosclerotic lesion is derived from a single smooth muscle cell that serves as a source of all the cells within that lesion. Thus, each plaque is a benign neoplasm derived from a cell that has been transformed by viruses, chemicals or other mutagens. (Benditt and Benditt, 1973).

It is hypothesised in "the response to injury" hypothesis that these processes occur as a consequence to some form of injury to the endothelium. Types of injury may include the effects of oxidised low density lipoprotein (oxLDL), mechanical stresses at branches associated with hypertension, viruses such as herpes and cytomegalovirus, toxins and immunological interactions with the endothelial cells.

One of the most likely "injuries" involve the oxidative modification of low density lipoprotein, a lipid particle with a central core of cholesterol ester and triglycerides, surrounded by an outer monolayer of phospholipid including polyunsaturated fatty acids (PUFAS), free cholesterol, several antioxidants, especially α-tocopherol and a protein apolipoprotein B (apo B) which is recognised by the LDL receptor. Human plasma has been shown to contain a low concentration of oxLDL, possibly as a result of reaction with oxidants such as hydrogen peroxide in the presence of transition metal ions (see 1.4). This oxidation causes lipid peroxidation, with subsequent modification of apo B moieties. OxLDL can no longer bind to the tightly controlled LDL receptor which prevents overloading the cell with lipid. Instead, it is avidly endocytosed via the scavenger receptor pathway of macrophages. This receptor is not down-regulated by the

presence of internalised cholesterol, leading to the overloading of the macrophage and the formation of foam cells. OxLDL contains lipid peroxidation products that are diffusible through the cell, biologically active and toxic, including inhibition of nitric oxide-induced smooth muscle relaxation, stimulation of endothelial cells to produce adhesion molecules and activation of T-lymphocytes and growth factors for monocytes and smooth muscle cells (Esterbauer et al. 1993).

Therefore formation of the fibrous plaque is a wound healing response to a chronic trauma. Also, some of the foam cells emigrate back to the blood stream by pushing apart the endothelial cells. These cells are thrombogenic and cause platelets to aggregate and adhere to the endothelial surface, precipitating thrombus formation and further releasing growth-regulatory molecules that effect the endothelium and smooth muscle cells.

1.2.3 Consequences of atherosclerosis and their clinical manifestation

1.2.3.1 Effect on coronary flow and endothelial vasoreactivity

As the fibrous plaque enlarges within the coronary artery, it begins to encroach on the luminal space, decreasing luminal diameter and effecting epicardial coronary flow (Ross, 1993). Simultaneously, the endothelium becomes dysfunctional, particularly as regards vasoactivity. Ludmer *et al* (Ludmer et al. 1986) showed that intra-coronary injection of the endothelium-dependent vasodilator acetylcholine induced significant vasoconstriction at the sites of angiographically visible coronary atherosclerosis, indicative of a loss of normal endothelial function at these sites and a direct vasoconstrictor effect on underlying smooth muscle. However, this endothelial dysfunction is not homogeneous throughout the coronary vasculature of any individual with atherosclerosis. This is consistent with the apparently patchy nature of many diseased coronary arteries (el Tamimi et al. 1994). This paradoxical vasoconstriction has been

shown to occur with a variety of physical stimuli including exercise (Gage et al. 1986; Gordon et al. 1989), cold pressor testing (Nabel et al. 1988) and mental stress (Yeung et al. 1991). In comparison, normal coronary arteries dilated under such circumstances, with an increase in coronary flow (Yeung et al. 1991; Gage et al. 1986; Gordon et al. 1989; Nabel et al. 1988). It is likely that these paradoxical vasoconstrictor responses to physical stimuli were mediated by an inability of the endothelium to release NO in response to acetylcholine. Whether there are other mechanisms for this vasomotor dysfunction is unknown (Maseri, 1991).

It has also been shown that the normal vasodilation of epicardial coronary arteries to increased blood flow is impaired, thereby decreasing coronary flow reserve (Nabel et al. 1990). Therefore the consequence of a combined fixed luminal obstruction due to a fibrous plaque and loss of normal vasodilator function, may cause coronary flow to decrease below oxidative requirements or have impaired reserve, resulting in myocardial ischaemia. In general, the clinical consequence of this is either angina pectoris, usually in a stable exertional or mixed pattern (Ludmer et al. 1986; Yeung et al. 1991).

1.2.3.2 Plaque rupture

However, the acute ischaemic syndromes such as unstable angina pectoris, acute myocardial infarction and sudden death of ischaemic origin are usually the clinical manifestations of a sudden change in the fibrous plaque. It is accepted that such acute ischaemic syndromes are generally the result of thrombus formation, either occlusive or non-occlusive, in the coronary artery. The reason for this sudden pathological event has been shown to be a fissure or rupture of a fibrous atherosclerotic lesion in the majority of cases (Davies and Thomas, 1984; Zamorano et al. 1994). At coronary angiography such lesions appear eccentric with ill-defined, often overhanging and

irregular margins (Ambrose et al. 1986). However, serial angiographic studies have shown that it is not usually the most severely obstructive plaques that rupture (Moise et al. 1984; Giroud et al. 1992). Furthermore, atherosclerotic lesions tend to progress in severity in a stepwise and unpredictable manner, rather than by gradual occlusion of the coronary lumen, consistent with the importance of plaque rupture in the progression of atherosclerosis as well as in the pathogenesis of acute ischaemia (Ambrose et al. 1988; MacIsaac et al. 1993).

Morphologically, a ruptured plaque involves a fissure through the intimal lining of a plaque, exposing the underlying contents. Thrombus occurs both in the sub-intimal space and within the intra-luminal space (Davies and Thomas, 1984; MacIsaac et al. 1993). Plaques that have fissured tend to have a higher extracellular lipid and macrophage content, and less collagen, smooth muscle cells and calcium than intact plaques (Falk, 1992; MacIsaac et al. 1993). Fissures tend to occur at the margins of plaques, where caps are necrotic, very thin and infiltrated by macrophages and lymphocytes. They often occur at the junction between the fibrous plaque and normal tissue (MacIsaac et al. 1993). Whether the sub-intimal thrombus is due to rupture of the venules within the plaque is unclear (MacIsaac et al. 1993). These thrombi contain erythrocytes, fibrin and platelets, rather than just erythrocytes. Davies noted that subintimal haemorrhage without plaque fissuring was universally common, in both patients with sudden cardiac ischaemic death and age-matched controls (Davies and Thomas, 1984). Exposure of subintimal collagen induces platelets to adhere, aggregate and activate and both arms of the coagulation cascade, leading to thrombus formation (MacIsaac et al. 1993). The extent of thrombus formation and its effect on coronary flow determines whether the individual develops symptomatic ischaemia (MacIsaac et al. 1993). The determinants of the extent of thrombus formation are unclear, although there are associations between this and the depth of the intimal fissure, a variety of pro-coagulant states and the relative

thrombogenicity of the various components within the plaque (MacIsaac et al. 1993; Fernandez Ortiz et al. 1994).

There are many studies that suggest that there may be physical and emotional "triggers" prior to an acute ischaemic event, mediated via an as yet unknown physiological response which leads to plaque rupture (MacIsaac et al. 1993; Muller et al. 1989; Meisel et al. 1991). A vulnerable plaque will rupture when the forces acting it exceed its tensile strength. Possible forces a summarised in Table 1.3. It is likely that the causes of plaque rupture are heterogeneous, and may be multiple in any individual. Falk (Falk, 1992) hypothesises that the vulnerability of the plaque may be an even more important issue, which is supported by the histological evidence (Lassila, 1993; van der Wal et al. 1994; MacIsaac et al. 1993).

1.2.3.3 Myocardial metabolism during myocardial ischaemia

As a result of impaired myocardial perfusion, the ischaemic myocardium is unable to adequately sustain oxidative metabolism. At rest, metabolism is the same as in the normal heart (Camici et al. 1991). With stress, coronary flow reserve is impaired, resulting in ischaemia. The first manifestation is an impaired replenishment of the small stores of ATP and creatine phosphate (CP) (Jennings and Reimer, 1981). This results in a vicious cycle of failing metabolic pathways. Metabolically as in normal stress, there is a marked increase in glucose uptake, but the extent of carbohydrate oxidation is small (Figure 1.4). Reduced nicotinamide adenine dinucleotide (NADH) is unable to be reoxidised in the mitochondrion and accumulates in the cytosol. NADH is therefore oxidised by activated lactate dehydrogenase (LDH), diverting pyruvate from the tricarboxylic acid cycle to produce lactate, which is subsequently released (Camici et al. 1991; Gertz et al. 1981). Also increased alanine is released from the transamination of pyruvate, with increased uptake of

TABLE 1.3 Hypothesised Triggers of Plaque Rupture

Triggers of plaque fissuring		reference
Change in cardiac haemodynamics	sudden rise in blood pressure	Falk, 1992
	catecholamine surge	Falk, 1992
	sympathetic surge	Falk, 1992
Change in coronary size and flow	coronary vasospasm	Alpert, 1985
Mechanical stresses and strains on atherosclerotic plaques	circumferential wall stress	MacIsaac et al. 1993
	shear stress of blood flow	MacIsaac et al. 1993
	turbulent flow through a stenosed vessel	MacIsaac et al. 1993
	deformability of the plaque material	MacIsaac et al. 1993
Inflammatory response within the plaque	inflammation	Lassila, 1993 van der Wal et al. 1994
	macrophage-borne proteases	Lendon et al. 1991

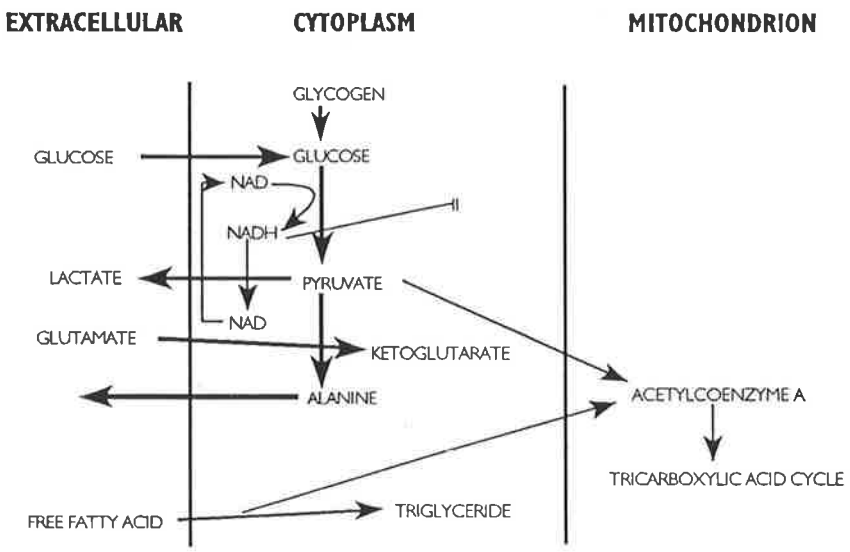


FIGURE 1.4 Myocardial metabolism during stress and ischaemia (Camici et al 1991).

glutamate as the NH_2 donor. Furthermore, glutamate may act as an anaerobic fuel, producing GTP on its conversion to succinate(Camici et al. 1991). As with pyruvate, utilisation of FFA for oxidation in the tricarboxylic acid cycle is impaired, resulting in increased storage of triglyceride (Camici et al. 1991).

The failure of the tricarboxylic acid cycle results in a decreased replenishment of ATP. The concentration of ATP required for the myocyte to remain viable is variable. It appears the critical issue is the availability of ATP supply in the direct vicinity of the metabolic pumps (Opie, 1993). When ATP stores at critical cellular sites are depleted, the cell is unable to regenerate high energy phosphate, maintain physiological ionic gradients and control their volume. Furthermore, after an episode of intense ischaemia, ATP stores may take hours to days to be restored to normal (Ellis et al. 1983; Reimer et al. 1981; DeBoer et al. 1980).

Another important cellular effect of ischaemia, is the accumulation of hydrogen ions causing a drop in pH. Low pH in combination with lactate, NADH and many other metabolites, inhibit glycolytic pathways, pyruvate's incorporation into the tricarboxylic acid cycle, the malate-aspartate cycle and other shuttle reactions (Rovetto et al. 1975; Hillis and Braunwald, 1977). Also, hydrogen ions activate the lysosomes which destroy intracellular proteins (Williamson et al. 1976).

An important consequence of impaired availability of ATP is impaired control of calcium flux in the myocyte. Calcium cannot be taken up by the sarcoplasmic reticulum or released from the cell. As a consequence it accumulates both in the cytosol and the mitochondrion, augmenting ATP usage. The introduction of the positive ions of calcium into the mitochondrion requires the removal of protons by ATP driven pumps (Opie, 1993; Marban et al. 1989). Raised intracytosolic

calcium activates enzymes such as phospholipase A_2 (Opie, 1993). The resultant lipolysis of membrane phospholipids and their subsequent interaction with free fatty acids to form lysophosphoglycerides micelles, which act as detergents and have been associated with arrhythmias (Corr et al. 1982; Opie, 1993). Furthermore, calcium overload has been associated with ischaemic contracture, increasing coronary vascular resistance and thereby intensifying the ischaemia to the cell (Marban et al. 1989; Opie, 1993).

Other cellular events associated with ischaemia include the activation of enzymes such as phospholipase A_2. Cellular enzymes which scavenge free radicals and antioxidants (see 1.4) such as GSH are depleted, resulting in increased accumulation of hydrogen peroxide and superoxide (Opie, 1993; Ferrari et al. 1991b; Janssen et al. 1993).

1.2.4 Changes in myocardial structure and function during myocardial ischaemia and infarction

1.2.4.1 Morphology

The earliest changes seen on electron microscopy (within 20 minutes of the onset of myocardial ischaemia) consist of reduction in the size and number of glycogen granules, intracellular oedema and swelling and distortion of the transverse tubular system, sarcoplasmic reticulum and the mitochondria (Kloner et al. 1980; Nayler and Elz, 1986). The histological changes that indicate irreversible injury are disruption of sarcolemmal membrane integrity and amorphous mitochondrial matrix densities (Farb et al. 1993; Jennings and Reimer, 1981; Farber et al. 1981). Other changes include aggregation and margination of nuclear chromatin, relaxation and disorientation of myofibrils, swelling of sarcoplasmic reticulum, enlarged, clumped and subsequently fragmented mitochondria, thinning and fractionation of myofilaments and disappearance of the

heterochromatin. These changes progress with time and ongoing infarction (Caulfield and Klionsky, 1959).

As necrosis proceeds, three main histological patterns may be seen (Baroldi, 1975). Firstly, coagulation necrosis, where the cells may be arrested in the relaxed state, with stretched myofibrils, mitochondrial damage and healing by phagocytosis by macrophages of the necrosed muscle cells. Secondly, coagulative myocytolysis occurs with hypercontracted contraction band necrosis, usually seen after reperfusion of a previously ischaemic territory. There is increased intracellular calcium, with hypercontracted myofibrils, contraction bands, mitochondrial damage, vascular congestion and healing by lysis of muscle cells. The third pattern of colliquative myocytolysis is characterised by oedema and cell swelling, early lysis of myofibrils, late lysis of nuclei and healing both by phagocytosis and lysis of myocytes (Baroldi, 1975).

Because of the perfusion gradient within the myocardium, there is a "wavefront" of cellular necrosis from subendocardium to epicardium over time after occlusion of a coronary artery (Reimer et al. 1977; Farb et al. 1993). However, the exact rate of necrosis in the human heart may vary greatly from other species and artificial models of infarction (Nayler and Elz, 1986; Virmani et al. 1992) and between individuals as will be discussed in 1.3.

1.2.4.2 Myocardial contractile function

With the onset of myocardial ischaemia in the human heart *in vivo* the initial effect on function is a change in diastolic function. During spontaneous, exercise-induced and pacing-induced myocardial ischaemia, there is an upward and rightward shift of the left ventricular pressure-volume loop (De Bruyne et al. 1993; Brutsaert et al. 1993; Sharma et al. 1983a; Carroll et al.

1983). The rightward shift appears to be related to impaired ventricular relaxation, whereas the upward shift is secondary to impaired compliance (Brutsaert et al. 1993; De Bruyne et al. 1993).

Impaired relaxation in the early phases of ventricular diastole, has been suggested to be a result of impairment of the 'triple control of relaxation' (Brutsaert et al. 1993). This includes impaired activation-inactivation related to impaired calcium homeostasis, changes in the sarcoplasmic reticulum pump and contractile proteins. Also excessive changes in cardiac load, or incoordination of load and activation-inactivation in time and space are involved (Brutsaert et al. 1993; Brutsaert and Sys, 1989).

With intense and ongoing ischaemia, systolic dysfunction develops with decreased contractility during contraction and early ejection and an inability to prolong systole. Peak systolic pressure drops and the region effected becomes hypokinetic, akinetic or even dyskinetic, depending on the severity of ischaemia and any pre-existing wall motion abnormality (Sharma et al. 1983a; Brutsaert et al. 1993). Therefore, the left ventricular ejection fraction may decrease, impairing the appropriate rise in cardiac output with exercise or stress (De Bruyne et al. 1993).

Extent and time course of recovery after an episode of myocardial ischaemia depend on the intensity and length of the ischaemic insult. After a short episode of mild ischaemia, recovery of normal contractile function occurs within minutes. However, mechanical dysfunction may persist for hours to days after return of normal coronary flow despite the absence of irreversible injury. This phenomenon has been called myocardial stunning (Bolli, 1990). Bolli (Bolli, 1990) proposed several pathophysiological mechanisms, with the important underlying molecular abnormality to be the generation of oxygen derived free radicals initiating enzyme inactivation and lipid

TABLE 1.4 (Bolli, 1990)

Proposed mechanisms for myocardial stunning
Generation of oxygen-derived free radicals
Excitation-contraction uncoupling due to sarcoplasmic reticulum dysfunction
Calcium overload
Insufficient energy production by mitochondria
Impaired energy use by myofibrils
Impairment of sympathetic neural responsiveness
Impairment of myocardial perfusion
Damage of the extracellular collagen matrix
Decreased sensitivity of myofilaments to calcium

peroxidation of organelle membranes. (Table 1.4). This is further discussed in 1.5.

1.2.4.3 Electrophysiological basis for electrocardiographic changes in acute evolving myocardial infarction

Myocardial ischaemia slows conduction of the cardiac action potential, prolongs the duration of recovery in the ischaemic zone, and diminishes the upstroke velocity, amplitude and duration of the action potential (Kleber et al. 1978). The first electrical change seen with myocardial ischaemia is an increased negative charge of the extracellular membrane and a more positive transmembrane action potential during phase 4. This induces a current flow towards the ischaemic area. As maximum ischaemia is within the subendocardial myocardium there is T-Q segment depression. This is then automatically shifted upward to the isoelectric control level by the alternating current electrocardiograph (ECG) machine, leading to relative S-T segment elevation (Fisch, 1992; Kleber et al. 1978). A further, but less significant contribution to S-T segment elevation occurs because of the shortened action potential, causing the injured subendocardial myocardium to undergo early repolarisation. Therefore the current moves towards the normal epicardial myocardium, leading to true S-T segment elevation (Kleber et al. 1978; Vincent et al. 1977). Of course, electrodes in the opposite orientation will show "reciprocal" S-T segment depression.

The polarity of the T wave depends on both the duration and moment of activation of the ischaemic action potential. If the ischaemic zone repolarises early, the T wave is upright. If the action potential is delayed to an extent to delay repolarisation to be later than normal myocardium, the T wave becomes inverted (Kleber et al. 1978).

The ionic and molecular explanation for this S-T segment elevation on ECG is due partly to

activation of ATP-sensitive, cycle independent K^+ channels via several mechanisms, including a decrease in intracellular ATP concentration (Deutsch et al. 1991). This results in an efflux of K^+, decrease in the action potential duration (Deutsch et al. 1991) and S-T segment elevation (Kleber et al. 1978). Increasing K^+ efflux has been shown to cause increasing action potential shortening (Nichols et al. 1991). Similarly, S-T segment elevation has been shown to be maximal when the activation recovery interval (an *in vivo* surrogate of action potential shortening) is maximal (Kubota et al. 1993). Therefore, the extent of S-T elevation appears to reflect the severity of myocardial ischaemia at the site being monitored, and the distribution of S-T elevation throughout the ECG, the myocardial distribution of the ischaemic zone (Nichols et al. 1991; Kubota et al. 1993).

1.2.5 Conventional therapy in the management of myocardial ischaemia and infarction

1.2.5.1 Anti-ischaemic and anticoagulant drugs

1.2.5.1.1 *Nitrates*

Organic nitrates such as glyceryl trinitrate (GTN), isosorbide dinitrate and isosorbide mononitrate are the most commonly utilised class of drugs for the treatment of symptomatic ischaemic heart disease. Sublingual preparations rapidly relieve myocardial ischaemia and oral preparations are utilised for the prevention of myocardial ischaemia in stable angina pectoris, usually in association with other anti-anginal medications (Flaherty, 1989). Intravenous GTN is established therapy for the relief of myocardial ischaemia in unstable angina pectoris (Curfman et al. 1983). For the management of acute myocardial infarction, intravenous GTN improves cardiac haemodynamics (Jugdutt, 1991; Stone et al. 1983), and has been shown to decrease infarct size, although these studies were carried out prior to widespread clinical utilisation of thrombolytic therapy (Jugdutt

and Warnica, 1988; Yusuf et al. 1988). However, isosorbide dinitrate has been shown to complement thrombolytic therapy in reducing intermittent coronary occlusion after intracoronary thrombolysis (Hackett et al. 1987).

After their metabolism, organic nitrates act as exogenous NO donors. Therefore their action relates to the effects of NO on vasculature and platelets, inducing both coronary and peripheral arterial and venous vasodilation, even in the presence of atherosclerosis (Horowitz and Henry, 1987). GTN markedly dilates collateral vessels (Cohn et al. 1977) and improves redistribution of coronary flow towards ischaemic regions of the subendocardium (Liu et al. 1985). This is partly mediated by a coronary vessel diameter-dependent effect of GTN (Harrison and Bates, 1993). GTN is a more potent dilator of large coronary arteries than microvessels, thus avoiding the problem of "coronary steal" (Patterson and Kirk, 1983). Further discussion of the proposed mechanisms of organic nitrate metabolism are discussed in 1.7.2.1.

The most significant problem related to the clinical use of nitrate therapy is nitrate tolerance, meaning a diminution of clinical effect of the drug, via reduced rates of NO generation, after continuous use over time (Henry et al. 1989b; Meredith et al. 1993; Boesgaard et al. 1994b). Factors that are related to the onset of tolerance include frequent dosage, large doses and continuous drug delivery without adequate "nitrate free periods" (Henry et al. 1989b; Abrams, 1991). The cause of nitrate tolerance remains unclear. Further discussion on this topic is in 1.7.3.2

1.2.5.1.2 *Calcium channel antagonists*

There are many calcium channel antagonists, but the agents most frequently utilised for the

treatment of myocardial ischaemic syndromes are verapamil, diltiazem and nifedipine. These agents are selective for the L-type slow calcium channels, interfering with the entry of calcium into myocytes and vascular smooth muscle cells. Nifedipine is a dihydopyridine derivative, binding to a specific receptor subclass, distinct from those associated with verapamil and diltiazem (Opie, 1990).

Nifedipine has a predominant effect on the peripheral and coronary vasculature, with no significant direct effect on chronotropic mechanism and only minor negative inotropic effects at the doses usually prescribed. Therefore, vasodilation induced by nifedipine can result in a reflex tachycardia, thereby limiting its clinical use as monotherapy in the management of myocardial ischaemia. Nifedipine has been shown to decrease the frequency of episodes of exertional angina (Higginbotham et al. 1986). However, in unstable angina pectoris, monotherapy with nifedipine was associated with either no effect (The Israeli SPRINT Study Group, 1988; Muller et al. 1984b; Muller et al. 1984a) or possibly an increased incidence of acute infarction (The HINT Research Group, 1986). Similarly, in the treatment of acute myocardial infarction, nifedipine had either no, or an adverse early effect, even in combination with other therapies including thrombolysis (Sirnes et al. 1984; Muller et al. 1984a; Erbel et al. 1988).

Verapamil and diltiazem have widely disparate structures and act on distinct receptors, but at appropriate dosages, the two drugs have clinically similar effects when used for the treatment of ischaemic heart disease. They can therefore be used and considered interchangeably . In contrast to the dihydropyridines, verapamil and diltiazem have negative chronotropic and inotropic effects in addition to their coronary and peripheral vasodilator effects (Opie, 1990). This avoids the reflex tachycardia seen when nifedipine is used. Both drugs are effective at reducing myocardial

ischaemia in exertional, mixed pattern and unstable angina pectoris (Khurmi and Raftery, 1987; Capucci et al. 1983; Theroux et al. 1985). In the setting of acute myocardial infarction, cardiac haemodynamics are favourably affected (Heikkila and Nieminen, 1984) and mortality reduced in patients in whom there was no significant left systolic ventricular dysfunction (The DAVIT Study Group, 1984; The Multicenter Diltiazem Postinfarction Trial Research Group, 1988). Therefore, subject to careful selection of patients and dosage (Arstall et al. 1992), these drugs appear to improve late outcome after myocardial infarction and are safe in the peri-infarction period. However, the early use of verapamil and diltiazem have not been established to be beneficial in combination with thrombolytic therapy.

1.2.5.1.3 *β-Adrenoceptor antagonists*

This large and varied class of drugs act to competitively inhibit the effects of neuronally released and circulating catecholamines on β-adrenoceptors. Different agents have varying selectivity for either the β_1 or β_2 receptor. As β_1 receptors predominate in the heart, selective β_1-adrenoceptor antagonists tend to be more commonly used for the management of myocardial ischaemic syndromes , thereby limiting but not eliminating the effects of β_2-receptor antagonism, such as bronchospasm and peripheral vasoconstriction. Many of these drugs are partial agonists, producing blockade by "shielding" the receptor from more potent agonists. This tends to lead to β-stimulation when sympathetic activity is low, such as at rest, and blockade when sympathetic activity is high under conditions of stress and exercise. This phenomenon is called intrinsic sympathomimetic activity. Other variations between the agents include relative potency, lipid solubility and presence or absence of associated α-adrenoceptor antagonist activity (Rutherford and Braunwald, 1992).

Despite these differences, most β-adrenoceptor antagonists have been shown to be extremely effective in the prophylaxis of exertional angina pectoris, reducing MVO_2 by reducing heart rate, blood pressure and contractility during times of increased sympathetic activity, despite their coronary vasoconstrictor effects (Rutherford and Braunwald, 1992). In unstable angina pectoris, most episodes of myocardial ischaemia are not precipitated by increased sympathetic activity, theoretically limiting the potential benefit of these agents (Figueras et al. 1979). However, the HINT study suggested benefit of metoprolol in reducing symptoms and the occurrence of myocardial infarction (The HINT Research Group, 1986). In the management of acute myocardial infarction β-adrenoceptor antagonist as monotherapy has been shown to decrease early mortality and myocardial salvage (ISIS-1 Collaborative Group, 1986; The International Collaborative Study Group, 1984; Roberts et al. 1984). However, in combination with thrombolytic therapy no improvement in myocardial salvage or mortality was seen, but ongoing ischaemia and re-infarction was reduced (The TIMI Study Group, 1989). Thus as with calcium antagonists, the early benefits of β-adrenoceptor antagonists in acute myocardial infarction are unclear in the thrombolytic era. In contrast, their role in secondary prevention following myocardial infarction is established, especially in the setting of left ventricular dysfunction, reducing the incidence of sudden death (BHAT Investigators, 1982; Pedersen, 1983; Herlitz et al. 1984).

1.2.5.1.4 *Perhexiline*

Perhexiline is an effective anti-anginal agent for the management of stable and unstable angina pectoris, improving exercise tolerance and quality of life (Horowitz and Mashford, 1979; Horowitz et al. 1986b; Cole et al. 1990). It produces incremental anti-anginal effects when added to other anti-anginal therapies (Cole et al. 1990), and because of its lack of significant inotropic effects, can be safely utilised in patients with severe left ventricular function (Silver, 1984;

Horowitz et al. 1995).

The mechanism of its efficacy is uncertain, although it may promote glucose utilisation while limiting lipid catabolism, thereby improving the efficiency of myocardial metabolism (Horowitz et al. 1995). Inhibition of lipid catabolism may predispose towards phospholipoidosis, which has been seen in the hepatocytes and Schwann cells of patients with clinical perhexiline toxicity (Albert and Lullmann Rauch, 1983).

There are currently no clinical trials to demonstrate its efficacy or otherwise in the setting of acute myocardial infarction.

1.2.5.1.5 *Aspirin*

Aspirin irreversibly inhibits the enzyme cyclooxygenase 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. The mechanism for this is unclear but may be due to rapid recovery of cyclooxygenase in vascular endothelial cells, or presystemic inhibition of platelet cyclooxygenase in the portal arterial system, where the concentration of aspirin may be higher than the systemic circulation (Pedersen and FitzGerald, 1984). Whatever the mechanism, the clinical response is an inhibition of platelet aggregation due to thromboxane A_2.

This inhibition of platelet aggregation has obvious beneficial possibilities in the management of atherosclerotic-induced ischaemic heart disease, especially the acute ischaemic syndromes. As a primary preventative measure, aspirin significantly decreased the risk of myocardial infarction, especially in those greater than 50 years of age. However, there was a trend towards increased risk

of haemorrhagic stroke (Steering Committee of the Physician's Health Study Research Group, 1989). No clear role of aspirin for the treatment of stable angina pectoris has been made, although many patients are prescribed aspirin as a preventative measure once the diagnosis of ischaemic heart disease is made. Aspirin has been shown to prevent myocardial infarction in the long-term management of unstable angina pectoris (Lewis, Jr. et al. 1983; Theroux et al. 1988) and death after acute myocardial infarction (ISIS-2 (Second International Study of Infarct Survival) collaborative group, 1988). Aspirin's benefit in myocardial infarction appears to be additive to that obtained from treatment with streptokinase (ISIS-2 (Second International Study of Infarct Survival) collaborative group, 1988).

1.2.5.1.6 *Anticoagulants*

Heparin and heparin-like compounds are a heterogeneous group of glycosaminoglycans, located in mast cells, endothelium and at extracellular sites in the lung and aorta. Heparin's anticoagulant effect relies on the presence of the endogenous coagulation inhibitors, heparin co-factor II and anti-thrombin III. Heparin greatly enhances the inactivation of thrombin and activated coagulation factors IIa, IXa, Xa and XIa which complex with antithrombin III. The reaction kinetics of the different factors with antithrombin III vary greatly.

Heparin is in widespread use in the management of the acute ischaemic syndromes, where coronary thrombus formation is one of the main underlying pathological changes. Intravenous heparin infusion has been shown to decrease the occurrence of refractory angina and myocardial infarction in unstable angina pectoris. It was found to be superior to treatment with aspirin, but no synergy was noted on combination of the two drugs (Theroux et al. 1988). For the management of acute myocardial infarction, heparin is commonly used either simultaneously with,

or immediately following thrombolytic therapy. It appears to prevent early re-occlusion of the reperfused infarct-related artery (Pasternak et al. 1992). However, it is unclear how long the heparin should continue and whether heparin should be administered intravenously or subcutaneously.

There are many new anticoagulant preparations (for example, direct anti-thrombins) currently being studied in clinical trials for the management of acute ischaemic syndromes. The results of clinical trials of these agents are largely incomplete at this stage.

Warfarin, an oral anticoagulant, acts as a competitive inhibitor of Vitamin K which is essential for the hepatic production of coagulation factors II, VII, IX and X, and Protein C. Warfarin, like aspirin has been shown to decrease the risk of death after myocardial infarction, although this was a placebo-controlled trial rather than a comparison with aspirin (Smith et al. 1990).

1.2.5.2 Thrombolytic therapy

The use of thrombolytic therapy for the management of evolving acute transmural myocardial infarction is well established. Its impact on the management of acute myocardial infarction and the role of coronary care units is profound. Although beneficial effects occur as a result of intracoronary thrombolytic drug infusion, (Ganz et al. 1981; Markis et al. 1981; Khaja et al. 1983) this form of therapy has never been widely utilised for logistic reasons. On the other hand, intravenous thrombolysis is now very widely available for treatment of acute infarction (ISIS-2 (Second International Study of Infarct Survival) collaborative group, 1988; Gruppo Italiano Per Lo Studio Della Streptochinasi Nell'infarto miocardico and GISSI, 1986; O'Rouke et al. 1988; AIMS Trial Study Group, 1988).

There are many thrombolytic agents available, including streptokinase (SK), urokinase, anisoylated plasminogen streptokinase activator complex (APSAC) and recombinant tissue-type plasminogen activator (rTPA). APSAC has the advantage that it can be intravenously administered rapidly over 2 to 5 minutes, whereas the other agents must be infused. Streptokinase is a purified exotoxin from type C streptococci. It forms a complex with free or fibrin-bound plasminogen which develops activator activity, initiating systemic fibrinolysis. Urokinase, is a product of renal tubular epithelial cells and excreted in the urine, whereas rTPA is a DNA recombinant form of endogenous tissue plasminogen activator. Both urokinase and rTPA directly convert plasminogen to plasmin. In the high doses required to obtain coronary thrombolysis from an intravenous infusion rTPA, like SK causes systemic rather than just thrombus-specific fibrinolysis.

Due to the initiation of systemic fibrinolysis, the major adverse event attributed to these agents is bleeding. Major bleeding requiring transfusion or leading to death or significant morbidity occurs in approximately 1-2% of patients treated (Califf et al. 1992; Fibrinolytic Therapy Trialists' (FTT) Collaborative Group, 1994; Maggioni et al. 1992). SK may also induce an immune response, leading to allergic reactions, such as anaphylaxis or inactivation of the drug by anti-streptokinase antibodies (Dykewicz et al. 1986; Buchalter et al. 1992). Another significant adverse event is early myocardial rupture, usually within the first 24 hours of thrombolytic therapy (Honan et al. 1990; Fibrinolytic Therapy Trialists' (FTT) Collaborative Group, 1994). As a consequence of these and other unidentified factors, thrombolytic therapy is associated with increased mortality for the first 24 hours of treatment (Fibrinolytic Therapy Trialists' (FTT) Collaborative Group, 1994). This topic is discussed in more detail in 5.2.

As regards the effects of thrombolytic agents on mortality post-myocardial infarction, the major

clinical trials have predominantly compared SK with rTPA. Both GISSI-II and ISIS-3 showed no mortality difference between either agents and similar haemorrhage rates (GISSI and Gruppo Italiano Per Lo Studio Della Sopravvivenza Nell'Infarto miocardico. 1990; ISIS-3 and Third International Study of Infarct Survival Collaborative group. 1992), despite rTPA being associated with higher infarct related artery patency rates at 90 minute coronary angiography (Lincoff and Topol, 1993). This may be have been a result of higher reocclusion rates with rTPA (Chesebro et al. 1987). The GUSTO trial (The GUSTO Investigators, 1993) was therefore performed to re-address the issue of possible differential effect between agents, utilising a more rapid rTPA infusion rate and aggressive heparinisation protocol, resulting in a small mortality advantage of rTPA over SK (The GUSTO Investigators, 1993). There have been no clinical trials that have suggested a survival benefit from the use of thrombolytic therapy in acute ischaemic syndromes other than myocardial infarction (Brunelli et al. 1991).

1.2.5.3 Non-pharmacological therapy

1.2.5.3.1 *Percutaneous transluminal coronary balloon angioplasty*

Percutaneous transluminal coronary balloon angioplasty (PTCA) is now a generally accepted and validated therapy for the treatment of symptomatic angina pectoris, both in patients with stable (Parisi et al. 1992) or unstable symptoms (Kamp et al. 1989; Steffenino et al. 1987), normal or mildly reduced left ventricular function and significant coronary stenoses in 1 or 2 vessels (not including the left main artery) (Rutherford and Braunwald, 1992; Baim, 1992; King and Schlumpf, 1993). Its main benefit is in the relief of symptoms, rather than improvement in survival (Mabin et al. 1985; Stammen et al. 1991; Parisi et al. 1992). The major limiting factor associated with this technique is restenosis of the lesion, which is in at least 25% of cases (Kamp et al. 1989; King and Schlumpf, 1993).

As an alternative or adjunct to pharmacological intervention in the treatment of acute myocardial infarction, revascularisation with PTCA has been assessed in many clinical trials involving a variety of clinical settings. It was found there was no advantage, from the point of view of myocardial salvage or early survival, in routinely performing PTCA immediately following thrombolysis as compared with deferring this procedure for 18-48 hours. In fact, PTCA carried out at the time of thrombolysis was associated with a lower procedural success rate and increased morbidity, particularly as regards bleeding complications (The TIMI Research Group, 1988; Califf et al. 1991; Simoons et al. 1988). Furthermore, there was no survival or myocardial salvage advantage seen in trials involving routine coronary angiography and PTCA on all high-grade residual stenoses at 18-48 hours, unless the patient had spontaneous or inducible ongoing myocardial ischaemia or other indications suggestive of a high risk prognosis (The TIMI Study Group, 1989; SWIFT Trial Study Group, 1991).

The role of PTCA as a "salvage" procedure, as a result of failed reperfusion with thrombolysis is not clear. "Rescue" PTCA is associated with a high morbidity and significant mortality and in general is reserved for patients with ongoing chest pain, haemodynamic instability or evidence of a large amount of myocardium at risk (St Goar and Stone, 1994).

Primary PTCA in evolving acute myocardial infarction (the immediate intention to perform PTCA without prior thrombolysis) has been shown to have similar outcomes to thrombolysis as regards myocardial salvage (Gibbons et al. 1993; Zijlstra et al. 1993; Grines et al. 1993) and less recurrent ischaemia or reinfarction (Zijlstra et al. 1993; Grines et al. 1993). This manoeuvre therefore, may be an option in patients with contra-indications to thrombolytic therapy or who are haemodynamically unstable. However, its widespread utilisation as standard therapy for acute

myocardial infarction remains logistically difficult in most hospitals (Lange and Hillis, 1993).

1.2.5.3.2 *Coronary artery bypass grafting*

Prior to PTCA coronary artery bypass grafting was the only means of revascularising individuals with coronary artery disease. Generally venous or internal mammary arterial conduits are utilised. Arterial conduits appear to be superior with improved survival and decreased recurrence of symptoms as compared venous grafts and predominantly correlates to patency rates (Loop et al. 1986; Cameron et al. 1988). Improvement in venous conduit patency rates has been achieved by the use of lifelong aspirin after surgery (Lorenz et al. 1984; Underwood and More, 1994).

Indications for surgery on coronary anatomical and cardiac haemodynamic grounds have been determined on the basis of a survival benefit over medical therapy in several clinical trials (The Veterans Administration Coronary Artery Bypass Surgery Cooperative Study Group, 1984; Alderman et al. 1990; Varnauskas, 1988). A failure of medical therapy also is an indication for revascularisation with coronary artery bypass grafting. Generally, these criteria apply to all clinical manifestations of ischaemic heart disease. Only one study has suggested a survival benefit with the utilisation of coronary artery surgery over medical therapy in early evolving acute myocardial infarction (DeWood et al. 1989). However, the logistical problems associated with this therapy inhibit its widespread utilisation.

1.3 Myocardial salvage after coronary occlusion

1.3.1 Selected methodologies for the assessment of myocardial salvage, infarct size and left ventricular function in the peri-infarction period in humans

1.3.1.1 Cardiac enzyme release

The release of myocardial intracellular enzymes into the peripheral circulation are part of the clinical 'triad' of diagnostic tools for the diagnosis of acute myocardial infarction; the other two criteria being chest pain and ECG changes consistent with myocardial ischaemia. The most frequently utilised enzymatic markers are creatine kinase (CK), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) (Lee and Goldman, 1986). Other intracellular substances liberated after myocardial cell death include myoglobin (Isakov et al. 1988), myosin light (Isobe et al. 1989)and heavy chains (Leger et al. 1990) and troponin T (Ravkilde et al. 1995; Efthymiadis et al. 1994). The biochemical marker that has been the most widely utilised in clinical practice and studies of myocardial infarct size determination is total creatine kinase release.

Creatine kinase has three isoenzymes, MM, MB and BB and several isoforms of the MM and MB isoenzymes have been identified (Roberts, 1987). In animal models of myocardial infarction, creatine kinase release with time correlated with depletion of the same from infarcted myocardium and the extent of infarction (Shell et al. 1971). However, creatine kinase is also found in other tissues including skeletal muscle, brain, intestinal tract, uterus and prostate (Lee and Goldman, 1986; Roberts and Sobel, 1973). Although the MM isoenzyme is the predominant enzyme in myocardial cells, the MB isoenzyme is also variably present. However, the MB isoenzyme is not specific for myocardium, although other organs such as skeletal muscle and small intestine only have low concentrations (Roberts and Sobel, 1973). Normal human myocardium also has very low MB isoenzyme content and activity, with increasing content and activity in hearts with left ventricular hypertrophy and/or coronary artery disease. Therefore, in patients with acute myocardial infarction the extent of MB release will depend not just on the extent of infarction but also on underlying coronary anatomy and left ventricular wall thickness. (Ingwall et al. 1985).

The time-activity curve of total CK in peripheral venous blood are influenced both by the rate of release and removal from the vascular compartment. Factors that change the rate of release include the extent of myocardial necrosis (Hackel et al. 1984; Blanke et al. 1984)regional myocardial perfusion and the rate of reperfusion (Blanke et al. 1984; Horie et al. 1986), local degradation of the enzyme within the heart, and exchange of enzyme between extravascular and vascular compartments (Sobel et al. 1977). Factors influencing the rate of disappearance from the circulation include varying plasma volume and low cardiac output (Norris et al. 1975). Furthermore, localised coronary vasoconstriction may result in a disproportionate extent of local lymphatic breakdown of creatine kinase, at the expense of elusion into the venous circulation. This may explain some of the effects of β-adrenoceptor antagonists on creatine kinase release (Peter et al. 1978). Therefore, both peak creatine kinase, time to peak creatine kinase (Horie et al. 1986) and in particular the area under the time-activity curve are correlates of myocardial infarct size (Rogers et al. 1977; Norris et al. 1975; Blanke et al. 1984). Conversely with the β-adrenoceptor antagonist effects, the use of thrombolytic agents tends to cause a "washout" of CK with higher CK peak at an earlier time after the onset of infarction, related to more rapid reperfusion (Lee and Goldman, 1986; de Zwaan et al. 1988). The rate of increase of CK activity in plasma has been shown to correlate with final infarct size (Devries et al. 1989). However, total cumulative CK or area under the time-activity curve still appears to correlate well with infarct size (Blanke et al. 1984; de Zwaan et al. 1988).

The ratio of early release of various isoforms of CK-MM and CK-MB may be useful in early diagnosis and timing of the onset of infarction (Puleo et al. 1990), or detecting myocardial necrosis in the absence of a peripheral venous CK elevation above the normal range (Jaffe et al. 1986). Also, the isoform profile of CK-MM, in particular the ratio of CK-MM_3 to total CK, changes

rapidly with reperfusion, thereby being a biochemical marker of the timing of this treatment endpoint (Devries et al. 1986; Puleo et al. 1987; Nohara et al. 1989).

1.3.1.2 Coronary angiography in the determination of infarct-related coronary artery patency

Coronary angiography allows an assessment of the coronary anatomy and has been extensively utilised in studies examining the early peri-infarction period. Visualisation of the coronary anatomy (on occasions in combination with the performance of PTCA) allows for the determination of the speed, frequency and effectiveness of recanalisation, and therefore examines the effectiveness of treatment for evolving acute myocardial infarction. Patency of the infarct related artery can be determined either before treatment, serially over several hours to days after treatment or at some time later. A grading of patency was developed by the TIMI group (TIMI Study Group, 1985). TIMI grade is widely utilised as a measure of efficacy of thrombolysis.

1.3.1.3 Left ventricular function

An important correlate of myocardial salvage is the residual cardiac function after myocardial necrosis. However, after acute myocardial infarction it is difficult to clearly distinguish between past and present necrosis and stunning in any individual. Therefore the measurement of cardiac function *in vivo* in humans after myocardial infarction is a fairly crude marker of myocardial salvage. In general, overall left ventricular ejection fraction and assessment of left ventricular size and regional wall motion abnormalities have become the most widely used parameters. Qualitative and quantitative techniques for their determination (of variable accuracy) include cineangiography (Dodge et al. 1960) echocardiography and radionuclide ventriculography .

1.3.1.4 Electrocardiography

The ECG can be utilised in several ways for the assessment of myocardial salvage. Continuous monitoring of S-T segment deviation over time during the early treatment of evolving acute myocardial infarction has been utilised to measure changes in the extent or intensity of myocardial ischaemia (Madias et al. 1975; Krucoff et al. 1993b; Kubota et al. 1993) and the speed of recanalisation. This methodology has been validated using simultaneous coronary angiography (Hackett et al. 1987; Krucoff et al. 1986; Krucoff et al. 1993b). Rapid resolution of S-T segment elevation has been shown to correlate with restoration of patency of the infarct related artery in patients receiving thrombolytic therapy (Krucoff et al. 1986; Krucoff et al. 1993b), greater myocardial salvage and improved survival (Schroder et al. 1994).

Different studies vary in the exact methodology for the measurement of S-T segment elevation. One method summates several leads' S-T deviation (Kwon et al. 1991; Schroder et al. 1994) and uses this when considering percentage change in S-T segment deviation, while for another method, one reference lead where S-T deviation is initially most pronounced (Krucoff et al. 1993a; Veldkamp et al. 1994) is followed and analysed over time. There is a technical and interpretative difficulty in summating S-T segment deviation, especially in inferior infarction. S-T depression in the praecordial leads, which may represent posterior myocardial injury, anterior ischaemia, reciprocal changes or a combination of all three, may possibly decrease the validity of the calculated sum S-T segment deviation. Another variation in the utilisation of this non-invasive technique is the definition of what extent of S-T segment resolution signifies recanalisation. Utilising a single reference lead method, the TAMI group (Veldkamp et al. 1994; Krucoff et al. 1993b), showed that a 50% resolution of S-T segment elevation, as compared to the previous peak S-T segment elevation correlated with recanalisation (Veldkamp et al. 1994; Krucoff et al. 1993b).

Another ECG marker of reperfusion of the infarct related artery may be early inversion of the T wave. Matetzky *et al* (Matetzky et al. 1994) showed that T wave inversion within 24 hours was associated with significantly increased probability of TIMI 3 flow in the infarct related artery and improved left ventricular function, suggesting improved myocardial salvage. Even at 3 hours after initiation of thrombolytic therapy, rapid reduction of T wave amplitude correlated with a patent infarct related artery (Richardson et al. 1988).

A method of "scoring" the QRS portions of 12 lead ECG, named the Selvester QRS scoring system, has been developed in an attempt to determine the size of a myocardial infarct from the ECG (Wagner et al. 1982; Ideker et al. 1982; Roark et al. 1983; Ward et al. 1984; Hindman et al. 1985; Freye et al. 1992; Sevilla et al. 1992). Points are accumulated in a 54 criteria/ 32 point scoring system, evaluating Q and R wave durations, R and S wave amplitudes, R/Q or R/S amplitude ratios and the presence of R wave notching. Each point represents approximately 3 percent of the left ventricle. The system was shown to be highly specific for myocardial infarction (Hindman et al. 1985) and in these small studies correlated well with autopsy determination of infarct size in anterior ($r = 0.80$) (Ideker et al. 1982), inferior ($r = 0.74$) (Roark et al. 1983), posterolateral ($r = 0.72$) (Ward et al. 1984) but not multiple infarcts ($r = 0.44$) (Sevilla et al. 1992). Similarly, bundle branch block and left ventricular hypertrophy decreased the sensitivity of the scoring system (Freye et al. 1992). ECG tracings from some period 1 day to several years after infarction were predominantly considered. Therefore, its application to the rapidly changing ECG of evolving acute myocardial infarction has not been validated.

Potentially the Selvester QRS scoring system could be applied to serial ECGs to measure both the maximal myocardial area at risk and final infarct size, in order to calculate the percentage of

myocardial salvage effected by a therapeutic manoeuvre. However, determining which of the rapidly changing ECGs should be considered to measure the myocardium at risk, and whether acute transmural myocardial ischaemia and injury, rather than final necrosis can be scored in a similar manner, is unclear.

1.3.2 Determinants of myocardial salvage during myocardial infarction

1.3.2.1 Collateral flow

As discussed in 1.1, the normal human heart has a variable amount of collateral vessels with little collateral flow. However, if an epicardial coronary artery develops an obstruction, a pressure gradient develops across the adjacent collateral vascular bed connecting the distal vessel with other epicardial coronary arteries. As a consequence, blood flow increases through the collateral vessels to the distal portion of the occluded vessel, with subsequent dilation of these vessels, making them visible at coronary angiography (Elayda et al. 1985). The mechanism whereby vessels dilate and new vessels grow is unclear. Schaper *et al* (Schaper et al. 1990) suggest that intermittent myocardial ischaemia induced by a progressive epicardial coronary arterial stenosis leads to the expression of mitogen activator which initiates fibroblast growth factor transcription in endothelial cells. This induces both mitosis and endothelial production of platelet derived growth factor, which stimulates smooth muscle cell mitosis. The stimulated endothelium may also cause monocytes and platelets to adhere and release various growth factors such as the angiogenetic peptide tumour necrosis factor. Whether other metabolic factors, such as adenosine, that influence autoregulation also influence the development of collateral flow is unclear (Schaper et al. 1990).

The extent of potential collateralisation varies between individuals, although this may be due to differing coronary haemodynamics. Important haemodynamic variables that determine the extent

of collateral blood flow include the severity of the coronary obstruction (usually greater than 90% stenosis) (Levin, 1974; Schwartz et al. 1984), the patency of feeding coronary arteries, the size and vascular resistance of the post-obstructive segment (Newman, 1981) and duration of ischaemia in the region supplied by the obstructed coronary artery (Mohri et al. 1989). There is also a temporal component to the development of collateral flow. If a coronary obstruction develops gradually, collateral vessels gradually increase and becomes potentially significant, even if it is not visible on coronary angiography, whereas a sudden coronary occlusion is generally associated with little collateral flow (Patterson et al. 1983; Sabri et al. 1991). The speed with which collateral vessels enlarge and flow becomes haemodynamically significant is unclear, but has been shown on coronary angiography to begin to be visible within hours after total coronary occlusion (Schwartz et al. 1984; DeWood et al. 1986).

The functional significance of collateral circulation has been shown to be of importance in patients with ischaemic heart disease. During temporary balloon occlusion of a coronary artery, distal coronary perfusion pressure is higher in the presence of well-developed collaterals (Probst et al. 1985; Meier et al. 1987). Furthermore, well developed collaterals are associated with less S-T segment elevation, less left ventricular asynergy and less chest pain during balloon coronary occlusion (Cohen and Rentrop, 1986). Similarly, well collateralised but totally occluded coronary arteries are not always associated with perfusion defects on exercise thallium-201 scintigraphy (Eng et al. 1982), suggesting adequate coronary blood flow via the collateral circulation in these cases. The haemodynamic and functional importance of collateral circulation has also been demonstrated at coronary angiography in individuals with a totally occluded epicardial coronary vessel. Those with well developed collaterals had better regional left ventricular contraction (Levin, 1974). However, even with well developed collaterals, coronary flow reserve is generally

decreased (Gregg and Patterson, 1980).

In the setting of acute myocardial infarction, the influence of collateral circulation on infarct size has been demonstrated (Freedman et al. 1985; Juilliere et al. 1990). Patients with well developed collateral circulation at the time of infarction had better preservation of myocardial function and improved survival. (Habib et al. 1991). In contrast, the size of the ischaemic zone is directly correlated with the final infarct size in the well collateralised dog heart. This possibly relates to decreasing effectiveness of collateral circulation to adequately perfuse increasingly larger ischaemic zones (Lowe et al. 1978).

1.3.2.2 Speed of reperfusion

As discussed in 1.2.4.1, myocardial necrosis begins in a progressive manner, approximately 20 minutes after the onset of coronary occlusion. The endocardium is the first area to necrose, but infarction generally continues towards the epicardium over several hours (Reimer et al. 1977), being partly limited by the extent of the collateral circulation to the myocardium at risk (Habib et al. 1991). It is therefore logical to consider that if blood flow can be restored prior to the completion of necrosis, myocardium may be salvaged. In human studies, evidence for salvage with rapid reperfusion comes from the thrombolytic clinical trials for the management of evolving acute myocardial infarction. These studies have shown that reperfusion with thrombolytic therapy correlates with better left ventricular function as compared with placebo therapy (The I.S.A.M.Study Group. 1986; White et al. 1987; O'Rouke et al. 1988; Sheehan et al. 1988). This appears to subsequently confer a survival benefit (White et al. 1987). In addition, mortality was least in groups treated with thrombolytic therapy within two to three hours of the onset of chest pain, and no consistent survival benefit was seen beyond twelve hours (ISIS-2 (Second

International Study of Infarct Survival) collaborative group, 1988; Gruppo Italiano Per Lo Studio Della Streptochinasi Nell'infarto miocardico and GISSI, 1986; The GUSTO Investigators, 1993; Lincoff and Topol, 1993). This is consistent with the progressive necrosis over the first few hours being curtailed by reperfusion of the infarct-related artery.

Another factor of importance is that recanalisation, if carried out with thrombolytic therapy, may be a "stuttering" process, with multiple transient episodes of coronary occlusion and patency over several hours prior to final recanalisation (Hackett et al. 1987). Although, the mechanisms may be multiple, this cyclic blood flow reduction has been related to episodic activated platelet-induced thrombus formation (Folts et al. 1982). Intermittent occlusion and patency has been shown to be associated with fluctuating S-T segment elevation on the surface ECG (Hackett et al. 1987; Krucoff et al. 1986) and was resolved by the intracoronary infusion of isosorbide dinitrate, which has known anti-platelet effects (Hackett et al. 1987). Recurrent transient S-T segment elevation in the first 10 hours after thrombolytic therapy has been shown to correlate with a delay in the time to peak creatine kinase, which infers decreased myocardial salvage (Kwon et al. 1991). Therefore, rapid and sustained recanalisation, without transient reocclusion are important for optimal myocardial salvage (Kwon et al. 1991; Schroder et al. 1994).

Furthermore, sustained coronary reocclusion occurs in a significant minority of patients and is associated with decreased left ventricular function (Lim et al. 1991)and greater in-hospital mortality (Ohman et al. 1990). Re-occlusion is not always predictable (Ellis et al. 1989), but has been shown in some studies to correlate with TIMI 2 flow (Kwon et al. 1991) or complex morphology of the coronary obstruction (Wall et al. 1989). Possible mediating factors include thrombolytic therapy-induced platelet activation (Golino et al. 1988; Fitzgerald et al. 1988) or the

increased thrombogenicity of the partially lysed thrombus (Owen et al. 1988; Gash et al. 1986).

1.3.2.3 Adequacy of reperfusion

Because the obstruction of the coronary artery in acute myocardial infarction is generally due to a combination of thrombus formation in the presence of a plaque rupture, effective thrombolysis may occur, but a high grade stenosis may remain. This may limit the effectiveness of coronary flow down the infarct related artery and thereby limit the extent of salvage of the myocardium at risk. In particular TIMI 2 flow at 90 to 240 minutes after onset of therapy is associated with decreased myocardial salvage as compared TIMI 3 flow (Karagounis et al. 1992), and increased risk of re-occlusion (Wall et al. 1989). Whether TIMI 2 flow represents a high grade residual stenosis, or extensive distal myocardial necrosis, vascular oedema and microvascular destruction may vary between individuals (Lincoff and Topol, 1993).

Another contentious aspect of reperfusion is whether adequate tissue perfusion returns after restoration of epicardial coronary artery patency. In animal studies, coronary occlusion followed by reperfusion was first associated with hyperaemia, then after several hours an absence of flow to areas of the myocardium at risk, despite a patent epicardial coronary artery (Ambrosio et al. 1989; Jeremy et al. 1990). This phenomenon has been called "no reflow". No reflow was demonstrated in humans after recanalisation for the treatment of evolving acute anterior myocardial infarction less than 6 hours duration (Ito et al. 1992). In this study 23% of patients showed a residual perfusion defect on myocardial contrast echocardiography after successful recanalisation. These patients subsequently demonstrated less myocardial salvage than those who fully reperfused. Most "no reflow" occurred at the centre of the myocardial area at risk (Ito et al. 1992; Kloner et al. 1974a). It is therefore uncertain whether this is due to significant microvascular

damage of already necrosed myocardium, or occurs within ischaemic myocardium which may survive if adequately reperfused (Kloner et al. 1980; Kloner et al. 1974a; Ito et al. 1992). Further discussion of this topic is in 1.5

1.3.3 Clinical relevance

The extent of myocardial infarction and subsequent cardiac dysfunction are important determinants of mortality after acute myocardial function (Geltman et al. 1979; Holman et al. 1978; The Multicenter Postinfarction Research Group, 1983; Sheehan et al. 1988). Furthermore, decreased cardiac function after myocardial infarction can result in decreased exercise tolerance, the onset of clinically manifest cardiac failure, which subsequently results in impaired quality of life and a poor long-term prognosis (The Multicenter Postinfarction Research Group, 1983; AIRE Study Investigators, 1993; Cohn and Rector, 1988). Therefore, limiting the extent of myocardial necrosis at the onset of evolving myocardial infarction is a high priority in the management of this syndrome.

1.4 Redox state, reactive oxygen species and antioxidant mechanisms

1.4.1 The chemistry of reactive oxygen species

Free radical formation is part of normal tissue biology. They are formed by homolytic cleavage of a covalent bond of a molecule (reaction A)

$$X{:}Y \rightarrow X^{\bullet} + Y^{\bullet} \qquad A$$

or by electron transfer with the loss or gain of a single electron (reaction B) (Cheeseman and Slater, 1993).

$$X + e^{-} \rightarrow X^{\bullet -} \qquad B$$

In the former reaction very high energy is required, such as high temperature, UV light or ionising

radiation. Therefore electron transfer is the more common source of free radicals which can positively or negatively charged, or electrically neutral. Some of the most biologically important free radicals are derived from molecular oxygen, which is a biradical with two unpaired electrons with parallel electron spins. With nonradical molecules, it is relatively nonreactive, but can be reduced to form a variety of free radicals.

Superoxide is formed by the reduction of oxygen with a single electron (reaction C).

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

In itself, superoxide is not very damaging to tissues, although it has been shown to be capable of inactivating the NADH dehydrogenase complex of the mitochondrial electron transport chain *in vitro*(Halliwell et al. 1992). Its main importance in myocardial cells appears to be as a substrate for the formation of hydrogen peroxide and the hydroxyl radical (McCord, 1985). It is capable of diffusing across cellular membranes (Goldhaber and Weiss, 1992).

Hydrogen peroxide is not a free radical but is called a reactive oxygen species because it can easily be reduced to a free radical. It can be produced by either a the reduction of oxygen by two electrons (reaction D)

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \qquad D$$

or the reaction of two superoxide molecules (reaction E).

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

This latter reaction, whereby free radical substrate forms non-radical product, is a dismutation reaction. It is a slow spontaneous reaction, but can be catalysed by a family of enzymes called superoxide dismutases (SOD). It appears that the major role of hydrogen peroxide is as a substrate for the production of other free radical species although it is an oxidising agent in itself (Fridovich,

1978). It is more lipophilic than superoxide and more stable with a longer half-life. It appears to be even more capable than superoxide of diffusing considerable distances from its site of generation(Ferrari et al. 1991b).

Hydrogen peroxide can then readily generate the hydroxyl radical either by reacting with transition metal ions ,in particular iron and copper (reaction F) in a Fenton reaction.

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+} \qquad F$$

Superoxide also reduces, in a reversible reaction the transition metal ions into their more reactive ferrous or cuprous forms (reaction G) (Halliwell et al. 1992; Fridovich, 1978; Halliwell and Gutteridge, 1990).

$$O_2^{\bullet-} + Fe^{3+} \rightleftarrows Fe^{2+} + O_2 \qquad G$$

The sum of these reactions is named the Haber-Weiss reaction (reaction H) (Cheeseman and Slater, 1993).

$$O_2^{\bullet-} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2 \qquad H$$

The Haber-Weiss reaction is dependent upon the presence of superoxide as a source of hydrogen peroxide. The hydroxyl radical is a highly reactive oxidising radical with an extremely short half-life, but can be very damaging within a small radius of its production, by reacting with most tissue components. (Cheeseman and Slater, 1993)

1.4.2 Carbon-centred free radicals

The next important set of free radicals are the carbon-centred radicals ($R^{\bullet}$). They arise from the reaction of an oxidising radical, such as the hydroxyl radical, with a biological molecule such as a lipid, protein, nucleic acid or carbohydrate (reaction I).

$$RH + {}^{\bullet}OH \rightarrow R^{\bullet} + H_2O \qquad I$$

These rapidly and spontaneously react with oxygen to form a peroxyl radical (reaction J),

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \qquad J$$

which can then become alkoxyl radicals ($RO^{\cdot}$). (Cheeseman and Slater, 1993).

1.4.2.1 Lipid peroxidation

The oxidation of lipids (lipid peroxidation) is of importance because of the prevalence of lipid in biological tissues and relevance to cellular function. The cell membrane, mitochondria and sacrolemma are rich sources of polyunsaturated fatty acids (PUFA). Lipid peroxidation sets off a self-perpetuating chain reaction that can damage the structure and therefore function of the membrane. The chemistry of lipid peroxidation is complex, but the same proportion of breakdown products for each type of lipid are found, whatever the initiating free radical species (Porter, 1984). The lipid peroxyl radical ($LOO^{\cdot}$) are the carriers of the chain reaction, oxidising further PUFA, produce lipid hydroperoxides (LOOH) that can further break down to other radical species or a variety of nonradical compounds including aldehydes, alkenals, alkanals, hydroxyalkenals, ketones and alkanes (Slater, 1984). These products, especially hydroxyalkenals, aldehydes and lipid peroxides can diffuse to other sites, causing damage to other cellular components, adversely affecting DNA and protein synthesis (Michiels and Remacle, 1991).

1.4.2.2 Malondialdehyde

Of particular interest to this thesis is the aldehyde product of lipid peroxidation, malondialdehyde (MDA). MDA results mainly from the degradation of poly-unsaturated fatty acids (PUFAS) with greater than two methylene-interrupted double bonds, such as arachidonic acid and docosahexaenoic acid. There are several proposed mechanisms for MDA formation (Table 1.5). Firstly, several studies have suggested that oxidised PUFAS with more than two double bonds

break down to MDA via bicyclo-endoperoxides or hydroperoxy epidioxides as intermediates (Pryor and Stanley, 1975; Frankel and Neff, 1983). A second possible source of MDA from the PUFAS with more than two double bonds involves the progressive degradation of the fatty acid chain to a hydroperoxyaldehyde which can become MDA by β-scission (Esterbauer et al. 1991). Lastly, in some tissues, MDA can be formed by enzymatic reactions. MDA is a byproduct of thromboxane A_2 synthesis by platelet thromboxane synthetase from several prostaglandins. Similarly, spermine is converted to 3-amino-propanol by polyamine oxidase, which is subsequently oxidised by an aminoxidase to MDA (Esterbauer et al. 1991).

In aqueous solution, MDA can exist in several forms, depending on pH (Figure 1.5). The enolate anion is the predominant species at pH 7.4 and is of low reactivity, but as the pH falls, the β-hydroxyacrolein species, which is more reactive, predominates. It is an electrophile which can react with nucleophiles. Similarly, at physiological pH, MDA reacts very slowly with amino acids and sulphydryls such as cysteine and glutathione (Esterbauer et al. 1991). This is in contrast to the rapid reaction of other α,β-unsaturated aldehyde products of lipid peroxidation such as 4-hydroxynonenal. However, MDA readily reacts with proteins under physiological conditions. MDA cross-links proteins and modifies several of the amino acid residues (Esterbauer et al. 1991). The reaction between MDA and the nucleosides guanosine, adenosine and cytidine occurs slowly, being accelerated by decreasing pH. MDA does not appear to react with thymidine. It has been proposed that, like protein, MDA can modify double-stranded DNA by crosslinks (Esterbauer et al. 1991).

MDA metabolism, which has been studied in the rat and mouse liver, involves oxidation to CO_2 and H_2O. Mitochondrial or cytosolic aldehyde dehygrogenases convert MDA to malonic acid

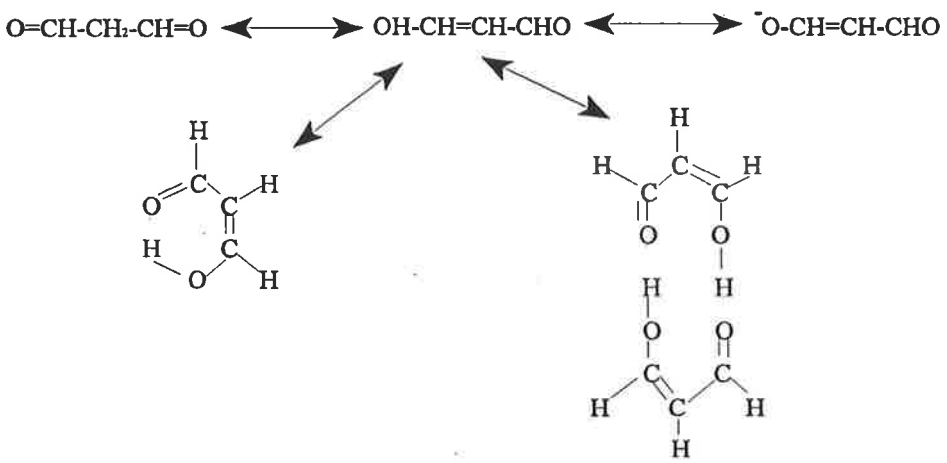


FIGURE 1.5 Structures of MDA in aqueous solution. Equilibrium is predominantly determined by pH (Esterbauer et al. 1991)

TABLE 1.5 Possible biological sources of malondialdehyde

Type of Reaction	Source Molecule of MDA Synthesis	Proposed mechanism of production
Free radical interaction	Polyunsaturated Fatty Acids with >2 methylene-interrupted double bonds	via bicyclo-endoperoxides or hydroperoxy epidioxides as intermediates
	eg. 1.Arachadonic acid 2.Docosahexaenoic acid	progressive degradation of the fatty acid chain to a hydroperoxyaldehyde by β-scission
Enzymatic reaction	prostaglandins PGH_2, PGH_3 & PGG_2	platelet thromboxane synthetase producing thromboxane A_2 and MDA
	spermine	polyamine oxidase produces 3-amino-propanol → oxidised by aminoxidase to MDA

semialdehyde, which spontaneously decarboxylates to acetaldehyde. This is further oxidised by the aldehyde dehydrogenases to acetate and then to CO_2 and H_2O. A small amount of MDA in the liver is converted to malonate, which is a substrate for malonyl-CoA and can then be carboxylated to acetyl-CoA (Esterbauer et al. 1991). Metabolic degradation products of MDA-modified proteins, nucleic acids and phospholipid bases can also be excreted in the urine. A very small but variable proportion of MDA is excreted in the free form (Draper and Hadley, 1990). The major source of urinary MDA comes from MDA-modified proteins ingested with food of animal origin, although there are studies showing increased excretion of the products after oxidative stress in animals (Esterbauer et al. 1991). Therefore, in most biological materials MDA exists predominantly in various covalently bound forms and little in the free state (Draper and Hadley, 1990).

1.4.2.3 Protein oxidation

Another carbon centred radical that forms within the myocardial cell during oxidative stress is derived from the protein myoglobin. The interaction of hydrogen peroxide with myoglobin leads to its oxidation to ferryl myoglobin. Metmyoglobin is more readily activated than oxymyoglobin to the ferryl state (reaction K).

$$(HM\text{-}Fe^{3+} + H_2O_2 \rightarrow {}^{\bullet}M\text{-}[Fe^{4+}{=}O] + H_2O) \qquad K$$

This has been shown to be the predominant radical present in electron paramagnetic resonance spectroscopic studies of isolated myocytes under oxidative stress. This radical is capable of initiating lipid peroxidation (Turner et al. 1991).

1.4.3 Sources of reactive oxygen species in the heart.

The presence of some free radicals is normal within myocardial cells under physiological

conditions (see Table 1.6). The reduction of oxygen occurs predominantly within the mitochondria. Mitochondrial enzymes are capable of reducing oxygen to water by tetravalent reduction without the production of radical intermediates. This accounts for about 95% of the oxygen consumption of tissues. Only 5% proceeds by the univalent pathway as described in 1.4.1, resulting in a "leakage" of electrons from the mitochondrial transport chain (Thompson and Hess, 1986; Ferrari et al. 1991b).

The autoxidation of hydroquinones, leukoflavins, catecholamines, sulphydryls and tetrahydropterins produces superoxide (Fridovich, 1978; Ferrari et al. 1991b). Furthermore, superoxide is slowly released when haemoglobin or myoglobin are converted to methaemoglobin and metmyoglobin respectively (Fridovich, 1978). Other sources include the microsomal cytochrome P_{450} system, and possibly prostaglandin synthesis (Ferrari et al. 1991b). The metabolism of arachidonic acid by cyclooxygenase, especially in endothelial cells (Maza and Frishman, 1988) has been shown to produce superoxide (Werns et al. 1986).

1.4.3.1 Xanthine oxidase system

Several oxidases, for example xanthine oxidase which oxidises hypoxanthine and xanthine to uric acid, produce superoxide (reaction L) (McCord, 1985).

$$\text{xanthine} + H_2O + 2O_2 \rightarrow \text{uric acid} + 2O^{\cdot}_2 + 2H^+ \qquad \text{L}$$

In the human myocardium, it is controversial as to whether the endothelial cells contain xanthine oxidase (Werns and Lucchesi, 1990; Halliwell and Gutteridge, 1990; Lazzarino et al. 1994; de Jong et al. 1990). In most species, this enzyme is not present in physiological conditions, but is produced during ischaemia by the catalytic conversion of xanthine dehydrogenase (Maza and Frishman, 1988). Normally, xanthine dehydrogenase catalyses the reduction of NAD^+ (reaction

TABLE 1.6 Sources of reactive oxygen species

Reactive Oxygen Species	Site of Production	Mechanism of Production
$O_2^{\bullet}$	Mitochondria	univalent reduction if O_2
$\rightarrow H_2O_2$		leakage of e^- from electron transport chain
$\rightarrow OH^{\bullet}$	Mitochondria & cytosol	autoxidation of hydroquinones, leukoflavins, catecholamines, sulphydryls and tetrahydropterins
	Cytosol (erythrocytes, myocardium, and muscle)	haemoglobin & myoglobin → methaemoglobin & metmyoglobin
	Cytosol (especially endothelial cells)	metabolism of arachidonic acid by cyclooxygenase
	Sarcoplasmic reticulum	microsomal cytochrome P_{450} system prostaglandin synthesis
	? Human endothelial cytosol	xanthine dehydrogenase ↓←protease←↑ calcium xanthine oxidase
NO	Endothelial cells, Vascular smooth muscle cells, Myocytes & Macrophages	constitutive and inducible NO synthase
$O_2^{\bullet}$ HOCl $\rightarrow OH^{\bullet}$ $\rightarrow H_2O_2$ →etc	Activated neutrophils	NADPH oxidase myeloperoxidase lipooxygenase

M) (McCord, 1985).

$$\text{xanthine} + H_2O + NAD^+ \rightarrow \text{uric acid} + NADH + H^+ \qquad \text{M}$$

The rise in intracellular calcium associated with myocardial ischaemia activates a protease which results in sulphydryl oxidation or limited proteolysis of xanthine dehydrogenase, catalytically converting the enzyme irreversibly to the oxidase. In the rat heart, xanthine oxidase concentration doubles after approximately eight minutes of total global ischaemia (McCord, 1985). Furthermore, during myocardial ischaemia cellular depletion of ATP leads to an elevated concentration of AMP, which is subsequently catabolised by purine nucleoside phosphorylase to adenosine, inosine and then finally hypoxanthine, the major substrate for xanthine oxidase, apart from oxygen (Werns et al. 1986; McCord, 1985).

1.4.3.2 Activated neutrophils and macrophages

Important sources of superoxide and other free radicals are activated phagocytes such as polymorphonuclear leukocytes, macrophages or monocytes (Fridovich, 1978; Ferrari et al. 1991b). When activated these cells exhibit a rapid, up to 20-fold increase in molecular oxygen consumption in a process called the respiratory burst. This results in the cellular production and release of a variety of oxidants. Stimuli for phagocytic activation are multiple, but include activated complement components , interleukin 1, platelet activating factor and tumour necrosis factor. Activation is mediated by a complex series of intracellular metabolic processes involving activation of the hexose monophosphate shunt, activation of enzymes that regulate calcium, lipid and high energy phosphate metabolism and granule fusion (Warren et al. 1989).

These changes, in combination with the increased oxygen consumption, are linked to the generation of a variety of oxygen-based oxidants. Superoxide is predominantly generated by a

plasma membrane-associated NADPH oxidase system. As discussed in 1.4.1 superoxide dismutates either spontaneously or via SOD to hydrogen peroxide. These oxygen-centred radical species are then substrates for the generation of other radical species, possibly by Fenton reactions, although this remains controversial. An important source of oxidants from activated phagocytes is the myeloperoxidase-hydrogen peroxide-halide system, which via the enzyme myeloperoxidase, produces hypohalous acids. The commonest form of this group of compounds *in vivo* is hypochlorous acid. (reaction N).

$$H_2O_2 + Cl^- \rightarrow H_2O + HOCl \qquad N$$

Myeloperoxidase is a haem-containing enzyme within the primary lysosomal granules of neutrophils and monocytes. Activated neutrophils discharge myeloperoxidase into phagocytic vacuoles or into the extracellular environment. Neutrophils also contain lactoperoxidase which can catalyse hypochlorous acid formation (Klebanoff, 1980). Hypochlorous acid is a fairly stable but potent oxidant that may give rise to other oxidants and radical species, such as chloramines, and the hydroxyl radical after reaction with superoxide. It also reacts with various bio-molecules including thioethers, sulphydryl groups, amino acids and unsaturated carbon chains (Albrich et al. 1981). N-chloroamines may inactivate leukotrienes and lysosomal enzymes and since they are lipophilic, may induce lipid peroxidation (Mehta et al. 1988; Warren et al. 1989). Lastly, singlet oxygen may be produced by activated phagocytes. It is a highly reactive, short-lived electrophile reacting with compounds with electron-rich double bonds or unsaturated heterocyclic rings (Koppenol, 1976).

1.4.3.3 Nitric oxide

Both the myocytes and endothelial cells produce nitric oxide (NO). NO has been implicated in a wide variety of cell signalling and cytotoxic functions (Culotta and Koshland, 1992). It is

synthesised from one of the terminal nitrogen atoms of L-arginine by the enzyme NO synthase (Palmer et al. 1988) and requires the cofactors NADPH, 6(R)-5,6,7,8-tetrahydrobiopterin, flavin adenine dinucleotide and flavin mononucleotide. Many isoforms of NO synthase have been isolated from a wide variety of cells (Forstermann et al. 1993). For example, 2 calcium- and calmodulin-dependent isoforms are expressed constitutively: a 'neuronal' constitutive NO synthase (Bredt et al. 1991) and an 'endothelial' constitutive NO synthase (Lamas et al. 1992). A calcium independent inducible isoform has been isolated in macrophages (Stuehr et al. 1991), neurons, hepatocytes, myocytes (Balligand et al. 1993) and vascular smooth muscle cells (Forstermann et al. 1993). Induction of these inducible isoforms is mediated by bacterial lipopolysaccharide and several cytokines, including interleukin-1 and interferon-γ (Geng et al. 1992; Tsujino et al. 1994).

Despite the fact that NO is a radical species, there is little evidence that it reacts with more than a small range of compounds *in vivo* or is destructive in the way other radicals such as hydroxyl are (Butler et al. 1995). In the aqueous phase at physiological concentrations, the reaction of NO with oxygen is less than 1 second, with NO_2^- being the one of the products obtained in these circumstances (Wink et al. 1993). This then spontaneously, but slowly be oxidised to NO_3^-.

It has been suggested that the proliferation of roles assigned to NO may be attributable partly to different redox states of NO and the subsequent reaction with other molecules, including other radical species. For example the oxidised form of NO, the nitrosonium ion (NO^+), has been hypothesised to be associated with the neuroprotective effects of NO (Lipton et al. 1993). The nitrosonium ion is a transient species in aqueous solution at physiological pH and therefore unlikely to exist *per se* in a cell. However, it is possible that naturally occurring nitrosothiols and S-nitrosoproteins, such as S-nitroso-albumin, S-nitroso-glutathione (GSNO) and S-nitroso-

S-nitrosoproteins, such as S-nitroso-albumin, S-nitroso-glutathione (GSNO) and S-nitroso-cysteine (SNC) act as biological sources of the nitrosonium ion with their ability to transfer NO^+ to a second sulphydryl or nucleophile in a transnitrosation reaction (reaction O) (Butler et al. 1995; Scharfstein et al. 1994).

$$R^1SNO + R^2S^- \rightarrow R^1S^- + R^2SNO \qquad O$$

The most biologically relevant reactions of NO involve its reaction to iron. The reversible binding to haem iron is responsible for the activation of soluble guanylate cyclase (Craven and DeRubertis, 1978), and its own inactivation in blood when binding to haemoglobin (Butler et al. 1995). NO will also bind to non-haem iron, such as iron-sulphydryl cluster in several enzymes involved in the respiratory cycle and DNA synthesis, including nitrogenase, mitochondrial aconitase, NADH:ubiquinone oxireductase and NADH:succinate oxireductase to from an Fe-S-NO complex, inhibiting their function (Reddy et al. 1983). This may be part of the cytotoxic action of NO. In addition, the cytotoxic effect of NO may be mediated by its reaction with superoxide to produce the powerful oxidant peroxynitrite, which has been suggested to decompose to form the hydroxyl radical (reaction P) (Beckman et al. 1990; Butler et al. 1995).

$$NO + O_2^- \rightarrow ONOO^-$$

$$ONOO^- + H^+ \rightarrow ONOOH \qquad P$$

$$ONOOH \rightarrow NO_2 + OH^\cdot$$

However, this formation of the hydroxyl radical has been challenged by Koppenol *et al* (Koppenol et al. 1992). Conversely, peroxynitrite has been shown to act as a NO donor, probably mediated via the formation of a S-nitrosothiol (Moro et al. 1994; Villa et al. 1994).

1.4.4 Endogenous antioxidant mechanisms in the heart

Because of the potentially damaging effects of radical species, there are a variety of antioxidant mechanisms used to either prevent their generation or intercept or "scavenge" those that are produced (Cheeseman and Slater, 1993).

1.4.4.1 Sequestration of transition metal ions

Potential for the Haber-Weiss reaction is reduced by the specific sequestration of transition metal ions. For example, the binding of iron to transferrin and ferritin, minimises the amount of "free" iron available in both the extra-cellular and intra-cellular environment respectively(Halliwell et al. 1992). However, some bound iron can remain catalytic because of its solubility, thus existing in a low-molecular weight pool and thereby putatively be a source of the hydroxyl radical (Crichton and Charloteaux Wauters, 1987). Copper is bound in plasma to both albumin and as Cu^{++} to its specific binding protein caeruloplasmin (Cotgreave et al. 1988; Halliwell and Gutteridge, 1990). However, like iron, some bound copper can remain active, participating in redox reactions.

1.4.4.2 Enzymatic antioxidant mechanisms

1.4.4.2.1 *Removal of peroxides*

Another antioxidant mechanism to minimise the potential for the Haber-Weiss reaction is the removal of peroxides. Catalase, predominantly located in peroxisomes, organelles which contain many hydrogen peroxide generating enzymes, reacts with hydrogen peroxide to form water (Cotgreave et al. 1988). Therefore, oxygen can be reduced to water and free radical generation from this substrate minimised via the combination of superoxide dismutase and catalase. In myocardial cells, the concentration of catalase is relatively low (Ferrari et al. 1991b; Turner et al. 1991). Glutathione peroxidase is found in the cytosol and reacts with both hydrogen peroxide and

TABLE 1.7 Proposed antioxidant mechanisms in the human

Mechanism	Site	Molecule
Sequestration of transitional metal ions. Avoid Fenton and Haber-Weiss reactions	Intracellular & Extracellular	1.transferrin 2.ferritin 3.caeruloplasmin 4.albumin 5.uric acid
Enzymatic & nonenzymatic Removal of peroxides	Intracellular	1.catalase 2.GSH peroxidase 3.myeloperoxidase 4.prostaglandin synthetase 5.haemoglobin 6.GSH S-transferases 7.phospholipid hydroperoxide GSH peroxidase
Enzymatic Catalyse $O_2 \rightarrow H_2O_2$	Intracellular & Extracellular	1.CuZn superoxide dismutase (cytosol) 2.Mn superoxide dismutase (mitochondria) 3.Cu superoxide dismutase (blood)
Nonenzymatic Break the "chain-reaction" of lipid peroxidation	Intracellular & extracellular	1.α-tocopherol (in lipid membranes) 2. Ubiquinol (mitochondria, nucleus) 3. ascorbic acid (cytosol & blood) 4. β-carotene (selected tissues) 5.bilirubin
Nonenzymatic Reduce oxidants	Extracellular & Intracellular	1.uric acid 2.glutathione 3.taurine (cytosol)
Remove &repair damaged molecules 1.protein 2.DNA 3.lipids	Intracellular	1.removed by proteolytic systems 2.repair enzymes 3.removal by lipases, peroxidases and acyl transferases

peroxide and cytosolic fatty acid hydroperoxides (reaction Q),

$$LOOH + 2GSH \rightarrow LOH + GSSG \qquad Q$$

once they have been cleaved from membrane phospholipids by a phospholipase (Cheeseman and Slater, 1993). GSH peroxidase contains selenium which confers its catalytic activity (Cotgreave et al. 1988). It is more abundant than catalase in the myocardial cell and thus is the predominant oxidiser of hydrogen peroxide(Ferrari et al. 1991b; Thompson and Hess, 1986). There are a variety of other peroxidases and molecules that may reduce hydrogen peroxide to water. These include myeloperoxidase, prostaglandin synthetase and haemoglobin in erythrocytes, where the levels of glutathione peroxidase are low(Cotgreave et al. 1988). Furthermore, there are a family of non-selenium containing glutathione S-transferases that metabolise low molecular weight organic hydroperoxides (Cotgreave et al. 1988). These substrates have been released from bound hydroperoxides by phospholipases. Another small selenium containing protein, called phospholipid hydroperoxide glutathione peroxidase directly catalyses the reduction of membrane-bound lipid hydroperoxides (Cotgreave et al. 1988; Hill and Burk, 1984; Sies, 1991; Meister and Anderson, 1983).

1.4.4.2.2 *Superoxide dismutase*

The superoxide dismutases (SOD) are a family of metalloenzymes whose structure is highly conserved and widely distributed in biological tissues. The prevalent enzyme is the CuZn SOD. It is mostly located within the cytosol, but may also be present within the nucleus. Another form, manganese SOD (MnSOD) which is located within the mitochondria, scavenges superoxide produced from electron leakage in the respiratory electron transport chain. A third form of SOD contains copper (CuSOD) and is predominantly found in plasma. It is therefore termed the extracellular SOD (Cotgreave et al. 1988). Because of its large size (MW ≈ 32 kDa), SOD is

prevented from diffusing from one intracellular compartment to another or being transported across the cellular membrane.

1.4.4.3 Nonenzymatic antioxidants

1.4.4.3.1 *α-Tocopherol*

Other endogenous free radical scavengers are not enzymes. Within cellular membranes, α-tocopherol, a member of the vitamin E family is an important "chain-breaking" antioxidant. It acts as a substrate in a reaction with lipid peroxyl radicals to form a tocopheroxyl radical which is relatively stable, and in itself unable to initiate lipid peroxidation (reaction R) (Cheeseman and Slater, 1993).

$$LOO^{\bullet} + \alpha\text{-tocopherol-OH} \rightarrow LOOH + \alpha\text{-tocopherol-O}^{\bullet} \qquad R$$

This α-tocopherol radical can then be reduced by ascorbate, ubiquinol if in the mitochondria(Halliwell et al. 1992), or enzyme systems such the phospholipases or glutathione peroxidase(Cotgreave et al. 1988).

1.4.4.3.2 *Ubiquinol*

Another lipid soluble chain-breaking antioxidant is Coenzyme Q_{10} otherwise called ubiquinol. This is a fat soluble quinone with a chemical structure similar to vitamin K. It is found in relatively high concentrations in the heart, and intracellularly is located predominantly in the mitochondria, nucleus and microsomes bound in its active form to a variety of binding proteins (Greenberg and Frishman, 1988). Ubiquinol appears to be synthesised in the endoplasmic reticulum and transported via the Golgi apparatus to its various cellular locations. It can pass across the cellular membrane to bile and plasma, where it binds to lipoproteins (Ernster and Forsmark Andree, 1993; Bowry et al. 1992). It serves a variety of functions including regulation of metabolic and enzymatic

activity, membrane stability and as an electron and proton carrier in the mitochondrial electron transport, preventing depletion of metabolites necessary for resynthesis of adenosine triphosphate (Greenberg and Frishman, 1988; Ernster and Forsmark Andree, 1993). Also, it acts as an antioxidant like α-tocopherol, reducing a radical to become one and subsequently be recycled in the electron transport chain(Halliwell et al. 1992). Furthermore, it appears to be the preferential antioxidant in plasma LDL particles after ascorbic acid and before α-tocopherol (Stocker et al. 1991).

1.4.4.3.3 *β-Carotene*

β-Carotene, a metabolic precursor of vitamin A, like α-tocopherol is a dietary dependent, lipophilic, chain-breaking antioxidant in membranes. It has been shown to react with superoxide, singlet oxygen and the peroxyl and alkoxy radicals especially in a low oxygen tension environment such as the retina. It is likely to act in synergism with α-tocopherol which in contrast, acts more efficiently as an antioxidant at higher oxygen concentrations (Cotgreave et al. 1988; Das and Maulik, 1994). Products of these oxidation reactions include epoxides, ketones and aldehydes. The biological role of these molecules is unclear. The concentration of β-carotene in myocardium is low as compared to organs such as the retina, liver, adrenal gland and testes (Sies et al. 1992).

1.4.4.3.4 *Ascorbic acid*

Water soluble ascorbic acid (vitamin C), present in most biological systems as ascorbate, acts as a powerful, and possibly the most important antioxidant within the cytosol and plasma (Sies et al. 1992; Frei et al. 1989). It has many antioxidant properties. It reacts rapidly with many reactive oxidising species, including superoxide, hydrogen peroxide, hypochlorite, singlet oxygen and the hydroxyl and peroxyl radicals, producing a semidehydroascorbate radical (Halliwell et al. 1992;

Sies et al. 1992), which like the α-tocopherol radical is poorly reactive. Ascorbate can also recycle α-tocopherol by reduction of the radical form of this vitamin, potentially transferring the oxidative challenge away from the membrane to the aqueous phase (Sies et al. 1992).

In extracellular fluids, there is little recycling of ascorbate, so concentrations rapidly drop during times of oxidative stress. However enzymatic systems, such as NADH-semidehydroascorbate reductase or GSH dependent dehydroascorbate reductase, exist in the cytosol to recycle ascorbate at the expense of NADH and GSH respectively. These enzymatic systems possibly exist because semidehydroascorbate can nonenzymatically react with glutathione, producing a variety of products including the cytotoxin oxalate (reaction S).

semidehydroascorbate + GSH → oxalate + $GS^{\bullet}$ S

In the presence of transition metal ions such as copper or iron, ascorbate can become a pro-oxidant initiating lipid peroxidation via a Fenton-type reaction.(Halliwell et al. 1992; Cotgreave et al. 1988; Das and Maulik, 1994). With effective physiological sequestration of metal ions in the oxidised state (ie Fe^{3+}, Cu^{2+}) the antioxidant properties of ascorbate seem to predominate(Frei et al. 1989).

1.4.4.3.5 *Uric Acid*

Uric acid (up to 300 μmol/L) is active within plasma and has been shown to react with radical species such as the hydroxyl radical. This non-enzymatic reaction is the sole source *in vivo* of a variety of stable products including allantoin and parabanic acid (Hicks et al. 1993a). Urate may also protect plasma ascorbate from oxidation (Cotgreave et al. 1988) by the formation of complexes with iron. The urate binds iron as Fe^{3+} thereby preventing a Fenton-type oxidation of

ascorbate and lipids (Hochstein et al. 1984).

1.4.4.3.6 *Bilirubin*

Another molecule within plasma and some tissues, that in physiological concentrations acts as a chain-breaking antioxidant is bilirubin. It reacts directly with the peroxyl radical and is thought to supplement the activity of β-carotene in many tissues (Cotgreave et al. 1988; Sies et al. 1992).

1.4.4.3.7 *Glutathione*

Glutathione, a sulphydryl-containing tripeptide, is present in high concentrations in the cytosol (0.5-10 mmol/L) (Cotgreave et al. 1988; Meister and Anderson, 1983) and only in the micromolar concentration range in extracellular compartments. In the intracellular space, 99% of glutathione is in the reduced form, while in plasma 90% is GSH (Meister, 1985; Meister and Anderson, 1983). Intracellularly, glutathione is insensitive to the normal peptidases due to it's γ-glutamyl peptide bond. The concentration of glutathione is regulated by a complex group of enzyme systems (see Figure 1.6) (Cotgreave et al. 1988; Ferrari et al. 1991b; Meister and Anderson, 1983). The synthesis of GSH by γ-glutamylcysteine synthetase and GSH synthetase is controlled by feedback inhibition of GSH on γ-glutamylcysteine synthetase and the cellular availability of substrate, in particular cysteine (Meister, 1985). GSH breakdown is via γ-glutamyl transpeptidase, with the major fraction of this enzyme positioned on the external surface of the cell membrane (Meister and Anderson, 1983). From animal studies (where GSH synthetase or γ-glutamyl transpeptidase are experimentally inhibited) it is clear that although intracellular GSH is the major substrate for γ-glutamyl transpeptidase, plasma GSH, predominantly excreted by the liver is also utilised. The major organ involved in plasma GSH uptake is the kidney (Meister and Anderson, 1983; Meister, 1985).

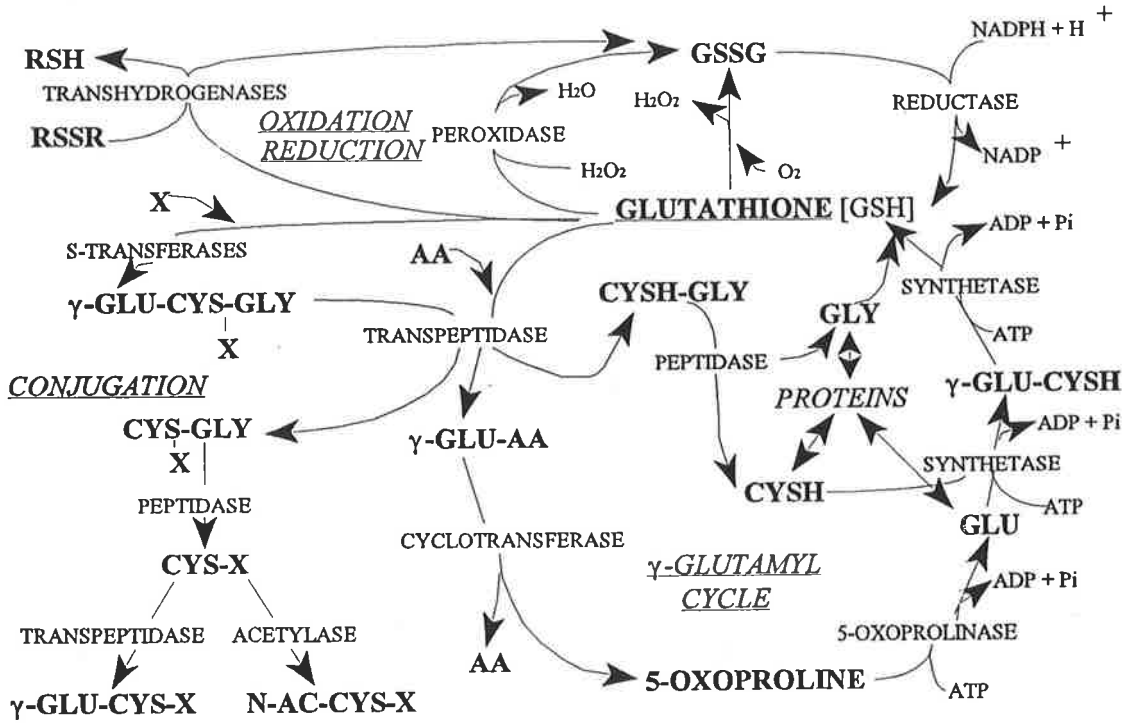


FIGURE 1.6 Glutathione metabolism (Meister and Anderson, 1983)

GSH is the major transcellular transport form of glutathione out of the cell (Lauterburg et al. 1984; Ishikawa and Sies, 1984), although at times of oxidative stress, when intracellular GSSG concentrations rise to potentially toxic levels, it appears that active transport of GSSG out of the cell takes place (Ishikawa and Sies, 1984; Meister, 1985; Janssen et al. 1993). Even then, GSH excretion remains predominant (Ishikawa and Sies, 1984).The maximum capacity of the myocardial GSSG transport system appears to be less than in the liver (Ishikawa and Sies, 1984; Lauterburg et al. 1984). However, release of GSH and GSSG from the myocardium into the extracellular environment have been shown to parallel changes in their intracellular concentrations during oxidative stress (Ferrari et al. 1992; Ishikawa and Sies, 1984; Janssen et al. 1993).

Cellular transport studies suggest that GSH is transported from the cytosol to an external cellular membrane site containing transpeptidase. Here it may interact with cystine to become γ-glutamylcystine. It is this molecule that can be transported back into the cytosol and subsequently reduced by dehydrogenation involving GSH to become γ-glutamylcysteine. Extracellular GSH can inhibit γ-glutamylcystine transport and intracellular GSH inhibits cysteine utilisation by γ-glutamylcysteine synthetase. GSH and GSSG are finally excreted mostly in the bile, but also in urine (Meister and Anderson, 1983; Meister, 1985).

Importantly, glutathione can reduce oxidants such as hydrogen peroxide to water predominantly via the enzyme glutathione peroxidase, and directly reduce such radicals as superoxide, hydroxyl radical and peroxyl radicals, becoming a thiyl radical ($GS^{\cdot}$) and eventually GSSG. Oxidised glutathione is returned to the reduced state by NADPH-linked glutathione reductase (reaction T).

$$GSSG + 2NAD(P)H \rightarrow 2GSH + 2NADP \qquad T$$

This enzyme is tightly linked to glutathione peroxidase in the cytosol and to the pentose phosphate

shunt, which is the source of NADPH. Furthermore, GSH reacts with electrophiles forming covalent adducts, such as disulphides with proteins(Cotgreave et al. 1988). Because of its ability to react with electrophiles, it modulates the activity of several enzymes(Ferrari et al. 1991b), is essential for the synthesis of some proteins and deoxyribonucleotide precursors of DNA. It is also able to conjugate with several drugs, hormones and cytokines (Meister and Anderson, 1983).

1.4.4.3.8 *Taurine*

The β-amino acid taurine is not available for incorporation into proteins and therefore accumulates to high intracellular concentrations. This is particularly so in cells that produce high concentrations of ROS or in cells rich in lipid membranes. One of its cellular roles may be as an endogenous antioxidant, reacting with ROS such as hypochlorous acid to form less reactive products (Cotgreave et al. 1988).

1.4.4.4 Repair mechanisms

Lastly, important antioxidant defense mechanisms by biological tissues are the repair processes that remove damaged molecules before they have an adverse effect on cellular function or viability. For example, oxidatively damaged nucleic acids are repaired by specific enzymes. Oxidised proteins are removed by proteolytic systems and peroxidised lipids by lipases, peroxidases and acyl transferases (Cotgreave et al. 1988; Cheeseman and Slater, 1993).

1.5 Reperfusion injury and oxidative stress following myocardial ischaemia

Reperfusion injury was originally defined by Hearse (Hearse, 1977) as cell death (or damage) caused by reperfusion, in contrast to cell death (or damage) caused by the preceding ischaemic episode. However, several cardiac events are exclusive to the onset of reperfusion after myocardial

ischaemia and do not necessarily involve cell necrosis. Therefore, the definition of reperfusion injury for this thesis has been expanded to remove the parentheses from Hearse's definition and include cellular damage that subsequently leads to cellular, vascular and haemodynamic events that have been demonstrated to be associated specifically with reperfusion.

Reperfusion injury in humans may potentially occur in the clinical settings of cardiopulmonary bypass for cardiac surgery, reperfusion in the management of acute myocardial infarction, coronary PTCA and preservation of donor hearts for transplantation. However, most of the evidence for the presence and extent of reperfusion injury comes from animal models, with variable correlation to the human setting due to differences in cardiac anatomy and collateral circulation, molecular and enzymatic content and experimental technique (Nayler and Elz, 1986).

1.5.1 Evidence for the occurrence of reperfusion injury

1.5.1.1 Overall biochemical and structural changes associated with myocardial ischaemia and reperfusion

The histological changes seen with progressive myocardial ischaemia are outlined in 1.2.4.1. The important change that indicates irreversible damage, or cell death, is disruption of sarcolemmal membrane integrity. In such irreversibly ischaemic cells, restoration of blood flow is associated with acceleration of already lethal damage (Nayler and Elz, 1986). Reperfusion exacerbates the loss of calcium, ionic and osmotic homeostasis due to lack of membrane integrity and depletion of ATP and phosphocreatine (PC), resulting in sodium and calcium overload (Jennings et al. 1990; Braunwald, 1982). There is extensive oedema resulting from this hyperosmolar intracellular environment (Tranum Jensen et al. 1981), and early, abrupt myofibrillar contracture, with contraction bands, sarcolemmal whorls and calcium deposits, in the form of hydroxyapatite, in the

mitochondria (Kloner et al. 1974b; Nayler and Elz, 1986; Virmani et al. 1992). In particular, the contraction bands and mitochondrial calcium deposits are specific to reperfusion (Nayler and Elz, 1986), although calcium deposits may be absent in cells near the subendocardium after severe ischaemia. These may represent irreversibly ischaemic cells with no mitochondrial function left at reperfusion (Kloner et al. 1974a). It has been suggested that such abrupt and violent contracture in association with cellular oedema may mediate mechanical disruption of sarcolemmal membranes and intercellular attachments (Ganote and Kaltenbach, 1979; Jennings et al. 1990). This process must be distinguished from the general spectrum of reperfusion injury, as it represents only an acceleration of the process of cell death.

If there is extensive cellular swelling and oedema, adjacent capillaries may be compressed, impairing blood flow to that locality. The survival of any reversibly ischaemic cells being supplied by these compressed capillaries may be compromised (Tranum Jensen et al. 1981). Furthermore, intramyocardial oedema increases coronary vascular resistance and impairs myocardial compliance, thus raising left ventricular diastolic pressure and further impairing myocardial perfusion, especially to the subendocardium (see 1.2.4.2) (Brutsaert et al. 1993).

Not only do myocytes suffer ischaemic damage, but so do endothelial cells. During ischaemia induced by coronary occlusion in the dog Kloner *et al* (Kloner et al. 1980) there was a considerable lag between the onset of myocyte damage as compared endothelial cell damage. Microvascular damage was only seen when there was already considerable underlying, often irreversible myocyte damage. Damage was understandably most prominent in the subendocardium. Histological changes included a loss of pinocytic vesicles, localised areas of endothelial swelling, occasional foci of haemorrhage, endothelial gaps and intra- and extravascular fibrin deposition.

Upon reperfusion of an ischaemic vascular bed, there is explosive endothelial cellular swelling. Cells lose pinocytic vesicles and become voluminous, with large intraluminal protrusions. Also activated neutrophils, erythrocytes and platelets obstruct capillaries. This capillary bed "plugging" may potentially limit the effectiveness of reperfusion to any surrounding, potentially viable myocytes (Kloner et al. 1974b).

Prolonged ischaemia followed by reperfusion invariably results in haemorrhage into the ischaemic territory. This is rarely seen with permanent coronary occlusion (Higginson et al. 1982). Haemorrhage implies loss of vascular integrity resulting from endothelial damage, especially in the capillary bed. The haemorrhage seen after reperfusion occurs almost exclusively in the subendocardium and is confined to regions of necrotic myocardium (Kloner et al. 1980; Higginson et al. 1982). This suggests that haemorrhage only occurs in areas where myocytes were irreversibly damaged prior to reperfusion and does not result in lethal reperfusion injury to reversibly ischaemic myocardium (Fishbein et al. 1980). However, haemorrhage like oedema, may increase myocardial stiffness with the consequent haemodynamic changes.

In reversibly ischaemic myocytes, ischaemia results in depletion of ATP and PC, but mitochondrial oxygen consumption and initial rate of mitochondrial ATP production are relatively well preserved. With reperfusion, mitochondrial oxygen consumption and ATP synthesis decline, with little early restoration of overall ATP and PC stores (Ferrari et al. 1986). This impaired restoration of ATP and PC synthesis may partly explain why reperfused myocytes are unable to maintain normal aerobic metabolism, resulting in impaired cellular functions including a loss of control of ionic and osmotic homeostasis. This may then result in subsequent cell death (Poole Wilson et al. 1984; Ferrari et al. 1986). However, it is difficult to distinguish which cells were lethally or

reversibly ischaemic at the time of reperfusion by histological examination of the tissue after this event. The final results are histologically identical (Nayler and Elz, 1986). Furthermore, reversibly ischaemic cells may not potentially become irreversibly damaged at reperfusion, but have incremental reversible damage prior to their subsequent recovery (Sharma et al. 1983b; Stern et al. 1985). This however could adversely effect normal myocardial electrical and contractile functional recovery, even if temporarily.

In the presence of regional ischaemia, necrosis generally occurs in a progressive manner from subendocardium to subepicardium (Reimer et al. 1977). However, in the globally ischaemic isolated heart, the progression to irreversible damage is more random. At any one time, it is possible to find cells that exhibit all the characteristics of ischaemic damage closely adjacent to relatively normal cells (Nayler and Elz, 1986). This has also been noted in isolated cell suspensions of myocytes (Stern et al. 1985). The time to the development of contracture of isolated myocytes within an hypoxic medium varied from cell to cell and was unpredictable.

In a single hypoxic myocyte cell preparation, it was shown that the cell abruptly contracted after a highly variable period. This was presumed to represent a rigor state and remained inert without further change in morphology. At reoxygenation after a period of contraction, the cells either partially recovered to a shortened form capable of stimulated twitches, or rounded up rapidly to a disordered, non-functional hypercontracted form. The longer the period in the contracted state during hypoxia, the more likely the cell would not recover (Stern et al. 1985). The hypercontracted cells at reoxygenation demonstrated the potential "fatal" nature of oxygen in this model.

1.5.1.2 Myocardial stunning

Myocardial stunning is defined as myocardial mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage. It is a fully reversible abnormality with time. However, it is distinct from myocardial "hibernation", where there is ongoing ischaemia and impaired coronary blood flow contributing to myocardial dysfunction in the presence of viable myocardium (Bolli, 1992).

In animal models, stunning can be produced after several manoeuvres. In the dog, coronary occlusion for less than 20 minutes does not result in myocardial necrosis but prolonged contractile dysfunction. Also, repeated brief occlusions, each of 5 - 10 minutes duration, have a cumulative effect with the largest decrease in contractile function occurring after the first occlusion, and additional decrements becoming progressively smaller. If the coronary occlusion occurs for more than 20 minutes, but less than 3 hours, subendocardial infarction results, with a variable amount of subepicardial stunning which may take weeks to fully recover. In isolated hearts, global ischaemia variably causes myocardial necrosis and reversible dysfunction on reperfusion, whereas global ischaemia in the intact animal, supported by different forms of cardioplegia, may be followed by prolonged contractile dysfunction without infarction. Lastly, myocardial ischaemia induced by myocardial stress, such as exercise in the setting of a flow-limiting epicardial coronary stenosis may result in contractile abnormalities, despite adequate coronary flow in the recovery period. Whether, the pathogenesis of these different forms of myocardial stunning are identical is unclear (Bolli, 1990).

The severity of postischaemic myocardial dysfunction is directly correlated to the severity (Bolli et al. 1988b) and duration (Preuss et al. 1987) of the ischaemic episode, with the subendocardium

generally more severely effected (Bolli et al. 1989). Therefore, even though this phenomenon is considered to be a form of reperfusion injury, it is the preceding ischaemic episode that "primes" the myocardium for its development (Bolli, 1990). A unique feature of myocardial stunning is the presence of considerable contractile and metabolic reserve. When stimulated by positive inotropic agents the depressed myocardial contractile function improves (Ellis et al. 1984).

Study of myocardial stunning in humans is more difficult than in animal models, because of the many factors that influence left ventricular function apart from myocardial contractility. Furthermore, the extent of infarcted as compared with stunned myocardium after reperfusion, the presence of ongoing ischaemia and therefore the possibility of hibernating myocardium all need to be considered.

With these limitations in mind, myocardial stunning has been studied in humans. During short periods of balloon inflation in coronary PTCA, regional systolic dysfunction has been noted, but recovery within 5 minutes, simultaneous with normalisation of myocardial lactate metabolism generally occurs (Serruys et al. 1984). However, in a minority of patients regional left ventricular wall stiffness remained abnormal for beyond 12 minutes of observation, although the left ventricular pressure-volume loop had returned to normal (Wijns et al. 1986). Therefore, in uncomplicated PTCA the duration of the ischaemic episode in inadequate to induce significant stunning at reperfusion.

In the clinical setting of stable and unstable angina pectoris, it is difficult to be certain whether a regional wall motion abnormality is due to stunning, ongoing ischaemia or hibernation. Therefore, coronary blood flow would need to be demonstrated as normal at the time of assessing left

ventricular function for myocardial stunning to be diagnosed, with the confounding problem of intermittent ischaemia falsely prolonging myocardial dysfunction (Bolli, 1992).

During thrombolytic therapy for the treatment of myocardial infarction, salvaged, reversibly ischaemic myocardium has been shown to exhibit some characteristics suggestive of myocardial stunning. Res *et al* (Res et al. 1986) utilised gated equilibrium radionuclide ventriculography to assess left ventricular ejection fraction in patients randomised to receive intracoronary streptokinase or not. The most marked differences were in patients with anterior infarction, with myocardial salvage being demonstrated when comparing no treatment versus thrombolytic treatment on day 2 (34 ± 13% (standard deviation) vs 39 ± 13%, p=0.01). Furthermore, left ventricular function only significantly improved in the treated group over the next 2 weeks (increments in ejection fraction 1.3 ± 8.7% vs 4.9 ± 9.4%, p=0.0006). Similar findings were also noted by Anderson *et al* (Anderson et al. 1983) in a smaller study assessing the effectiveness of intracoronary streptokinase. Left ventricular ejection fractions were similar between control and treated groups at day 1 (43 ± 11% vs 42 ± 13%, p=ns), but at day 10 the treated group showed considerable improvement in myocardial function (47 ± 9% vs 39 ± 12%, $p<0.05$). Ito *et al* (Ito et al. 1993) utilised myocardial contrast echocardiography to determine the myocardial area at risk (defined as a contrast defect after right and left coronary injections with hand-agitated Haemaccel), in patients successfully reperfused with intracoronary urokinase for evolving anterior myocardial infarction. The endocardial length demonstrating akinesis and/or dyskinesis was determined as a ratio of the area at risk. Over 14 days, this ratio progressively decreased and then plateaued with no further improvement at 28 days (ratio 1.00 ± 0.02 day 1 vs 0.73 ± 0.10 day 14, $p<0.01$ vs 0.72 ± 0.10 day 28). This improved function over time is consistent with the presence of myocardial stunning in patients early after reperfusion.

Another clinical setting in which myocardial stunning has been investigated is cardioplegia for cardiac surgery as this represents a period of significant global ischaemia followed by reperfusion. Because of the frequent use of positive inotropic drugs in the postoperative period, comparisons of left ventricular function over time may be confounded. Despite this, Roberts *et al* (Roberts et al. 1980) demonstrated a transient, but significant decrease in left ventricular ejection fraction early after cardiac surgery (50 ± 4% preoperative vs 38 ± 3% 2 hours postoperative vs 48 ± 4% 24 hours postoperative vs 57 ± 4% 7 days postoperative).This phenomenon occurred in 36 of the 40 patients studied. Similarly results were observed by Breisblatt *et al* (Breisblatt et al. 1990), with a fall of left ventricular ejection fraction from 58 ± 12% preoperatively to 37 ± 10% at 262 ± 116 minutes post-bypass, prior recovering to 55 ± 13% 426 ± 77 minutes post-bypass and full recovery by 24 to 48 hours. Furthermore, Ferrari *et al* (Ferrari et al. 1990) separated patients undergoing coronary artery bypass grafting into two groups according to the duration of the cross-clamping time. Cardiac index progressively improved from 2.69 ± 0.24 L/min/m^2 preoperatively to 3.80 ± 0.19 L/min/m^2 24 hours postoperatively in the group with a cross-clamping time less than 30 minutes (mean 25 ± 2 minutes). However, in the group with a cross-clamping time greater than 30 minutes (mean 55 ± 3 minutes) cardiac index transiently deteriorated from 2.66 ± 0.21 L/min/m^2 preoperatively to 2.32 ± 0.25 L/min/m^2 2 hours postoperatively, prior to recovering at 24 hours to 3.81 ± 0.21 L/min/m^2. This consistent demonstration of early transient depression of myocardial contractile function after cardiac surgery is most likely a manifestation of myocardial stunning.

1.5.1.3 Arrhythmias

In animal models, brief periods of myocardial ischaemia are frequently associated with significant ventricular arrhythmias, such as ventricular tachycardia and fibrillation, within seconds of the

return of coronary flow (Hale et al. 1984; Kloner, 1993). This correlation between arrhythmias and reperfusion after brief episodes of ischaemia is now undisputed (Tosaki and Das, 1994; Euler, 1994). The electrophysiological mechanisms include membrane depolarisation, abnormal automaticity, action potential prolongation, early and late after-depolarisations and triggered activity (Goldhaber and Weiss, 1992). These findings suggest that reperfusion arrhythmias are non-reentrant in nature (Pogwizd and Corr, 1987). The length of the ischaemic period is associated with a bell shape curve for the frequency of malignant arrhythmias. Hearse *et al* (Hearse and Tosaki, 1987) demonstrated a maximum frequency of reperfusion-induced ventricular fibrillation after 10 minutes of regional ischaemia to the territory of the left anterior descending artery. Longer periods of ischaemia correlated with a lower frequency of ventricular fibrillation. Furthermore, after long periods of ischaemia arrhythmias may occur during the ischaemic period, without a dramatic increased incidence after the onset of reperfusion (Hale et al. 1984; Kloner, 1993).

In contrast to animal models, reperfusion arrhythmias after myocardial ischaemia in humans do not appear to be of great clinical significance, although two characteristic patterns are seen which are quite specific for reperfusion in the setting of evolving acute myocardial infarction. Goldberg *et al* (Goldberg et al. 1983) examined the incidence of reperfusion arrhythmias in 20 patients being treated with intracoronary streptokinase for evolving acute myocardial infarction of less than 6 hours duration. In 14 patients an arrhythmia occurred within seconds to minutes of restoration of antegrade flow through the infarct-related artery. Most commonly, idioventricular rhythm without haemodynamic compromise was observed. This arrhythmia appears to be specific for reperfusion. In patients with inferior myocardial infarction, sinus bradycardia associated with transient hypotension was noted on occasions. This arrhythmia has been attributed to the activation of the

Bezold-Jarisch reflex (Koren et al. 1986).

Hackett *et al* (Hackett et al. 1990) also noted arrhythmias to occur within minutes of reperfusion for the treatment of acute myocardial infarction of less than six hours duration. However, arrhythmias were just as likely to occur when the infarct-related artery remained occluded. They therefore concluded that arrhythmias in the setting of evolving acute myocardial infarction were neither specific nor sensitive for reperfusion in humans. Further support for the low incidence of malignant reperfusion arrhythmias after acute myocardial infarction in humans was seen in the significant decrease of in-hospital ventricular fibrillation in thrombolytic treated patients as compared placebo (Gruppo Italiano Per Lo Studio Della Streptochinasi Nell'infarto miocardico and GISSI, 1986; ISIS-2 (Second International Study of Infarct Survival) collaborative group, 1988).

The reason why reperfusion arrhythmias are not of high incidence or significance in humans with acute myocardial infarction is unclear. Differences between animal and human settings include the prolonged ischaemic period associated with significant infarction whereas in animal models infarction has not occurred. Also, reperfusion is likely to occur more slowly in patients mechanically or pharmacologically recanalised, in contrast to the almost instantaneous reperfusion in animal models (Opie, 1989; Kloner, 1993).

1.5.1.4 Myocyte necrosis

It has been methodologically difficult to clearly demonstrate lethal reperfusion injury. This is because there is no clear differentiator of irreversibly ischaemic myocytes after reperfusion that determines whether or not they were only reversibly ischaemic at the onset of reperfusion. This

has no doubt been partly responsible for the controversial nature of both the existence and significance of reperfusion injury. Generally, lethal myocardial injury may be inferred from studies comparing the extent of myocardial infarction in animals treated with of without agents that are hypothesized to limit reperfusion injury. However results are variable, possibly due to factors such as insensitive methodology, differences in collateral flow, extent of ischaemia, time of reperfusion and effectiveness of the pharmacological agent (Miura, 1990). The different agents and their effects are discussed in detail in 1.6.

Hofmann *et al* (Hofmann et al. 1980) utilised a dog heart model, occluding two small coronary arteries within the same animal for either 3 or 6 hours of ischaemia with or without 60 or 90 minutes of reperfusion. There was no evidence of increasing infarct size attributable to reperfusion. However, the dog has a well collateralised heart and this was not determined during the study in order to exclude this confounding effect on infarct size.

In a similar study of the comparison of two coronary artery occlusions in the same dog, Ganz *et al* (Ganz et al. 1990) were unable to demonstrate extension of infarction with 90 to 240 minutes of ischaemia followed by 5 minutes of reperfusion in one artery only. The heart was then removed and stained with triphenyltetrazolium chloride (TTC). In viable myocytes TTC is reduced to formazan pigments by diaphorases that utilise NADH or NADPH as electron donors. Thus, infarcts have been accurately identified as TTC staining defects (Klein et al. 1981) after 180 minutes of reperfusion in rabbits (Shirato et al. 1989) and 90 minutes in dogs (Schaper et al. 1979). Ganz *et al* (Ganz et al. 1990) used electron microscopy to confirm the validity of TTC staining very early after reperfusion in their model.

However, there are several limitations to this study. In preliminary experiments, the model was unable to detect an increased infarct size despite 5 incremental minutes of ischaemia, suggesting it was somewhat insensitive. Also, the prolonged ischaemia time may have resulted in a majority of the myocytes at risk being irreversibly damaged prior to reperfusion. Furthermore, collateral flow was slightly higher in the reperfused region. Lastly, the reperfusion period was only 5 minutes, which may have been too short for lethal reperfusion injury to be completely manifest. Lethal reperfusion injury may not be instantaneous, but progress over several hours after reperfusion (Miura, 1990).

The only study to show histological evidence for lethal reperfusion injury was by Farb *et al* (Farb et al. 1993). To avoid the confounding effects of collateral circulation, this study used the poorly collateralised, open chest rabbit heart model. Differing histological stains were used in the same animal, in the same ischaemic zone after 30 minutes of ischaemia at the onset of reperfusion, and after 180 minutes of reperfusion. Horseradish peroxidase (HRP) is a fine structural tracer protein molecule which detects ultrastructural changes in membrane permeability in experimental models of myocyte necrosis, therefore being specific for irreversibly injured myocytes . HRP was intravenously infused at reperfusion and stained ischaemia-induced irreversibly injured cells. After 180 minutes, TTC was infused through the just-resected, retrogradely perfused heart. Calculated infarct size was similar for both methods if HRP and TTC were simultaneously infused after 180 minutes of reperfusion. However, HRP staining at the onset of reperfusion indicated an infarct size of 45.3 ± 2.8% (standard error of mean) of the area at risk, as compared TTC staining after 180 minutes infusion, which demonstrated 59.8 ± 3.3% of the area at risk was infarcted ($p=0.0002$). This demonstrated that infarct size had increased about 15% after the onset of reperfusion in the absence of collateral flow. The distribution of the HRP-positive cells was greatest in the central

mid-myocardium, with sparing of the subendocardium and subepicardium. Infarct extension proceeded towards the epicardium as indicated by TTC staining after 180 minutes reperfusion.

Infarct extension may have been overestimated by the comparison of the stain-positive HRP area and a stain-negative TTC area. However, TTC staining is reliable measure of infarct size in the rabbit after 90 minutes of reperfusion (Shirato et al. 1989), TTC and HRP staining gave the same estimates of infarct size when infused simultaneously, and electron microscopy validated their results in selected sections.

By a more indirect methodology, Frame *et al* (Frame et al. 1983) used 60 minutes of coronary occlusion followed by 45 minutes of reperfusion in the dog heart to demonstrate increasing binding of radiolabeled anticardiac myosin antibody during reperfusion, a marker of myocyte membrane disruption. This suggested that in the well collateralised dog heart there was increasing lethal reperfusion injury over time after reperfusion, similar to the rabbit.

1.5.1.5 "No reflow"

As discussed in 1.5.1.1. myocardial ischaemia and reperfusion results in considerable endothelial and microvascular damage in areas of extensive myocardial damage, especially in the subendocardium (Kloner et al. 1980; Kloner et al. 1974b). Activated neutrophils, erythrocytes and platelets obstruct these damaged capillaries, resulting in capillary bed plugging, which limits the effectiveness of reperfusion. This phenomenon of "no reflow" has been discussed briefly in 1.3.2.3. It does not occur in myocardium in the absence of infarction (Johnson et al. 1988).

Kloner *et al* (Kloner et al. 1974a) showed that "no-reflow" 20 minutes after reperfusion only

appeared after 90 minutes of coronary artery occlusion in the dog and not after 40 minutes despite the presence of significant myocardial damage at this time. Lack of reperfusion occurred in the subendocardium, at the centre of the ischaemic zone. Significant endothelial damage and "plugging" was confined to areas of no-reflow and adjacent myocytes frequently contained no calcium granules in the mitochondria, suggesting severe irreversible ischaemia prior to reperfusion. Myocyte swelling was similar at 40 and 90 minutes, suggesting that this did not significantly contribute to no-reflow. The areas of no-reflow were often haemorrhagic and packed with erythrocytes and neutrophils. Therefore some early flow must have been present for a short period at reperfusion, probably inducing endothelial damage with downstream obstruction leading to rouleaux formation of red blood cells, and subsequent increased viscosity and stasis.

Ambrosio *et al* (Ambrosio et al. 1989) and Jeremy *et al* (Jeremy et al. 1990) confirmed these findings in their dog models, showing that the extent of the perfusion defect increased with time from the onset of reperfusion. In general, initial reperfusion within the first few minutes was hyperaemic with a subsequent decrease in flow to poorly collateralised regions over several hours. Areas with late impairment of flow showed contraction band necrosis and calcium granules in the adjacent myocytes, in contrast to the pattern of coagulation necrosis observed in the areas where a perfusion defect was present early after reperfusion. Also capillary plugging was most marked in areas of delayed impairment of flow.

The extent of the perfusion defect after reperfusion correlated well with subsequent infarct size in both animal and human models (Johnson et al. 1988; Jeremy et al. 1990; Ito et al. 1992). Patients exhibiting "no reflow" 15 minutes after reperfusion of an acute myocardial infarct of less than six hours duration, had similar left ventricular ejection fractions prior to recanalisation as

compared patients not exhibiting a perfusion defect (34.7 ± 8.9 vs 42.3 ± 11%). However, there was no significant improvement of left ventricular ejection fraction after 4 weeks, in contrast to the significant improvement in ejection fraction in patients with no reperfusion defect (42.7 ± 8.9 vs 56.4 ± 13.4%) (Ito et al. 1992). The authors believed the results suggested patients with a perfusion defect had a larger proportion of irreversibly ischaemic myocardium prior to reperfusion, rather than the perfusion defect being responsible for subsequent lethal myocardial injury of reversibly ischaemic myocytes(Ito et al. 1992).

These pathological findings suggest that progressively increasing "no reflow" represents an effect of reperfusion injury. Whether the impairment of flow leads to further lethal myocyte injury, or whether the endothelial damage occurs as a late sign, with all adjacent myocytes irreversibly damaged prior to the onset of "no reflow" is uncertain. However, endothelial damage appears to correlate with its occurrence and this occurs at a later time to myocardial damage, suggesting that "no reflow" is a consequence of reperfusion injury rather than a cause. Furthermore, reperfusion injury is likely to occur within minutes rather than hours after reperfusion. Therefore, the relatively slow onset of "no reflow" is against its being an important causative factor.

1.5.1.6 Microvascular damage and endothelial dysfunction

Structural damage to the endothelium is also accompanied by changes in function. The activation of endothelial phospholipase A_2 due to raised intracellular calcium concentration induces the endothelial production of platelet activating factor (PAF), leukotriene B_4 (LTB_4), thromboxane A_2 (TXA_2) into the intravascular lumen. These factors are all potent mediators of inflammation acting as chemoattractants, mediating neutrophil activation, adherence and diapedesis (Lehr et al. 1991). Furthermore, PAF induces the expression of endothelial adhesion molecules, such as

integrin neutrophil adhesion molecule CD11/18 (Kusuoka and Marban, 1992; Lefer and Lefer, 1993). Myocardial and endothelial damage also induces the activation of complement, in particular $C1_q$ and C5a with subsequent activation of the complement cascade, facilitating chemotaxis and adhesion of neutrophils to the ischaemic-reperfused endothelium (Rossen et al. 1985; Shandelya et al. 1993). At reperfusion after ischaemia, neutrophils are activated, adhere and migrate into the adjacent tissue. In the activated state, neutrophils generate oxidants and leukotrienes which induce the endothelial expression of other adhesion molecules such as ICAM-1 and E-selectin. This amplification of the inflammatory process promotes endothelial leakage of protein into the tissues (Lehr et al. 1991; Kurose et al. 1994a), endothelial and myocyte damage and necrosis (Cooke and Tsao, 1993) (see 1.5.2.3)

Reperfusion after ischaemia has been associated with impaired vascular relaxation to endothelial-dependent vasodilators in canine epicardial coronary artery rings, but preservation of the relaxation response to the NO donor nitroprusside (Ku, 1982; VanBenthuysen et al. 1987). Sobey *et al* (Sobey et al. 1990) showed that both epicardial and resistance vessels had impaired vasodilation. In contrast, Quillen *et al* (Laurindo et al. 1991) showed that after ischaemia and reperfusion, canine large conduit arteries were not altered in their response from normally perfused vessels. Three hours of ischaemia without reperfusion mildly impaired small vessel (110 - 220 μm) endothelial-dependent relaxation, but reperfusion after 1 hour of ischaemia induced marked impairment, but with preservation of relaxation to GTN. This attenuated endothelial-dependent relaxation during reperfusion after ischaemia appears to be correlated to a markedly diminished endothelial release of both NO and prostacyclin. This has been shown to occur within minutes of reperfusion and precedes neutrophil infiltration (Tsao et al. 1990; Ma et al. 1993; Hempel et al. 1990; Kurose et al. 1994b).

However, vasomotor tone is incrementally affected by activated neutrophils in the reperfused myocardial vasculature. In an isolated cat heart model Tsao *et al* (Tsao et al. 1992) showed that the vasodilator response to acetylcholine was incrementally impaired by activated neutrophils at reperfusion after myocardial ischaemia as compared reperfusion with Krebs buffer, whereas the endothelial-independent vasodilator response of GTN remained intact. This adverse response to acetylcholine was almost completely ameliorated by the simultaneous infusion of SOD or an antibody to the neutrophil adherence glycoprotein. Similarly, the addition of LTB_4-activated neutrophils to canine coronary artery rings was shown to induce vasoconstriction and impaired relaxation to endothelial-dependent acetylcholine. This effect was attenuated by the sulphydryl-containing NO donor SPM-5185 (Lefer et al. 1993).A putative mechanism by which activated neutrophils mediate this vasoconstriction may be by the inactivation of NO by superoxide (Gryglewski et al. 1986), although NO has also been shown to inhibit neutrophil NADPH oxidase (Clancy et al. 1992), suggesting a complex interaction between NO and neutrophils. Furthermore, NO has been shown to prevent neutrophil adherence to the endothelial surface, probably by modulation of the neutrophil adhesion molecule CD11/18β (Kubes et al. 1991).

Significance of the cytoprotective effect of NO has been examined in several studies. Infusion of exogenous NO donors during reperfusion decreased the extent of neutrophil adherence and migration (Lefer et al. 1993; Kurose et al. 1994b; Weyrich et al. 1992), platelet-leukocyte aggregation and mast cell degranulation (Kurose et al. 1994b). As a consequence, NO donors decreased endothelial leakage (Kurose et al. 1994b) and extent of myocardial infarction and impairment of left ventricular function in dogs (Lefer et al. 1993). Therefore, the interaction between endothelium and leukocytes is of great significance in the development of reperfusion injury.

1.5.2 Potential mechanisms of reperfusion injury

1.5.2.1 Oxidative stress

The term oxidative stress refers to increased concentration of oxidants relative to antioxidants in an intracellular and/or extracellular environment. Oxidative stress has been demonstrated during myocardial ischaemia and in particularly during reperfusion. It appears that oxidative stress plays a pivotal role in the pathogenesis of reperfusion injury. Supporting data to this effect come from a variety of models in both animals and humans.

Garlick *et al* (Garlick et al. 1987) used a Langendorff perfused rat model of 15 minutes total, global ischaemia followed by anoxic and/or aerobic reperfusion. Utilising the spin-trap N-tert-butyl-α-phenylnitrone (PBN) (see 2.1.1.2) a burst of PBN-spin adduct formation was seen soon after aerobic reperfusion, with a peak formation at 4 minutes after onset of reperfusion. If the heart was reperfused with an anoxic buffer for 10 minutes then an oxygenated buffer, a significant free radical burst occurred only after oxygenated reperfusion. The electron spin resonance (ESR) signals were consistent with spin-trapping by PBN of either carbon-centred species or an alkoxyl species. It was concluded that these were secondary radical products of the initial free radical reactions with lipid membranes. The initial short-lived radicals were considered to be short-lived oxygen-centred species, such as superoxide or the hydroxyl radical. Furthermore, increasing PBN concentration by scavenging free radicals correlated with an extension of the duration of ischaemia tolerated before the heart became vulnerable to reperfusion-induced ventricular fibrillation (Hearse and Tosaki, 1987) and an overall decreased frequency of these arrhythmias (Hearse and Tosaki, 1987; Tosaki and Braquet, 1990). However, this was a blood-less model with high xanthine oxidoreductase activity . Other species, especially humans, should be studied in the presence of blood reperfusion, in view of the likely importance of neutrophils as sources of radical species and

a paucity of myocardial xanthine oxidase.

Bolli *et al* (Bolli et al. 1988a) examined free radical generation associated with 15 minutes of regional ischaemia followed by 3 hours of reperfusion in the open-chest dog model. PBN adduct formation increased slightly during the ischaemic period, but a burst of free radical activity was seen within 1 minute of reperfusion. This burst abated after approximately 10 minutes, but persistent release of PBN adduct continued for beyond 3 hours of reperfusion. Again, the ESR signals were characteristic of oxygen- and carbon-centred adducts. There was an inverse linear relationship between cumulative PBN adduct release and the extent of collateral blood flow as determined by radioactive microspheres. As with the rat model, PBN infusion was associated with reduced impact of reperfusion. In this case PBN infusion was associated with better myocardial contractility in the reperfusion period as compared dogs not receiving PBN. Hence, a burst, followed by sustained release of free radicals occurs in dogs when reperfused after ischaemia with blood. Furthermore, scavenging of free radicals in the reperfusion period and less intense ischaemia were both associated with less myocardial stunning.

Spin trap agents cannot be utilised *in vivo* in man, because of toxicity. However, in a single case study of coronary sinus blood withdrawal and addition of a spin trap *ex vivo* was carried out during immediate PTCA for acute myocardial infarction to document the presence of a free radical burst with reperfusion (Grech et al. 1993). PBN was again utilised as the spin trap. The patient had an increased ESR signal amplitude prior to reperfusion as compared non-ischaemic controls, but within minutes of reperfusion there was an approximately 3 fold increase in ESR signal amplitude. This free radical burst diminished dramatically with the onset of acute spontaneous reocclusion 1 hour after the initial reperfusion, but another burst was noted on restoration of

arterial patency. The burst of free radical generation gradually abated over 4 hours after the second reperfusion, but then gradually increased to plateau over the next 24 hours, persisting beyond this time. This case report parallels the time course of changes seen in animal models (Bolli et al. 1988a).

To further explore the overall role of free radicals, and the individual role of different radical species in the pathogenesis of reperfusion injury, a variety of studies determining the effect of the introduction of an oxidant flux to the myocardium have been reported. The main limitation to these studies relates to the difficulty of establishing that the concentrations of oxidants generated are similar to those seen during ischaemia followed by reperfusion (Goldhaber and Weiss, 1992).

Goldhaber *et al* (Goldhaber et al. 1989) exposed isolated perfused rabbit intraventricular septa to either hydrogen peroxide or superoxide for 30 minutes and demonstrated progressive cellular loss of potassium ions, action potential duration shortening leading to inexcitability, loss of developed systolic force, progressive rise in diastolic force and depressed high-energy phosphate concentrations. These changes are similar to those seen during myocardial ischaemia. Furthermore, a 4 minute exposure of isolated perfused rat hearts to the hydroxyl radical was associated with a rapid rise in intracellular calcium concentration, which preceded major reductions in tissue ATP and PC concentrations (Corretti et al. 1991). Exposure of isolated hearts and myocytes to oxidant fluxes produced arrhythmias and electrophysiological changes similar to those seen with reperfusion after short periods of ischaemia (Pallandi et al. 1987; Barrington et al. 1988; Hearse et al. 1989).

In a study involving isolated myocytes exposed to a superoxide flux, there was an increase in

hypercontracted cells within 5 minutes, followed by a progressive increase in the number of round cells. Biochemically, lipid peroxidation progressively increased between 10 and 20 minutes as did intracellular sodium and calcium concentrations. Cellular ATP concentrations were significantly lower and AMP concentrations higher after 20 minutes (Kirshenbaum et al. 1992). Therefore oxidative stress may contribute to the development of calcium overload, high energy phosphate depletion and arrhythmias seen in reperfusion injury.

As mentioned earlier, the ischaemic period prior to reperfusion has great influence on the extent of reperfusion injury. Of note, the ischaemic period *per se* does not appear to be associated with significant free radical activity (Bolli et al. 1988a). A possible explanation for this relationship is that antioxidant mechanisms within the myocardium are progressively depleted with increasingly intensive and prolonged ischaemia, resulting in an increasing inability to protect the myocardium from the oxidative effects of a return to aerobic metabolism and the possible influx of free radicals after reperfusion. The mechanism by which such antioxidant mechanisms may be depleted remains unclear.

Ferrari *et al* (Ferrari et al. 1992) examined the effect of myocardial ischaemia and reperfusion on the intracellular concentrations of a variety of endogenous antioxidants. In the isolated perfused rabbit heart mitochondrial MnSOD concentration progressively decreased to 50% of its pre-ischaemic concentration after 90 minutes of low flow global ischaemia, while cytosolic CuZnSOD, glutathione peroxidase and glutathione reductase concentrations remained unchanged. With reperfusion, no further change in these enzymes concentrations were seen. Myocardial tissue GSH concentrations progressively fell during myocardial ischaemia without an equivalent rise in tissue GSSG. With reperfusion, tissue GSH concentrations continued to be depleted and were associated

with a significant rise in tissue GSSG concentrations, markedly decreasing tissue GSH:GSSG. In contrast to tissue changes in glutathione, release of GSH and GSSG remained low and unchanged during ischaemia. With reperfusion there was a significant rise in GSH release and a greater rise in GSSG release, resulting in a rise in GSH:GSSG. However, the depletion of mitochondrial MnSOD during myocardial ischaemia and reperfusion has been disputed by Subramanian *et al* (Subramanian et al. 1993). They demonstrated no change in mitochondrial MnSOD concentration or cytosolic CuZnSOD concentration during increasing periods of global ischaemia followed by reperfusion in an isolated working rat heart model.

In isolated perfused rat hearts depleted of myocardial glutathione, recovery of systolic function on reperfusion after global ischaemia was impaired as compared to normal hearts (Chatham et al. 1988; Blaustein et al. 1989). In addition, the rise in perfusion pressure and chamber stiffness on reperfusion was greater in glutathione-depleted rat hearts. This effect was ameliorated by supplementing the buffer with glutathione (Blaustein et al. 1989). However, no significant metabolic consequences on purine metabolism were demonstrated in glutathione-depleted rats (Chatham et al. 1988). These studies indicate that not only is the redox state of gluathione a useful marker of oxidative stress, but an important protective mechanism against reperfusion injury.

The effect of myocardial ischaemia and reperfusion on endogenous antioxidant activity and other indirect biochemical markers of free radical activity have also been assessed in humans during the setting of cardioplegia for cardiac surgery. The results of several studies, summarised in Table 1.8, vary somewhat due to variation in surgical technique, cardioplegic solutions utilised, biochemical parameters measured and methodology for their assay (see 2.1). Furthermore, it is not always possible to clearly determine the source of oxidative stress. Many of the studies suggest

that there is significant oxidative stress in many organs during cardiopulmonary bypass surgery, not only the heart. For example, the study reported by Roysten *et al* (Royston et al. 1986) observed a significant increase in peripheral thiobarbituric acid reactive substances (TBARS) concentration after removal of the aortic cross-clamp associated with a significant sequestration of neutrophils into the lungs at this time. Therefore, without an assessment of transcoronary gradients of plasma markers of oxidative stress, the source of such markers is not always apparent. This was demonstrated by Coghlan *et al* (Coghlan et al. 1993) who showed a significant increase in peripheral plasma TBARS concentration after final removal of the aortic cross-clamp, with no associated increase in the transcoronary gradient of plasma TBARS concentration suggesting that the most significant site of oxidative stress at this time was not the heart.

Despite the variation in experimental and operative methodology , the trend appears to confirm the animal data. Overall, these studies suggest that there is both a depletion of endogenous antioxidants during peripheral and myocardial ischaemia and an inability of these antioxidants to adequately protect the myocardium against the oxidative load exposed to the myocardium on reperfusion.

Evidence for oxidative stress during evolving acute myocardial infarction in humans is also available from many studies, and are summarised in Table 1.9. Earlier studies did not relate changes in lipid peroxidation products with reperfusion. In later studies there was a significant rise in the concentration of a variety of biochemical markers of oxidative stress after reperfusion as compared to no change in patients whose infarct related artery remained occluded. These changes reflect oxidative stress at reperfusion as in the animal models. Only 1 study noted a correlation between extent of oxidative stress and cardiac dysfunction at reperfusion (Davies et al. 1993).

TABLE 1.8 Extent of oxidative stress during coronary artery bypass grafting

Reference	No. of Patients	Biochemical marker	Results
Cavarocchi et al. 1986b	15	1. Activated complement 2. Neutrophil count 3. H_2O_2	1. Peripheral plasma C3a increased during and after cardiopulmonary bypass. C4a increased at reperfusion after protamine injection. No change on C5a. 2. Increased circulating neutrophils and band forms after reperfusion. 3. Peripheral plasma H_2O_2 increased after reperfusion.
Chenoweth et al. 1981	15	1. Activated complement 2. Neutrophil count	1. Increased C3a, but not C5a during and after cardiopulmonary bypass. 2. Significant neutrophilia during bypass. 3. Incubation of blood with the nylon-mesh liner of the bubble oxygenator and vigorous oxygenation of blood activates complement.
Royston et al. 1986	18	1. Plasma TBARS 2. Transpulmonary neutrophil count	1. Increased peripheral TBARS after removal of aortic cross-clamp. 2. Sequestration of neutrophils into lungs after removal of aortic cross-clamp.
Weisel et al. 1989	10	1. Plasma conjugated dienes 2. Tissue α-tocopherol	1. Conjugated diene release into coronary sinus on reperfusion after cross-clamping. 2. α-tocopherol unchanged during ischaemia and depleted after 30 minutes reperfusion.
Ferrari et al. 1990	22	Plasma GSH:GSSG	1. Increase in GSH and GSSG release into coronary sinus on reperfusion after cross-clamping. 2. Changes more profound when ischaemic period >30 minutes. 3. Decreased cardiac index and LV stroke work index up to 4 hours after cross-clamping if ischaemic period >30 minutes.
Bical et al. 1991	33	1. Tissue TBARS 2. Tissue adenine nucleotides	1. No significant change in tissue TBARS with ischaemia or reperfusion. 2. Decrease in ATP at end of cross-clamping and further decrease after 30 minutes reperfusion.
Prasad et al. 1992	21	1. TBARS 2. Luminol chemiluminescence of PMN	1. Increased peripheral TBARS and PMN free radical release just prior to cross-clamping and 24 hours post-operatively. 2. Decreased stroke volume after closing chest and 24 hours post-operatively.
Lazzarino et al. 1994	15	1. MDA 2. Uric acid	1. Increased myocardial release of MDA and uric acid when blood cardioplegia is delivered intermittently into the aorta and prepared grafts during cross-clamping period, and post-operatively. 2. No association between LV functional recovery and extent of myocardial MDA release.

Reference	No. of patients	Biochemical marker	Results
Davies et al. 1990b	15	1. TBARS 2. Conjugated dienes	1. Significant increase in peripheral TBARS and conjugated dienes after reperfusion, with little change in CS concentrations.
Ferreira et al. 1988	6	1. H_2O_2 chemiluminescence 2. Succinic dehydrogenase	1.Increased myocardial H_2O_2 chemiluminescence and decreased succinic dehydrogenase activity 10 minutes after reperfusion as compared pre-ischaemia. 2. Electron microscopic evidence of mitochondrial swelling and myofibrillar disorganisation after reperfusion.
Coghlan et al. 1993	10	1. TBARS 2. α-Tocopherol	1. Increased release of peripheral but not myocardial TBARS after final removal of aortic cross-clamp. 2. Increased myocardial release of α-Tocopherol after final removal of aortic cross-clamp.

Legend: TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; LV, left ventricle; PMN, polymorphonuclear leukocytes; GSH, reduced glutathione; GSSG, oxidised glutathione.

A third clinical setting where evidence of oxidative stress has been sought is that of myocardial ischaemia. Although no definitive evidence for myocardial stunning is available in humans, biochemical evidence suggests oxidative stress occurs during myocardial ischaemia. These studies are presented in Table 1.10. No correlation with the extent of ischaemia was demonstrated in the study by Oldroyd *et al* (Oldroyd et al. 1992), whereas the other studies did not attempt to correlate these phenomena with haemodynamics. Importantly when PTCA was the means of inducing myocardial ischaemia, the source of lipid peroxides may have been from a disrupted atherosclerotic plaque rather than myocardium (Oldroyd et al. 1992). However, it may be that these biochemical changes are a sensitive indicator of oxidative stress, even if that stress is not intense enough to be associated with an adverse effect on function. Furthermore, as in other studies, the release of lipid peroxide products occurred in the recovery or "reperfusion period" consistent with previous data.

From the large amount of data available it therefore appears that oxidative stress plays a pivotal role in the pathogenesis of reperfusion injury. By mechanisms unexplained, endogenous antioxidant systems are progressively depleted during myocardial ischaemia and reperfusion is associated with both a further decrease in these systems and a probable influx of oxidants. These oxidants then oxidise cellular membranes, proteins and even DNA, resulting in reperfusion injury.

1.5.2.2 Sources of oxidants

The sources of the free radical burst after the onset of reperfusion are probably multiple and related to a return to aerobic metabolism in a situation of impaired endogenous antioxidant activity, a modification of myocyte and endothelial cellular function resulting in additional free radical release and an introduction of free radical sources in the restored oxygenated perfusate.

TABLE 1.9 Oxidative stress during acute myocardial infarction in humans

Reference	No. Patients	Biochemical Marker	Therapy	Results
Dousset et al. 1983	70	TBARS	nil	1. Elevated peripheral venous [TBARS] day 1 AMI compared to normals 2. Rising peripheral venous [TBARS] over 3 days post-AMI
Rao and Mueller, 1983	10	TBARS	nil	Myocardial release of MDA from CS 5 hours post-AMI onset as compared no release in normals.
Aznar et al. 1983	26	TBARS	nil	Rising peripheral venous [TBARS] post- AMI peaking day 6-8 as compared to normals.
Davies et al. 1990a	50	1. TBARS 2. conjugated dienes	thrombolysis	1.Rise in peripheral venous [TBARS] 1 hour post-SK in patients who reperfuse and fall in patients with occluded artery. 2. No change in conjugated dienes at 1 hour post-SK.
Loeper et al. 1991	34	1. TBARS 2. SOD 3. GSH.Px	nil	1. Elevated peripheral venous [TBARS] day 2 & 12 post-AMI compared to normals. 2. Higher [TBARS] in patients with larger AMI. 3. Lower erythrocyte [SOD] post-AMI compared to normals. 4. Lower erythrocyte [GSH.Px] 12 days post-AMI as compared normals.
Young et al. 1993	67	1. MDA 2. Ascorbate 3. α-tocopherol 4. retinol	thrombolysis	1. In patients reperfused at 90 minutes: a) Rise in peripheral venous [MDA] during first 90 minutes post-rTPA. b) Fall in peripheral [α-tocopherol] during first 90 minutes post-rTPA c) Fall in peripheral venous [retinol] for 6 hours post-rTPA d) Fall in peripheral venous [ascorbate] at 6 and 24 hours post-rTPA. 2. No change in patients with occluded artery at 90 minutes.
Giardina et al. 1993	20	1. MDA 2. oxypurines	thrombolysis	1. In patients who reperfuse: a) Progressive rise in peripheral venous [MDA] over 24 hours. b) Rise in peripheral venous [xanthine] over 6 hours post-AMI. c) Progressive rise in peripheral venous [adenosine] over 24 hours post-AMI. 2. elevated plasma [MDA] in AMI patients pre-thrombolysis as compared normals. 3. No change in peripheral venous [MDA] or [oxypurines] in patients with occluded artery

Reference	No. of patients	Biochemical marker	Therapy	Results
Davies et al. 1993	72	1. TBARS 2. Total thiols	thrombolysis	1. In patients who reperfuse: a) Rise in peripheral venous [TBARS] 2 hours post-SK. b) Fall in peripheral venous [total thiols] at 2 hours post-SK. c) Inverse correlation between LV ejection fraction and [TBARS]. d) Direct correlation between LV ejection fraction and [total thiols]. 2. In patients with occluded artery: a) Fall in peripheral venous [TBARS] 2 hours post-SK. b) No change in peripheral venous [total thiols].
Grech et al. 1994	9	1. conjugated dienes 2. TBARS	primary PTCA	1. Rise in coronary sinus conjugated dienes from 2-30 minutes after reperfusion. 2. No change in coronary sinus [TBARS] over 24 hours post reperfusion.

Legend: AMI, acute myocardial infarction; CS, coronary sinus; GSH.Px, glutathione peroxidase; MDA, malondialdehyde; No., number; PTCA, percutaneous transluminal coronary angioplasty; SK, streptokinase; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

TABLE 1.10 Oxidative stress associated with myocardial ischaemia in the absence of infarction

Reference	No. of Patients	Mechanism of ischaemia induction	Biochemical marker	Results
Roberts et al. 1990	10	PTCA to LAD	TBARS	Transient rise in CS TBARS immediately following balloon deflation, returning to baseline after 1 minute.
Oldroyd et al. 1990	26	pacing <180 bpm	TBARS	Myocardial TBARS release 5 minutes after ischaemia when lactate released.
De Scheerder et al. 1991	10	PTCA to LAD (x4 inflations)	1.Oxypurines 2.TBARS	1. Transient elevation CS hypoxanthine between balloon inflations. 2. Progressive elevation of CS uric acid, peaking at 5 minutes after 4th inflation. 3. Progressive rise in CS TBARS peaking at 5 minutes recovery. 3.All parameters returning to baseline by 15 minutes recovery.
Oldroyd et al. 1992	15	PTCA to LAD	1. Lipid peroxides	1.Increased lipid peroxides in CS after each balloon inflation. 2.No correlation with CS lactate or S-T segment elevation.
McMurray et al. 1992	25	Unstable angina pectoris	1.TBARS 2.Total thiols	1.Elevated peripheral venous TBARS as compared stable angina and normals. 2.Decreased peripheral venous total thiols as compared normals.

Legend: CS, coronary sinus; LAD, left anterior descending coronary artery; No., number; PTCA, percutaneous transluminal coronary angioplasty; TBARS, thiobarbituric acid reactive substances.

Possible sources are presented in Table 1.6 (see 1.4.3), although, the extent of involvement by each putative source is unclear. In the isolated rat heart perfused with a superoxide flux, the myocyte mitochondrium was the most vulnerable organelle to structural damage on histological examination after 5 and 10 minutes of exposure to superoxide, whereas the endothelium demonstrated only sparse damage (Hegstad et al. 1994a). It is likely mitochondrial function would deteriorate in association with structural damage. Mitochondrial function was examined in a Langendorff-perfused rabbit heart (Ambrosio et al. 1993) and demonstrated to be an imporant source of free radical species during reperfusion after global ischaemia. Inhibition of mitochondrial respiration at reperfusion was associated with a significant reduction in the extent of free radical release. However, although these studies demonstrated the mitochondia as an important soucre of radical species during oxidative stress, and that the mitochondria are themselves vulnerable to damage by these radical species, the extent of other sources of oxidants *in vivo* remains uncertain. Of importance, there are likely to be inter-species differences (de Jong et al. 1990). Furthermore, it is possible that the extent of the preceding ischaemia may influence the subsequent source of free radicals in the reperfusion period. Also, the exact identity of the radical species involved is uncertain. Again, it is possible that several species are primarily involved, setting off a chain reaction for the formation of other free radicals and their products. As it appears that the return of oxygen is essential to the oxidative burst, it is likely that the primary free radical species are oxygen-centred, such as the superoxide and hydroxyl radicals (Garlick et al. 1987).

One possible, but controversial source of superoxide is the xanthine oxidase system (see 1.4.1.3). The xanthine oxidase enzyme system is a plausible source of superoxide. One substrate, hypoxanthine, progressively accumulates during myocardial ischaemia and oxygen, the other necessary substrate, is introduced at reperfusion. The enzyme has been shown in a variety of

animals and organs to be produced in association with raised intracellular calcium concentration during myocardial ischaemia. Therefore, immediately on the reintroduction of oxygen at reperfusion, superoxide formation is possible.

Against this hypothesis, is an uncertainty as to whether the human heart can produce adequate concentrations of this enzyme during myocardial ischaemia. DeJong *et al* (de Jong et al. 1990) found relatively little xanthine oxidase in perfused explanted hearts from cardiac transplant donor recipients. However, these hearts are not necessarily representative all human hearts. Also, the hearts were not made ischaemic prior to the assessment of oxidase activity, so activation of xanthine oxidase was not assessed. Therefore, it remained possible that the enzyme existed in the inactive state. Terada *et al* (Terada et al. 1988) showed that xanthine oxidase can be inactivated by oxygen metabolites. In support of de Jong's findings, however, other studies reported low enzyme activity of xanthine oxidase in the human heart (Grum et al. 1989). Conversely, Jarasch *et al* (Jarasch et al. 1986) demonstrated large amounts of xanthine oxidase in human coronary endothelium and high activity has been demonstrated in autopsy homogenate of human heart (Wajner and Harkness, 1989). Even if there is little xanthine oxidase activity within the human myocardium it may be present in circulating or migrating neutrophils or from other sources in the bloodstream. (Grum et al. 1987). A few studies have obtained evidence of myocardial metabolism of uric acid from hypoxanthine after episodes of myocardial ischaemia followed by reperfusion. The exact source of these substrates and products is unclear, but were associated with malondialdehyde production (De Scheerder et al. 1991; Lazzarino et al. 1994).

Activated neutrophils are likely to be an important source of a variety of free radicals and oxidants during reperfusion after prolonged ischaemia (see 1.4.1.3) (Hansen, 1995). Neutrophils are seen

in the myocardium after prolonged myocardial ischaemia (Engler et al. 1986b), with rapid incremental accumulation within minutes of reperfusion (Engler et al. 1986a). As discussed in 1.5.1.5, activated neutrophils are seen in the "plugged" capillaries in areas of "no reflow" after reperfusion (Kloner et al. 1974a; Johnson et al. 1988), are associated with reperfusion-induced microvascular dysfunction (see 1.5.1.6) and their extravascular emigration closely linked to albumin leakage induced by ischaemia-reperfusion (Kurose et al. 1994a). Neutrophils activated by zymosan have been shown to decrease cardiac contractility and cardiac output in anaesthetised dogs (Prasad et al. 1990). Also, activated neutrophils incrementally impaired recovery of left ventricular developed pressure in isolated Langendorff-perfused rat hearts when infused just before 20 minutes of ischaemia and immediately after reperfusion as compared those only perfused with Krebs buffer (Shandelya et al. 1993). Furthermore, in animal models, depletion of neutrophils or inhibition of neutrophil adhesion to endothelium has been shown to result in decreased reperfusion arrhythmias (Engler et al. 1986a), myocardial stunning (Westlin and Mullane, 1989) and infarct size (Romson et al. 1983; Simpson et al. 1990; Ma et al. 1991). However, there have also been negative studies (Tanaka et al. 1993), especially when the time of ischaemia is short (Bolli, 1993) and insignificant neutrophil accumulation has been demonstrated (Go et al. 1988).

1.5.2.3 Mechanisms of neutrophil activation and extravasation during reperfusion injury. Neutrophils appear to have multiple independent and interactive effects in the pathogenesis of reperfusion injury. As previously discussed, there is extensive evidence for a pathogenetic role for activated neutrophils in the microvascular dysfunction and damage of reperfusion injury (see 1.5.1.6) and as a source of oxidants in other manifestations of reperfusion injury (see 1.4.3 and 1.5.2.2). Furthermore, products other than oxidants released by activated neutrophils contribute to both their direct effect and their interaction with other cells, especially platelets and endothelial

cells.

Several proteolytic enzymes are released via degranulation at the time of neutrophil activation (Weiss, 1989). They include elastase which can hydrolyse many proteins in both plasma and the extracellular matrix (Janoff, 1985) and collagenase which variably cleaves the different collagen types (Weiss and Peppin, 1986). Migration of neutrophils through the vascular endothelium on reperfusion after ischaemia may be dependent on elastase (Zimmerman and Granger, 1990).

In addition, activated neutrophils also release a variety of proinflammatory mediators such as metabolites of arachidonic acid (for example LTB_4 and TXA_2) and PAF (Hansen, 1995). LTB_4 and PAF are potent amplifiers of neutrophil activation, stimulating chemotaxis, adhesion to the endothelium, oxidative metabolism and degranulation. Other leukotrienes, PAF and TXA_2 are coronary vasoconstrictors (Ezra et al. 1983; Feuerstein et al. 1984) and depress left ventricular function (Benveniste et al. 1983). The effects of PAF are likely to be mediated via PAF-induced aggregation and degranulation of platelets (Montrucchio et al. 1989). There is recent evidence to suggest that release of endothelin-1 from microvascular endothelium during ischaemia/reperfusion is a potent stimulator for PAF release from neutrophils (Lopez-Farre et al. 1995).

The extravasation of neutrophils seen after myocardial ischaemia and reperfusion is mediated by a sequence of steps:

(1) rolling of neutrophils along the endothelium,

(2) neutrophil activation, adhesion then cessation of rolling, and lastly

(3) transendothelial migration (Springer, 1994; Adams and Shaw, 1994).

Rolling is mediated by the selectins. L-selectin is constitutively expressed on the surface of

neutrophils but is rapidly shed on activation. E-selectin is expressed on the surface of cytokine-stimulated endothelial cells and P-selectin is stored in platelets and endothelial cells and mobilised to the cell surface in response to a variety of stimuli, including thrombin and oxygen-centred free radicals and reperfusion after myocardial ischaemia(Bevilacqua and Nelson, 1993; Lefer et al. 1994). Firm adhesion of neutrophils is mediated by neutrophil β2 integrins which are heterodimeric glycoproteins possessing a common β2 chain (CD18) and one of three α chains (CD11a, CD11b or CD11c). Neutrophils constitutively express β2 integrins, but there is a transient upregulation of CD11b/CD18 during chemotaxis which is essential for firm attachment to the endothelial surface and subsequent diapedesis (Adams and Shaw, 1994; Springer, 1994). The integrins bind to a counter-receptor on the endothelial surface, namely intercellular adhesion molecule 1 (ICAM-1) (Diamond et al. 1990) which is upregulated by cytokine stimulation (Bevilacqua et al. 1985). ICAM-1 upregulation has also been inferred in the post-ischaemic heart during early reperfusion (Kukielka et al. 1993; Kurose et al. 1994a). Furthermore, NO may have a regulatory role in the expression of endothelial adhesive mechanisms: hence increased neutrophil adhesion after NO synthase inhibition (Niu et al. 1994). The binding of CD11b/CD18 with ICAM-1 potentiates adherence-dependent neutrophil oxidant production (Entman et al. 1990).

In the setting of myocardial ischaemia followed by reperfusion, activation of neutrophils by activated fragments of the complement cascade appears to be of great importance. Prolonged myocardial ischaemia induces complement activation (Rossen et al. 1985; Rossen et al. 1988) which then appears to be essential in subsequent activation of neutrophils after reperfusion (Shandelya et al. 1993). $C1_q$, C5a and C3bi have all been implicated in the various steps involved with neutrophil activation in this setting (Hansen, 1995). Interleukin-8 production by endothelial cells is also an important mediator of neutrophil activation. After stimulation with tissue necrosis

factor or interleukin-1, interleukin-8 has been shown to be a potent neutrophil chemoattractant, inducing rapid shedding of neutrophil L-selectin, upregulation of neutrophil integrins and transendothelial neutrophil migration (Huber et al. 1991; Strieter et al. 1989; Wozniak et al. 1993). Therefore during reperfusion after myocardial ischaemia there are many putative interacting factors, cytokines, leukotrienes and other molecules that both activate neutrophils and amplify or modify their response to this activation.

Correlations between neutrophil activation and myocardial ischaemia-reperfusion in the clinical setting are few. De Servi *et al* (de Servi et al. 1990) demonstrated that immediately following PTCA there was increased coronary sinus plasma elastase concentration, and post-PTCA coronary sinus-harvested neutrophils demonstrated decreased release of superoxide and elastase after stimulation *ex vivo*. These results suggest that the neutrophils had undergone activation and degranulation within the myocardial vascular bed either as a response to endothelial trauma related to balloon dilatation or secondary to myocardial ischaemia-reperfusion. In the clinical setting of coronary artery bypass surgery, Fabiani *et al* (Fabiani et al. 1993) showed a decrease in coronary sinus neutrophil count in the early reperfusion period, suggesting trapping of neutrophils within the myocardial vascular bed. Furthermore, there was an increased myocardial release of elastase and lactoferrin during reperfusion after cross-clamping. This was associated with increased myocardial MDA release and increased chromosomal aberrations in blood cells harvested from the coronary sinus after reperfusion. Therefore, oxidative stress was associated with neutrophil activation and degranulation at some time during cross-clamping and reperfusion. Bell *et al* (Bell et al. 1990) studied the time course of peripheral venous plasma elastase and conjugated diene concentrations in a small number of patients presenting with acute myocardial infarction. All patients had elevated plasma concentrations of neutrophils, elastase and conjugated dienes as

compared normal controls. Patients non-randomly treated with thrombolytic therapy demonstrated an earlier peak (8 hours from the onset of symptoms) in plasma elastase concentration than those not treated (peak at 40 hours). However, no such difference was seen for plasma conjugated diene concentrations, although the first blood samples were not taken for 8 hours and no attempt was made to determine the success or failure of reperfusion. Quantitative myocardial imaging with labelled neutrophils within 18 hours of infarction demonstrated decreased uptake in patients treated with thrombolytic drugs, suggesting no amplification of the inflammatory response by thrombolytic therapy.

1.5.2.4 Calcium overload

The role of calcium in ischaemia-reperfusion is paramount. However, it is unlikely that calcium overload is the primary causative event of reperfusion injury. It is more likely, that its role is secondary to a prior cellular insult, such as lipid membrane damage by free radicals. However, increased intracellular calcium adversely effects cellular metabolism and probably is extremely important in promoting cell death.

On reperfusion of reversibly ischaemic myocytes there is a massive early influx of calcium into the cell, probably through normal physiological channels, as this occurs even in the presence of the intact sarcolemmal membranes (Marban et al. 1990; Poole Wilson et al. 1984; Nayler and Elz, 1986). There appears to be decreased ATP dependent uptake of calcium by the sarcoplasmic reticulum and decreased extrusion from the cell. This may reflect a loss of balance between active calcium uptake via Ca^{++}-ATPase and sequestration via Ca^{++} channels in the sarcoplasmic reticulum. Several studies have shown that both processes are decreased in activity, even after short periods of ischaemia, and persist despite reperfusion (Zucchi et al. 1994; Krause et al. 1989).

Also, decreased ATP stores interfere with trans-sarcolemmal sodium-potassium exchange and decreasing pH increases sodium-hydrogen ion exchange, which raises intracellular sodium. This induces increased sodium-calcium exchange (Braunwald, 1982). Sympathetic stimulation may further contribute to this uncontrolled calcium entry (Virmani et al. 1992; Nayler and Elz, 1986; Sharma et al. 1983b).

Postulated metabolic effects of raised intracellular calcium concentration include activation of enzymes such as phospholipase A_2 (Opie, 1993; Virmani et al. 1992) and proteases. One of the calcium-activated proteases in endothelial cells converts xanthine dehydrogenase to xanthine oxidase which produces superoxide during the metabolism of hypoxanthine and xanthine. These purine substrates accumulate during ischaemia as ATP is degraded (McCord, 1985; Virmani et al. 1992; Werns et al. 1986; Maza and Frishman, 1988). Calcium accelerates the depletion of ATP stores through calcium-activated ATPases. Increased mitochondrial calcium increases the proton load, both depleting electrochemical potential essential for ATP production and inducing ATP-dependent pumps to remove other cations from the mitochondria (Virmani et al. 1992).

The functional effect of calcium overload may be important in the pathogenesis of myocardial stunning. The decreased amount of calcium stored in the sarcoplasmic reticulum could diminish contractile protein activation through attenuated calcium release during systole (Krause et al. 1989). This excitation-contraction uncoupling has been demonstrated by Hanich *et al* (Hanich et al. 1993) in open chest dogs with 15 minutes of coronary occlusion then reperfusion. Epicardial electrocardiograms showed recovery to baseline at approximately 20 minutes with persistent systolic thinning and dyskinesis in the same regions.

1.6 Limitation of myocardial reperfusion injury with antioxidant drugs

1.6.1 Potential anti-oxidant drugs in animal models of reperfusion injury

Many studies have assessed the effect of a variety of antioxidant agents on the different manifestations of reperfusion injury in cellular and animal models. Although the results are not in complete concordance, they generally support the hypothesis that reperfusion injury exists in many models of myocardial ischaemia and reperfusion, and that antioxidant agents have a role in ameliorating the biochemical sequelae, cardiac haemodynamic effects and extent of myocardial necrosis attributable to such injury. A summary of some of the studies is compiled in Table 1.11.

1.6.2 Clinical trials of antioxidant therapy for potential reperfusion injury in humans

Clinical investigations of myocardial ischaemia and reperfusion which have been utilised for the assessment of antioxidant intervention are cardioplegia for coronary artery bypass grafting and reperfusion for the treatment of acute myocardial infarction. Apart from their putative antioxidant effects, some of the agents utilised have multiple pharmacological actions which may effect interpretation of the results. Furthermore, study design varies greatly with variable quality protocols and data presentation. Despite these significant limitations, it is important to review the available data in an attempt to determine any trends, either adverse or beneficial.

1.6.2.1 Antioxidant therapy during cardioplegia

Allopurinol, a competitive xanthine oxidase antagonist, has been assessed in several trials involving cardioplegia for cardiac surgery. Six randomised placebo-controlled studies utilised oral allopurinol prior to cardioplegia (Adachi et al. 1979; England et al. 1986; Johnson et al. 1991; Coghlan et al. 1994; Rashid and William Olsson, 1991; Tabayashi et al. 1991). These studies reported improved early post-operative cardiac performance (Johnson et al. 1991; Coghlan et al.

TABLE 1.11 Antioxidant drugs assessed in animal models of myocardial ischaemia-reperfusion

Antioxidant	reference	Model	Ischaemia+ reperfusion time	Timing of agent	Cardiac parameter assessed	Effect
			ENZYMES			
SOD	Neya et al. 1993	dog LAD occ	2 hrs + 1 hr	5 min before reflow	LV function	+
	Villari et al. 1993	rabbit Cx occ	30 min + 5.5 hr	at reflow	infarct size	+
	Koerner et al. 1991	rabbit Cx occ	15 min + 3 hr	from before ischaemia	LV function	-
	Werns et al. 1985	dog Cx occ	90 min + 6 hr	before ischaemia to 15 min rep	infarct size	+
	Johnson et al. 1990	cat LAD occ	1.5 hr + 4.5 hr	from 30 min ischaemia	infarct size	-
	Werns et al. 1988	dog LAD occ	90 min + 6-24 hr	15 min before occ to 15 min after rep	a) infarct size b) PMN accumulation	a) + b) -
	Naslund et al. 1990	pig LAD occ	a) 30 min + 24 hr b) 60 min + 24 hr c) 90 min + 24 hr	just before rep for 1 hr	infarct size	a) + b) + c) -
catalase	Werns et al. 1985	dog Cx occ	90 min + 6 hr	before ischaemia to 15 min rep	infarct size	-
SOD + catalase	Gross et al. 1986	dog LAD occ	15 min + 3 hr	from before ischaemia	LV function	
	Jolly et al. 1984	dog Cx occ	90 min + 24 hr	from before ischaemia	infarct size	+
	Koerner et al. 1991	rabbit Cx occ	15 min + 3 hr	from before ischaemia	LV function	+
	Przyklenk, Kloner, 1986	dog LAD occ	15 min + 3 hr	from before ischaemia	a) LV function b) ATP stores	a) + b) -
	Myers et al. 1985	dog LAD occ	15 min + 2 hr	1 hr from 15 min before ischaemia	LV function	+
	Richard et al. 1988	dog Cx occ	90 min + 1 hr	1 hr from 25 min before rep	infarct size	-
Peroxidase	Menasche et al. 1986	rat Langendorff global + cardioplegic solution	a) 90 min + 45 min	a) initial 2 min of ischaemia	LV function	+

Antioxidant	reference	Model	Ischaemia+ reperfusion time	Timing of agent	Cardiac parameter assessed	Effect
XANTHINE OXIDASE ANTAGONISTS						
Allopurinol	Headrick et al. 1990	rat Langendorff global	15 min + 20 min	from before ischaemia	a) LV function b) arrhythmias c) myocardial [high energy phosphate]	a) + b) + c) -
	Charlat et al. 1987	dog LAD occ	15 min + 4 hr	from before ischaemia	LV function	+
	Reimer & Jennings, 1985	dog Cx occ	40 min + 4 days	30 min before ischaemia to 40 min after	infarct size	-
Oxypurinol	Richard et al. 1988	dog Cx occ	90 min + 1 hr	bolus 25 min before rep	infarct size	-
MANNITOL						
	Bernier & Hearse, 1988	rat Langendorff LAD occ	10 min + 3 min	from before ischaemia	arrhythmia	+
METAL ION SEQUESTRATION						
Desferriox-amine	Bolli et al. 1987a	dog LAD occ	15 min + 4 hr	from before ischaemia	LV function	+
VITAMIN						
U74006F (α-tocopherol analogue)	Ovize et al. 1991	dog LAD occ	2 hr + 6 hr	from 1 hr after ischaemia	a) infarct size b) limit plasma conjugated dienes	a) - b) +
ubiquinol	Atar et al. 1993	pig LAD occ	8 min + 2 hr	chronic oral pretreatment	LV function	+
PMN ACTIVATION INHIBITORS						
Anti-CD 18	Tanaka et al. 1993	dog Cx occ	90 min + 3 hr	bolus before ischaemia & 30 min after rep	a) PMN accumulation b) limit no reflow c) infarct size	a) + b) + c) -
PAF receptor antagonist (CV-3988)	Sawa et al. 1994	pig global + cardioplegic solution	60 min + 60 min	from onset of rep for 15 min	a) MDA in CS b) tissue [ATP] c) LV function d) spontaneous defibrillation	a) + b) + c) + d) +

Antioxidant	reference	Model	Ischaemia+ reperfusion time	Timing of agent	Cardiac parameter assessed	Effect
			SULPHYDRYLS			
captopril	Miki et al. 1993	rabbit Cx occ	30 min + 72 hr	bolus before ischaemia	infarct size	-
	Westlin & Mullane, 1988	dog LAD occ	15 min + 3 hr	a) bolus before ischaemia b) bolus 2 min before rep	LV function	a) + b) +
	Koerner et al. 1991	rabbit Cx occ	15 min + 3 hr	bolus 5 min before rep & 60 min after	LV function	+
N-2-mercapto-propionyl glycine	Myers et al. 1986	dog LAD occ	15 min + 4 hr	from before ischaemia	LV function	+
	Koerner et al. 1991	rabbit Cx occ	15 min + 3 hr	bolus 5 min before rep & 60 min after	LV function	+
	Horwitz et al. 1994	dog LAD occ	90 min + 48 hr	a) 15 min before rep to 4 hr after b) 30 min to 4 hr after rep c) 15 min before rep to 1 hr after	infarct size	a) + b) + c) -
dimethyl-thiourea	Bolli et al. 1987b	dog LAD occ	15 min + 4 hr	before ischaemia to end of ischaemia	LV function	+
mono (2-mercapto-ethanesulph-onate)	Cargnoni et al. 1992	rabbit Langendorff global	60 min + 30 min	from before ischaemia	a) LV function b) infarct size c) maintenance of ATP stores d) limit GSSG release	a) + b) + c) + d) +

Antioxidant	reference	Model	Ischaemia+ reperfusion time	Timing of agent	Cardiac parameter assessed	Effect
			SULPHYDRYLS			
NAC	Forman et al. 1988	dog LAD occ	90 min + 24 hr	60 min before rep to 3 hr after	a) infarct size b) LV function c) limit myocardial oxidative stress	a) - b) + c) -
	Ceconi et al. 1988	rabbit Langendorff global	60 min + 30 min	a) from before ischaemia b) from reperfusion	1) infarct size 2) LV function 3) limit oxidative stress 4) mitochondrial function	1a) + b) - 2a) + b) - 3a) + b) - 4a) + b) -
	Kingma & Rouleau, 1989	rabbit Cx occ	45 min + 3 hr	from before ischaemia to end ischaemia	infarct size	-
	Sochman et al. 1990	dog LAD occ	2 hr + 2 hr	from 3 min before rep to 30 min after	a) infarct size b) arrhythmia	a) + b) +
	Tang et al. 1991	rat working-heart LAD occ	20 min + 30 min	from 10 min before and throughout	heart rate x aortic pressure	+
	Qiu et al. 1990	rat Langendorff LAD occ	a) 10 min + 10 min b) 30 min + 20 min	from before ischaemia	1) arrhythmia 2) LV function	1a) + b) + 2b) +
	Menasche et al. 1992	rat Langendorff cold cardio-plegic arrest	3 hr + 1 hr	in cardio-plegic solution	LV function	+

Legend: Cx, circumflex coronary artery; hr, hour; LAD, left anterior descending coronary artery; LV, left ventricle; min, minute; occ, occlusion; PMN, polymorphonuclear leukocyte, rep, reperfusion.

1994; Rashid and William Olsson, 1991), less perioperative infarction (Rashid and William Olsson, 1991; Tabayashi et al. 1991) and arrhythmias (Rashid and William Olsson, 1991; Adachi et al. 1979) in the allopurinol-treated patients. Mortality was significantly lower in the allopurinol treated patients in only one study (Johnson et al. 1991), but overall mortality in this study was quite high in both groups.

Biochemical evidence of oxidative stress was sought in two studies (England et al. 1986; Coghlan et al. 1994). Coghlan *et al* (Coghlan et al. 1994) monitored serial plasma TBARS and α-tocopherol concentrations in arterial and coronary sinus blood in 20 of the 50 randomised patients. Plasma TBARS concentration increased in both arterial and coronary sinus blood after reperfusion with no significant arterocoronary sinus gradient in either group. However, area under the coronary sinus plasma TBARS concentration time curve was significantly lower in the allopurinol-treated patients. Elevation of arterial concentrations of TBARS suggested oxidative stress in other organs. In the absence of any evidence of net myocardial production of TBARS, effects of allopurinol could not be evaluated. Plasma α-tocopherol concentrations showed no significant difference between the two treatment groups. England *et al* (England et al. 1986) demonstrated a decrease in systemic arterial plasma hydrogen peroxide concentration in allopurinol (or mannitol) treated patients immediately after cardiopulmonary bypass as compared to control patients with no correlation to clinical outcome or evaluation of tissue source of hydrogen peroxide.

Fabiani *et al* (Fabiani et al. 1993) utilised allopurinol in the cardioplegic solution in 14 randomised patients undergoing coronary artery bypass grafting. The post-operative course was uneventful in all, but chromosomal aberrations in blood drawn from the coronary sinus 20 minutes after aortic unclamping were significantly decreased in allopurinol-treated patients, suggesting decreased

oxidant-induced DNA damage.

The biochemical effects of supplemental α-tocopherol prior to cardioplegia were assessed by Cavarocchi *et al* (Cavarocchi et al. 1986a) and both α-tocopherol and ascorbic acid by Barta *et al* (Barta et al. 1991), without reference to clinical outcome. Cavarocchi *et al* (Cavarocchi et al. 1986a) reported no significant rise in arterial hydrogen peroxide concentration after removal of the cross-clamp in the 10 α-tocopherol treated patients as compared the significant rise seen in the 20 control patients. This was associated with significantly increased ascorbic acid and decreased α-tocopherol arterial concentrations in the control patients, which was not apparent in the α-tocopherol treated group. Barta *et al* (Barta et al. 1991) demonstrated that the extent of ischaemia as determined by peripheral and myocardial lactate release was the same between treated and control groups. However, plasma TBARS concentration in arterial and peripheral venous blood was significantly lower in the vitamin-treated group during cardioplegia and immediately after aortic declamping. Plasma TBARS concentration in the coronary sinus was significantly lower in the treated group immediately after aortic declamping. Unfortunately, no vascular gradients were derived from these data, although the results suggest lipid peroxidation occurring in both the peripheral and myocardial vascular beds. Furthermore, no corrections for coronary sinus blood flow were made, although some correction was made for the haemodilution associated with crystalloid cardioplegic solutions. Therefore, these two studies infer a beneficial biochemical effect from the use of α-tocopherol either alone or in combination with ascorbic acid, although it is not clear as to whether the amelioration of oxidative stress is within the myocardium or other tissues. Furthermore, there is no information as to clinical correlates of the decreased oxidative stress associated with vitamin supplementation.

One study of 40 patients assessed the effect of 7 days oral pretreatment with ubiquinol prior to cardiac surgery (Chello et al. 1994). Ubiquinol treatment was associated with decreased plasma TBARS, conjugated dienes and creatine kinase concentrations as compared to controls. There was a direct correlation between plasma TBARS and creatine kinase concentrations, suggesting that the measured decrease in oxidative stress resulted in less perioperative infarction. Furthermore, the decrease in oxidative stress related to ubiquinol pretreatment was associated with significantly less ventricular arryhthmias and an overall lower dosage of dopamine required for inotropic support in the post-operative period as compared to placebo-treated patients.

The effect of desferrioxamine administered intravenously and/or in the cardioplegic solution has been assessed in several studies (Menasche et al. 1988; Drossos et al. 1995; Ferreira et al. 1990). Drossos *et al* (Drossos et al. 1995) and Ferreira *et al* (Ferreira et al. 1990) both demonstrated decreased free radical production in myocardium biopsied during the early reperfusion period in desferrioxamine-treated patients as compared to controls. Treatment with desferrioxamine was associated with less severe mitochondrial damage (Ferreira et al. 1990) and decreased myocardial TBARS concentration (Drossos et al. 1995). Menasche *et al* (Menasche et al. 1988) demonstrated decreased *ex vivo* production of superoxide by phorbol myrisate acetate (PMA) and formyl-methionyl-leucyl-phenylalanine (fMLP)-activated neutrophils from venous blood after cardiopulmonary bypass as compared to control patients. They proposed that less "priming" of neutrophils had occurred, secondary to less amplification of the inflammatory response by iron-mediated radical generation in the desferrioxamine treated patients. However, the converse may also be true. The neutrophils of desferrioxamine treated patients may have had a decreased *ex vivo* stimulatory response because neutrophil NADPH oxidase had been partially pre-activated *in vivo*. No assessment of cardiac haemodynamic function was made in any of these studies, although there

were no differences noted between groups as regards perioperative outcome.

Taurine, a normal dietary, sulphur-containing amino acid was infused intravenously prior to cardioplegia in 12 patients in a randomised placebo-controlled study(Milei et al. 1992). Comparisons of myocardial biopsies before and 10 minutes after cross-clamping revealed a significant increase in hydrogen peroxide-initiated chemiluminescence, severely damaged mitochondria and necrotic myocytes in the placebo-treated group. No such increase was observed in the taurine-treated group. Although no haemodynamic measurements were taken, all patients recovered satisfactorily.

Two studies assessing different sulphydryl-containing compounds have been assessed in the setting of cardioplegia. The angiotensin converting enzyme inhibitor captopril was added to the cardioplegic solution in a randomised double blind placebo controlled study of 54 patients having coronary artery bypass grafting without adverse effects in the captopril-treated group (Di Pasquale et al. 1993). Biochemically, there was a decrease in plasma noradrenaline and creatine kinase concentrations from the coronary sinus after reperfusion in the captopril-treated group as compared placebo. Again, no haemodynamic assessment was made during this study, but the biochemical results suggested better cardioplegia as a result of captopril administration. Lastly, a preliminary report of the effect of the thiol-containing agent N-acetylcysteine (NAC) randomised in six patients, was presented by Ferrari *et al* (Ferrari et al. 1991a). NAC was infused intravenously before and after cardioplegia and into the arterial line of the pump during cardiopulmonary bypass. Myocardial release of plasma GSSG after reperfusion was less in NAC-treated patients and was associated with more rapid improvement of cardiac output over the first 12 hours after reperfusion.

From these small studies, allopurinol consistently demonstrated a cytoprotective effect of clinical relevance without adverse effect. Also, biochemical and clinical data concerning ubiqunol, taurine, captopril and NAC are promising but somewhat limited at this stage. The clinical relevance of the decreased oxidative stress associated with Vitamin C and E supplementation and desferrioxamine is not clear from these studies.

1.6.2.2 Antioxidant therapy during acute myocardial infarction

Superoxide dismutase (SOD) has been widely utilised in animal models of ischaemia-reperfusion (See Table 1.11). A human recombinant form of SOD is available and has been assessed in the setting of reperfusion for the treatment of acute myocardial infarction. SOD is unable to enter cells, limiting its intracellular availability. Thus, if a large amount of oxidative stress is derived from intracellular sources, SOD cannot afford cytoprotection. Secondly, as a catalyst of superoxide dismutation, producing hydrogen peroxide, it does not limit oxidant damage by this product or by other types of non-oxygen-centred radical species. Therefore, its specificity to oxygen-centred radical species may limit its clinical effect.

In a study reported by Murohara *et al* (Murohara et al. 1991) 34 patients presenting with evolving acute anterior myocardial infarction of less than 6 hours' duration treated with intracoronary rTPA or urokinase, were randomised to receive intravenous SOD as a bolus prior to thrombolysis followed by a 2 hour infusion or just thrombolysis. No adverse events were attributable to SOD. There was a trend towards less non-sustained ventricular tachycardia and significantly less ventricular premature beats per hour over the first 11 hours after reperfusion in the SOD-treated group. Not surprisingly, given the small size of this study, there was no difference in ejection fraction between the 2 groups at 3 to 4 weeks although ejection fraction was not assessed early

after reperfusion in an attempt to determine the extent of myocardial stunning in the 2 groups. No biochemical assessment of oxidative stress was sought in this study. A similar lack of incremental myocardial salvage, as assessed by paired early and late left ventriculograms was also reported in a larger prospective placebo-controlled trial of 120 patients with evolving acute myocardial infarction treated with immediate PTCA and a 60 minutes intravenous infusion of human recombinant SOD (Flaherty et al. 1994). Furthermore, there was no significant difference between groups as regards early reperfusion arrhythmias. These results do not support a beneficial role of SOD in human myocardial infarction. Apart from the proposed limitations already discussed, another possible reason for failure to improve myocardial salvage is the short duration of SOD infusion, especially in the light of the evidence that free radical activity is increased in the reperfused human heart for over 24 hours (Grech et al. 1993) and that SOD has an elimination half-life of less than 30 minutes.

Two studies (Forman et al. 1991; Wall et al. 1994) have utilised the perfluorochemical Fluosol at the time of reperfusion for evolving acute myocardial infarction. These small particle size, low viscosity and high oxygen-carrying capacity agents have potent anti-neutrophil effects, inhibiting neutrophil activation. In a pilot study of 12 patients with anterior infarction reported by Forman *et al* (Forman et al. 1991) a 30 minute intracoronary infusion of Fluosol after primary PTCA showed evidence of increased myocardial salvage as compared those treated with PTCA alone. Wall *et al* (Wall et al. 1994) subsequently reported a larger prospectively randomised open-labelled study of rapid intravenous infusion of 15 mL/kg Fluosol in combination with intravenous rTPA and atenolol in 430 patients presenting with evolving acute myocardial infarction. There was no evidence of myocardial salvage, as determined by no difference in infarct size on thallium imaging at 7.2. ± 1.5. days or left ventricular ejection fraction at 8.6 ± 2 days between patients

receiving Fluosol or not. Clinically, Fluosol-treated patients had a higher incidence of non-fatal cardiac failure and decreased recurrent ischaemia. The authors of this study believe that more effective early delivery of the drug, possibly in association with immediate PTCA as a means of recanalisation may be necessary to determine its clinical potential.

Captopril has also been assessed in this setting of thrombolysis for acute myocardial infarction (Di Pasquale et al. 1990; Nabel et al. 1991; Kingma et al. 1994; Di Pasquale et al. 1994). As this agent exerts both specific anti-oxidant effects via its sulphydryl content, as well as acting as an angiotensin converting enzyme inhibitor, it is not possible to delineate mechanisms of therapeutic effect. As captopril is not generally available in an intravenous form, oral captopril was utilised, starting at a dose of 6.25 mg and rapidly titrating dosage upwards to 100 mg daily for approximately 3 months, although one study began with an intravenous bolus of 2 mg (Nabel et al. 1991). In one of the most recent studies, a prospective randomised double-blind placebo-controlled method was utilised in 298 patients (Kingma et al. 1994), with the captopril being administered simultaneouly with intravenous streptokinase in patients with early evolving acute myocardial infarction. Accelerated idioventricular rhythm and non-sustained ventricular tachycardia was less frequent in captopril-treated patients. Furthermore, infarct size as measured via creatine kinase release was significantly smaller with a non-significant trend towards smaller left ventricular volumes. This was accompanied neurohumorally by a decrease in norepinephrine plasma concentrations 1 hour after onset of treatment. Clinically, captopril-treated patients developed more early hypotension, but less clinical cardiac failure on 3 months follow-up.

Di Pasquale *et al* (Di Pasquale et al. 1994) have also reported a study on the use of oral captopril in the same manner as Kingma *et al* (Kingma et al. 1994) for patients (n = 259) with evolving

acute myocardial infarction simultaneously treated with intravenous urokinase. The control group received captopril after 3 days. Again, benign reperfusion arrhythmias were less common with a smaller plasma CK peak concentration and shorter plasma CK normalisation time in the early captopril-treated group as compared the late captopril-treated group. Left ventricular ejection was no different at three weeks between the 2 groups. This group also reported (Di Pasquale et al. 1992) a double-blind study of 98 patients, in which captopril and intravenous glutathione for the first 2 hours of treatment resulted in incremental improvement in preservation of infarct size and decreased ventricular arrhythmias compared to captopril or glutathione alone.

It is impossible to discern a clinically significant antioxidant effect from the results of these studies. A reduction of reperfusion arrhythmias has been reported for both antioxidants and the non-thiol containing angiotensin converting enzyme inhibitor enalaprilat (Westlin and Mullane, 1988). Similarly effects on cardiac haemodynamics and the neurohumoral response to infarction may alter infarct size and CK release from the myocardium. Concurrent assessment of oxidative stress may assist in at least determining the effectiveness of captopril as an antioxidant in this setting. Furthermore, intravenous administration may assist adequate loading of the drug in the early phases of reperfusion, although the hypotensive effects of captopril may limit this strategy.

As regards N-acetylcysteine (NAC), there is only one report of a single case (Sochman and Peregrin, 1992) describing the therapeutic use of NAC during acute myocardial infarction in combination with intravenous streptokinase and PTCA to the left anterior descending artery 3 hours later. NAC was administered without adverse effect as a bolus intravenous dose of 2g during thrombolytic therapy. Initial echocardiography prior to successful PTCA revealed a large akinetic anterior segment with ejection fraction of 45%. At 14 days post-infarction, ejection

fraction was 64% with only minor anterior hypokinesis. It is therefore impossible to reach any firm conclusions about the extent of contribution of NAC to clinical outcome in this case.

The lack of positive results attributable to amelioration of reperfusion injury from these trials could be attributed to several possibilities, not all of which apply to each antioxidant tested:

1. The antioxidant agent chosen cannot reach the site of oxidative stress.
2. The antioxidant agent does not reach the site of oxidative stress in time or at adequate concentration.
3. The antioxidant agent is not administered for long enough to give sustained benefit.
4. The antioxidant agent is too specific to protect against a variety of oxidants from several sources.
5. Reperfusion injury contributes very little to either myocardial necrosis or functional impairment after acute myocardial infarction.

In view of possibilities 1 to 4, and the limited range of antioxidants so far examined, further assessment of the optimal management of putative reperfusion injury in human acute myocardial infarction is required before abandoning the use of antioxidants as clinically ineffective.

1.7 N-acetylcysteine and its use in myocardial ischaemia and reperfusion injury

1.7.1 Basic pharmacokinetics

The thiol-containing agent N-acetyl-l-cysteine (NAC) is available in intravenous, inhalable and oral forms. In the oral form peak plasma concentration is reached in approximately 1 to 2 hours after a single dose with a bioavailability of less than 10%. This low bioavailability appears to be predominantly related to extensive first pass metabolism, with a small contribution from rapid

oxidation in the gastrointestinal tract (De Caro et al. 1989; Olsson et al. 1988). In animal models only 3% of NAC was excreted unchanged in faeces (Holdiness, 1991). The apparent volume of distribution has been shown to be between 0.33 to 0.47 L/kg, suggesting moderately extensive tissue uptake (Olsson et al. 1988; Borgstrom et al. 1986). In plasma, NAC has been shown to be present in both the reduced and oxidised form, free and bound by labile disulphide linkages as NAC or cysteine onto protein peptide chains (Holdiness, 1991; De Caro et al. 1989). It is extensively metabolised and has been shown to increase plasma and various tissue concentrations of reduced glutathione, including liver, bronchoalveolar lavage fluid and myocardium(Ceconi et al. 1988; Ruffmann and Wendel, 1991; Traber et al. 1992). De Caro *et al* (De Caro et al. 1989) proposed the metabolic pathways of NAC (see Figure 1.7). In particular NAC is readily transported to the intracellular space, where it is a substrate for GSH production (Ferrari et al. 1991b) (see Figure 1.6). Furthermore, plasma GSH concentrations have been shown to be significantly increased 1.5 hours after 200 mg oral NAC in healthy volunteers (Cotgreave et al. 1987). Excretion of metabolites is 70% non-renal with the major urinary excretion product an inorganic sulphate. After bolus intravenous administration of 200 mg, total NAC declined in a triphasic manner with a terminal elimination half-life ($t_{½}$) of 5.58 hr, whereas reduced NAC had a terminal $t_{½}$ of 1.95 hr (Olsson et al. 1988). Elimination $t_{½}$ of NAC after prolonged intravenous infusion has not previously been determined.

Adverse effects after oral and intravenous administration include vomiting and diarrhoea (Holdiness, 1991). Asthma and respiratory arrest have also been reported after large intravenous doses suggesting an anaphylactoid reaction to NAC (Reynard et al. 1992; Ho and Beilin, 1983). Furthermore, the malodour of the sulphur component of NAC is a disadvantage when inhaled, with the potential for the induction of nausea, vomiting and bronchospasm (Holdiness, 1991).

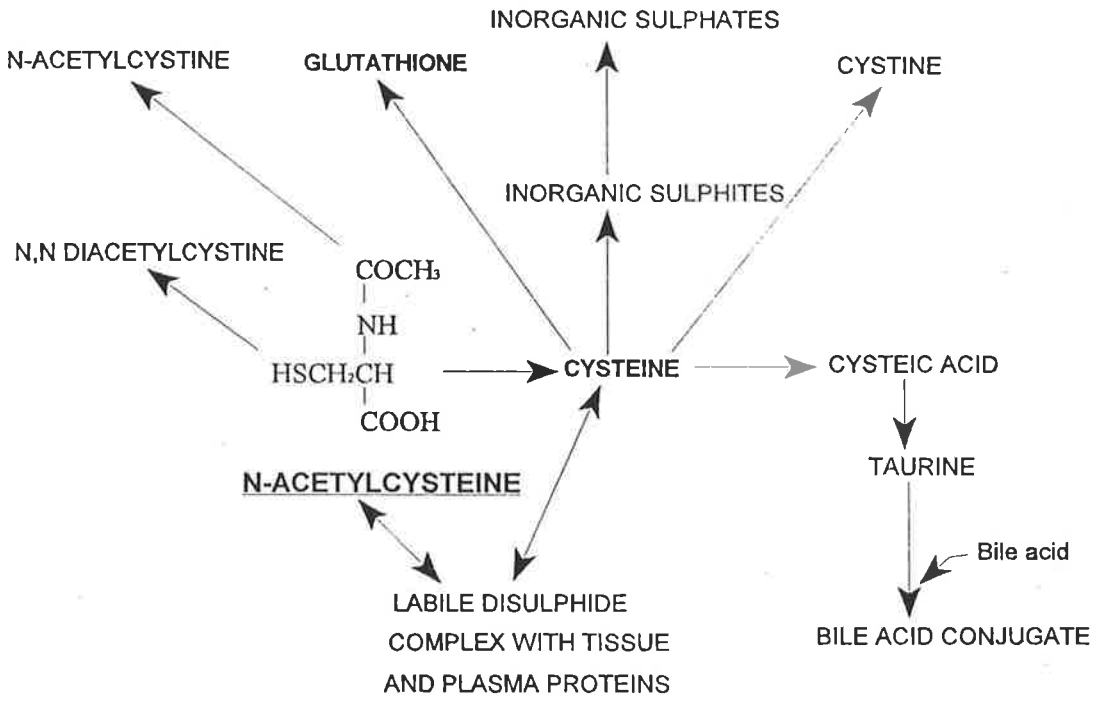


FIGURE 1.7 Proposed metabolic pathways of NAC (De Caro et al. 1989)

Apart from its proposed cardiovascular applications, NAC is in extensive clinical use in limiting hepatotoxicity after paracetamol overdose (Prescott, 1983) and has been utilised as a mucolytic agent (Olivieri et al. 1985).

1.7.2 Interaction with nitrates

1.7.2.1 Proposed mechanisms of organic nitrate action

Despite the fact that organic nitrates have been widely utilised in the management of myocardial ischaemia, the mechanisms by which nitrates are metabolised remains unclear. It has been shown that organic nitrates are pro-drugs and are indirect donors of NO (Schror et al. 1991), which like endogenous NO, activates soluble guanylate cyclase (see 1.1.2 and 1.4.3.3) (Feelisch and Noack, 1987). However, the precise mechanisms by which NO is produced are not fully elucidated. 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).

NO is released from GTN by a three electron reduction (Bennett et al. 1989). It appears that a thiol-dependent metabolic step is necessary. There is strong evidence that this sulphydryl-dependent step is at least in part enzyme mediated (Seth and Fung, 1993; Wheatley et al. 1994), but the nature of the enzyme involved remains uncertain. Kenkare *et al* (Kenkare et al. 1994) have shown a strong correlation between the rate of metabolism of GTN and enzyme activity of the *mu* isoenzyme of glutathione S-transferase. This enzyme catalyses the attachment of GSH to one of the nitrate groups of GTN which forms an unstable thionitrate. A subsequent reaction with another GSH yields glycerol dinitrate, a nitrogen oxide (either NO or nitrite) and GSSG (Harrison and Bates, 1993; Keen et al. 1976). Sulphydryls are also necessary to facilitate formation of S-nitrosothiols from released NO. While it was initially thought that S-nitrosothiols are intermediates

of NO formation (Ignarro and Gruetter, 1980), it is more likely that their formation proceeds in equilibrium with that of NO (Horowitz and Henry, 1987).

As discussed in 1.2.5.1.1, the coronary vasodilator effect of GTN has been shown to be dependent on vessel diameter with a marked attenuation of vasodilator effect in vessels less than 200 μm (Sellke et al. 1990). The vasodilator response was improved in these coronary microvessels by the addition of S-nitroso-cysteine, N-acetylcysteine or NO suggesting a decreased ability of the coronary microvessels to metabolise GTN to NO due to a decreased availability of thiols in this environment (Sellke et al. 1991). Furthermore, this effect of exogenous thiols was blocked by the glutathione-depleting agent buthionine sulfoximine, suggesting that they may undergo trans-sulphydryl exchange with glutathione (Wheatley et al. 1994). However, the physiological and pharmacological implications of this finding are uncertain. For example, it is not clear whether either sulphydryl or S-nitrosothiol concentrations are adequate after nitrate administration to exert significant effects on microvascular tone *in vivo*. The majority of available results suggest that in the absence of sulphydryl supplementation, there is only minimal GTN effect on such small vessels.

Isosorbide dinitrate is metabolised, again by a thiol-dependent step to two active metabolites isosorbide-5-mononitrate and isosorbide-2-mononitrate, which is further metabolised in a thiol-dependent manner to NO, as for GTN (Straehl and Galeazzi, 1985). In contrast, the cyanoferrate nitroprusside only requires a one electron reduction. It was originally considered to be a direct and spontaneous source of NO, but in biological fluids and in darkness (nitroprusside releases NO in a photochemical reaction) this does not appear to be the case (Butler et al. 1995; Harrison and Bates, 1993). When nitroprusside is exposed to a variety of reducing agents such as thiols, hepatic microsomes, erythrocyte and smooth muscle membranes, cyanide loss followed by NO release

occurs, suggesting a multi-step biotransformation (Harrison and Bates, 1993) which may involve thiyl radical intermediates (Butler et al. 1995). Furthermore it has been shown that release of NO from sodium nitroprusside is partially enzymatically catalysed (Kowaluk et al. 1992).

1.7.2.2 Potentiation of organic nitrate effects by N-acetylcysteine

Not only does the bioconversion of exogenous organic nitrates appear to be dependent on the presence of thiols, but several studies and models have demonstrated a potentiation of the effect of nitrates by exogenous supplementation with thiols. Oral NAC at a dose of 200 mg/kg alone has been shown to have little haemodynamic effect in patients with cardiac failure (Packer et al. 1987). The potentiation of organic nitrates by sulphdryl donors was first proposed by Needleman and Johnson (Needleman et al. 1973). Ignarro (Ignarro and Gruetter, 1980)then demonstrated that the addition of cysteine to GTN in a broken cell preparation induced increased guanylate cyclase activation, confirming this interaction. The potentiation of GTN by NAC was then studied in humans *in vivo*. Horowitz et al (Horowitz et al. 1983) demonstrated the GTN intravenous infusion rate required to induce a 10% fall in mean arterial blood pressure and a 30% fall in mean pulmonary capillary wedge pressure in humans was significantly less when NAC was infused just prior to GTN infusion as compared GTN infusion alone. This was further examined in the human coronary circulation *in vivo* by Winniford et al (Winniford et al. 1986). A significant incremental increase in coronary sinus blood flow, implying potentiation of dilatation of the coronary resistance vessels was noted after intravenous co-infusion of GTN and NAC as compared GTN alone. Therefore, both the coronary and peripheral vasculature, show a potentiated response to GTN when NAC is simultaneously infused.

A number of investigations have suggested that NAC alters the spectrum of vasodilator effects of

nitrates, with relative lack of potentiation of large arterial dilatation. Only peripheral venodilatation and subcutaneous blood flow were significantly incrementally increased by NAC when added to GTN intravenous infusion in normal humans (Boesgaard et al. 1994b). This result is to some extent consistent with the investigations of Harrison's group, who are concerned that in the presence of NAC, GTN may become a predominantly small coronary artery dilator, with the possible risk of induction of "coronary steal" (Wheatley et al. 1994; Harrison and Bates, 1993). However, Vekshtein *et al* (Vekshtein et al. 1990) demonstrated that NAC potentiated large coronary artery dilatation by GTN in man. Furthermore, the temporal artery is more responsive to the potentiation of GTN effect by NAC as compared the radial artery, which is associated with a higher incidence if severe headache in individuals treated with both intravenous NAC and GTN as compared GTN infusion alone (Iversen, 1992).

Potentiation of other nitrates apart from GTN was considered by Mehra *et al* (Mehra et al. 1994). Intravenous NAC potentiated the haemodynamic effects of orally administered isosorbide dinitrate in a randomised cross-over study of 14 patients with severe cardiac failure due to left ventricular systolic dysfunction. As compared a single oral dose of 40 to 120 mg isosorbide dinitrate alone, NAC infusion prior to this dose was associated with incremental effects on right atrial pressure, mean pulmonary artery pressure, mean pulmonary capillary wedge pressure and cardiac output over a 6 hour monitoring period.

The differential roles of intracellular and extracellular thiols was assessed by Boesgaard *et al* (Boesgaard et al. 1993) in a study utilising chronically catheterised, unrestrained rats. The effect of intravenous GTN on mean arterial blood pressure was increased by concomitant infusion of NAC, which had increased both intracellular and extracellular cysteine and GSH concentrations.

In contrast, oxothiazolidine which increased intracellular cysteine and GSH concentrations only, had no incremental effect on GTN-induced hypotension. Furthermore, inhibition of γ-glutamylcysteine synthetase by buthionine sulfoxamine, which results in intracellular GSH depletion, attenuated the hypotensive effect of GTN. This loss of GTN effect was avoided by co-infusion of NAC (Boesgaard et al. 1993). Therefore, GTN effect was dependent on both extracellular and intracellular thiol stores and attenuated when these stores are depleted.

The antiplatelet effect of organic nitrates is also potentiated by NAC. Platelet preparations *in vitro* have demonstrated the potentiation of the inhibition of platelet aggregation by GTN with NAC (Loscalzo, 1985; Chirkov et al. 1993a). This effect can be mimicked by the S-nitrosothiol S-nitroso-N-acetylcysteine (Loscalzo, 1985), but the concentration of S-nitroso-N-acetylcysteine generated in platelet-rich plasma after 10 minutes co-incubation of GTN and NAC was found to be less than 1% of that theoretically attainable by complete conversion of GTN to this S-nitrosothiol. This suggests that although S-nitrosothiols are potent anti-platelet agents, their formation is not an obligatory step mediating the potentiation of GTN by NAC (Chirkov et al. 1993a). Furthermore, there was a potentiation of the dose-dependent GTN effect on platelet disaggregation in the presence of NAC (Chirkov et al. 1993a). The concentrations of GTN required *in vitro* to achieve these anti-platelet effects are higher than those occurring in the clinical setting. However, GTN has been demonstrated to exert an *ex vivo* antiplatelet effect after 300 μg sublingual GTN in humans (Chirkov et al. 1993b). Furthermore, incubation with increasing concentrations of NAC to *ex vivo* platelet preparations of patients receiving intravenous GTN exhibit an increased anti-aggregatory response (Stamler et al. 1988). It can then be inferred that NAC is likely to exert an *in vivo* potentiation of this antiplatelet effect. Consistent with this, NAC potentiated the effects of GTN in inhibiting platelet aggregate induced cyclic flow reductions in

stenosed dog coronary arteries (Folts et al. 1991).

The therapeutic implications of this potentiation was assessed in a prospective double-blind placebo-controlled trial of the management of unstable angina pectoris by Horowitz *et al* (Horowitz et al. 1988). In 46 patients with severely symptomatic unstable angina pectoris receiving treatment with intravenous GTN infusion beginning at 5 µg/min 24 were randomised to receive intravenous NAC as a 5g bolus every 6 hours. Although there were no differences between the two groups as regards frequency of episodes of chest pain and increments in GTN infusion rate for pain control, the NAC/GTN group had a significantly lower incidence of acute myocardial infarction. The problem of hypotension induced by bolus infusions of NAC during GTN infusion was eliminated by continuous infusion of NAC at a rate of 10g/day.

1.7.2.3 Limitation of nitrate tolerance

One of the most significant limitations related to the clinical use of nitrate therapy is nitrate tolerance. This is defined as a diminution of clinical and biological effect of the drug after continuous use over time (Meredith et al. 1993; Boesgaard et al. 1994b). Factors that are related to the onset of tolerance include frequent dosage, large doses and continuous drug delivery without adequate "nitrate free periods" (Henry et al. 1989b; Flaherty, 1989). The cause of nitrate tolerance is not fully elucidated but is associated with reduced rates of bioconversion to yield nitric oxide (Horowitz and Henry, 1987; Henry et al. 1989a; Abrams, 1991) and unrelated to increased clearance of the drug (Axelsson and Ahlner, 1987).

Tolerance to organic nitrates has been demonstrated in the coronary (May et al. 1987; Meredith et al. 1993) and peripheral circulation (Packer et al. 1987; Boesgaard et al. 1994b; Ghio et al.

TABLE 1.12

Possible Mechanisms for the Interaction Between N-acetylcysteine and Organic Nitrates
Intracellular sulphydryl repletion as a co-factor for enzymatic bioconversion of nitrates (? via mu glutathione-S-transferase)
Increased non-enzymatic thiol-dependent bioconversion of GTN/isosorbide dinitrate
Increased production of S-nitrosothiols
Inhibition of the production of superoxide from endothelium, thereby decreasing peroxynitrite concentrations
Stabilisation of soluble guanylate cyclase

1994), but not conclusively in platelets. This tolerance has been manifest in patients with stable angina pectoris as a diminished time to 1mm S-T segment depression on ECG during exercise testing as compared to acute treatment (Boesgaard et al. 1991a). Of importance, the induction of tolerance to one exogenous nitrate results in "cross-tolerance" to other nitrates, although nitroprusside is only minimally affected (Abrams, 1991; Packer et al. 1987). Similarly, the vasodilator effect of S-nitrosothiols in nitrate-tolerant isolated coronary arteries is minimally affected (Henry et al. 1989a).

As with the acute effects of nitrates, the extent of tolerance varies in different vascular beds (Ghio et al. 1992; Boesgaard et al. 1994b; Ghio et al. 1994). Susceptibility to the development of nitrate tolerance may be greater in the venous than in the arterial circulation (Ghio et al. 1992). Significant tolerance has been shown in humans after the intravenous infusion of GTN within the peripheral circulation at an infusion rate of only 0.1 μg/kg/hr GTN for 23 hours (Boesgaard et al. 1994b) and 24 ± 3 μg/min for 20 hours in the coronary circulation (Meredith et al. 1993), making this an highly clinically relevant problem.

Nitrate tolerance has been hypothesised to be caused at least in part by a depletion of the intracellular and/or extracellular thiol pool (Horowitz, 1991). This hypothesis is based largely on previous demonstrations of increased cGMP formation by nitrates in broken cell preparations in the presence of sulphydryls (Ignarro and Gruetter, 1980). Many studies of nitrate tolerance have reported an attenuation of the diminution of nitrate effect by thiol donors such as NAC (Boesgaard et al. 1994b; May et al. 1987) or captopril (Meredith et al. 1993). Some human *in vivo* studies have demonstrated at least partial preservation of the potentiation of GTN effect after the induction of nitrate tolerance (Packer et al. 1987; Boesgaard et al. 1991b; Ghio et al. 1992; May

et al. 1987; Vincent et al. 1992; Ghio et al. 1994), whereas other such studies have shown partial attenuation of the extent of nitrate tolerance by concomitant use of GTN and intravenous or high dose oral (2.4 g to approximately 15 g daily) NAC or captopril throughout the experiment (Tsuneyoshi et al. 1989; Boesgaard et al. 1991a; Boesgaard et al. 1992; Boesgaard et al. 1994b; Meredith et al. 1993). However, while potentiation of GTN effect by NAC is extensively reported, there is no consensus as regards the attenuation of nitrate tolerance by NAC. In two studies measuring the extent of nitrate tolerance in patients with stable angina pectoris utilising exercise duration on treadmill testing, concurrent low oral dose NAC (1.2 g daily) failed to prevent tolerance to transdermal GTN(Hogan et al. 1990) and an intravenous bolus of NAC after isosorbide dinitrate tolerance induction (Parker et al. 1987) did not reverse tolerance. Also in a study of tolerance in patients with congestive cardiac failure, simultaneously infused intravenous NAC did not prevent or reverse most measures of tolerance (Dupuis et al. 1990). Similarly, nitrate tolerance as assessed by the diminished vasodilator response of epicardial coronary arteries in conscious dogs was not reversed by NAC (Munzel et al. 1989).

In order to assess the role of changes in thiol status in nitrate tolerance, Boesgaard *et al* (Boesgaard et al. 1994a) measured aortic and vena caval intracellular, and plasma cysteine and GSH concentrations in GTN tolerant, chronically catheterised unrestrained rats. No such depletion of thiols was shown. However, co-infusion of NAC after the induction of tolerance was associated with increased intracellular and extracellular cysteine and GSH concentrations and a potentiation of the hypotensive effect of GTN. The acute biochemical response to the intracellular sulphydryl donor oxothiazolidine was markedly attenuated in GTN-tolerant rats, implying the existence of impaired intracellular sulphydryl metabolic pathways in the presence of nitrate tolerance. This suggested that a marked depletion of total thiol stores is unlikely to be the mechanism of the

induction of nitrate tolerance *in vivo*, although more subtle changes of thiol availability occur.

Because exogenous thiols only appear to partially prevent or reverse nitrate tolerance, a multifactorial pathogenesis is likely *in vivo*. This was demonstrated by Dupuis *et al* (Dupuis et al. 1990)in a study of tolerance to intravenous GTN over 24 hours in supine and sedentary patients with congestive cardiac failure. Various neurohormonal changes were noted, including increased plasma epinephrine and renin concentrations and decreased plasma atrial naturetic peptide concentration within the first hour of treatment, returning to normal within 6 hours, despite increasing nitrate tolerance. This neurohormonal response to organic nitrate therapy suggests a secondary vasoconstrictor response and has been suggested as a possible mechanism both of nitrate "pseudotolerance" and of the "rebound" phenomenon, clinically manifest as increased myocardial ischaemia on abrupt withdrawal of nitrate therapy (Figueras et al. 1991).

Recently, several studies have suggested that a possible mechanism of nitrate tolerance involves the interaction of NO with superoxide. Münzel *et al* (Munzel et al. 1995) demonstrated an increased concentration of superoxide in GTN tolerant rabbit aortic rings. This was partially abolished by the removal of the endothelium, CuZn SOD, diphenylene iodonium (an inhibitor of flavoprotein containing oxidases), but not oxpurinol or inhibition of NO synthase. They suggested that nitrate tolerance induced increased superoxide production from a tissue intimately related to the endothelium, if not the endothelium itself. The hypothesised source of superoxide was an increased activity of vascular bound NADPH oxidase, activated by angiotensin II (Griendling et al. 1994). In their study, cAMP-dependent relaxation remained unchanged. It is proposed that superoxide reacts with NO to form peroxynitrite which although it activates soluble guanylate cyclase, has a shorter half life than NO. Furthermore, it may subsequently form other radical

species such as hydroxyl which induce vascular injury. Peroxynitrite has been shown to be a coronary vasodilator in isolated rat heart preparations (Villa et al. 1994). This effect was inhibited by oxyhaemoglobin, indicating that it acted as a source of NO . However, it appeared to be partly toxic to the vasculature, inhibiting the subsequent vasodilator responses to acetylcholine, isoprenaline and SNAP. This toxic effect was inhibited by the co-infusion of non-haemodynamically significant concentrations of prostacyclin or SNAP. Therefore, a possible mechanism of effect of NAC in the amelioration of nitrate tolerance may be as a scavenger of the increased radical species potentially generated (see 1.7.3).

1.7.3 The anti-oxidant effect of N-acetylcysteine in the myocardium

1.7.3.1 *In vitro* and *ex vivo* studies

The antioxidant activity of NAC has been studied in a variety of models, including cell-free oxygen-derived free radical generating systems, suspensions of polymorphs (PMN) activated by several methods and the *ex vivo* activation of human PMN after oral ingestion of NAC.

In a xanthine / xanthine oxidase system (superoxide as the product reduces cytochrome c in a concentration-dependent reaction), NAC at concentrations of 50 µmol/L to 1.5 mmol/L at physiological pH was a poor scavenger of superoxide in several studies (Aruoma et al. 1989; Betts et al. 1993) Aruoma *et al* (Aruoma et al. 1989) determined that rate constant of the reaction between NAC and superoxide was slow at $< 10^3$ mol/L/sec. Similarly, Westlin *et al* (Westlin and Mullane, 1988) reported that NAC was a weak scavenger of superoxide in a purine / xanthine oxidase system and a superoxide generating system utilising the autoxidation of epinephrine at pH 10.2, with a concentration of NAC required for 50 % inhibition of maximum response (IC_{50}) of 250 µmol/L. No rate constant was determined in these experiments. Therefore, NAC is likely to

be a poor scavenger of superoxide and if it does react with superoxide, the reaction proceeds slowly at physiological pH .

In contrast, Aruoma *et al* (Aruoma et al. 1989) found NAC at physiological pH to be a powerful scavenger of hypochlorous acid at concentrations of NAC ≥ 96 µmol/L, and the hydroxyl radical, with a rate constant of 1.36×10^{10} mol/L/sec. Gressier *et al* (Gressier et al. 1994) utilising PMA-activated human PMN to measure hypochlorous acid and hydroxyl radical production by the clorination of taurine and the oxidation of deoxyribose, reported the IC_{50} of NAC was 14 µmol/L and 480 µmol/L respectively. In an free-cell Fe^{++}-EDTA-H_2O_2 hydroxyl-generating system the IC_{50} for the oxidation of deoxyribose by NAC was 380 µmol/L (Gressier et al. 1994). NAC was also noted to be a scavenger of hydrogen peroxide. Hydrogen peroxide production by human PMA-activated PMN, determined by the peroxidase-dependent oxidation of phenol red demonstrated that NAC had an IC_{50} of 77 µmol/L (Gressier et al. 1993), but was associated with a slow rate constant of 0.85 mol/L/sec (Aruoma et al. 1989).This suggests that the reaction of NAC with hydrogen peroxide may not be significant at the low concentrations of hydrogen peroxide likely to be present *in vivo* (Aruoma et al. 1989).

Luminol chemiluminescence has been utilised in several models, as it detects a variety of oxygen-centred radical species generated by the myeloperoxidase system (Ohman et al. 1992). The tripeptide fMLP activates both the NADPH and myeloperoxidase system to produce hypochlorous acid (Paulsen and Forsgren, 1989). The IC_{50} of NAC for luminol chemiluminescence of fMLP-activated PMNs was shown to be 50 µmol/L (Betts et al. 1993; Paulsen and Forsgren, 1989). However when zymosan, a stimulant of predominantly intracellular PMN metabolic activity (Paulsen and Forsgren, 1989) was utilised the luminol chemiluminescence IC_{50} of NAC was 30

mmol/L (Kharazmi et al. 1988; Paulsen and Forsgren, 1989). Therefore, NAC appears to be a potent scavenger of oxygen-centred radicals generated by the myeloperoxidase system of the activated PMN.

As regards superoxide production as measured by cytochrome c reduction by canine PMA-activated PMN, the IC_{50} of NAC was noted to be 25 µmol/L (Westlin and Mullane, 1988). Betts *et al* (Betts et al. 1993) using parallel experiments in human PMN, showed that while NAC had no superoxide scavenging effect in a xanthine / xanthine oxidase system, when fMLP-activated PMN were utilised as a source of superoxide, NAC inhibited both cytochrome c reduction (IC_{50} = 820 µmol/L) and luminol chemiluminescence (IC_{50} = 50 µmol/L). This suggested that NAC was not only a scavenger of a variety of oxygen-centred radical species, excluding superoxide, but also inhibited the production of superoxide from fMLP activated PMNs. The mechanism of this inhibition is unknown.

Apart from the inhibition of superoxide production and scavenging of predominantly myeloperoxidase generated radical species, high concentrations of NAC have been shown to inhibit chemotaxis (IC_{50} = 6 mmol/L) (Kharazmi et al. 1988) and enhance receptor-mediated phagocytosis (Ohman et al. 1992). Conversely, Paulsen *et al* (Paulsen and Forsgren, 1989) showed no influence of NAC on chemotaxis or bactericidal capacity. Therefore, it is uncertain whether such PMN functions would be influenced by NAC at concentrations achievable *in vivo*.

The effect of orally , but not intravenously administered NAC has been assessed on human PMN function *ex vivo*. Healthy human PMN function, before and 90 minutes after a single oral 400 mg dose of NAC, demonstrated chemotaxis to be unaffected, but luminol chemiluminescence of the

zymosan-activated PMN was significantly inhibited by NAC (Jensen et al. 1988). Drost *et al* (Drost et al. 1991) demonstrated no inhibition of PMA-activated PMN release of hydrogen peroxide or superoxide, but enhanced spontaneous generation of hydrogen peroxide by PMN after 5 days of 600mg daily oral NAC. However, this dose of NAC did not result in a significant increase in plasma or PMN glutathione concentrations, although plasma cysteine concentration rose significantly. These minimal changes on sulphydryl plasma concentrations are consistent with the low bioavailability of oral NAC, and may explain the lack of effect on PMN function. Optimally, plasma concentrations of NAC should have been related to anti-oxidant effect. These results suggest that NAC may have an antioxidant effect *in vivo* with higher concentrations of NAC than those achieved after a single 400 mg oral dose.

The mechanisms by which NAC exerts its antioxidant effect appear to be multiple. It is uncertain how NAC inhibits superoxide production in fMLP-activated neutrophils. However, the radical scavenging effect of NAC is mediated by the strong reducing property of the sulphydryl group. It is likely to have a beneficial effect both by replenishing GSH, and itself reacting as a substitute for GSH in non-enzymatically mediated reactions with radical species (Moldeus et al. 1986). *In vitro* interactions between NAC and GSH result in their respective thiyl radicals as detected on electron spin resonance. Although the fate of the thiyl radical is unclear *in vivo*, it is likely that the thiyl radical interacts with oxygen, forming a sulphonyl hydroperoxide before subsequently forming a disulphide (Moldeus et al. 1986). The thiyl radicals of NAC and glutathione can also react with PUFAs in the presence of oxygen to produce conjugated dienes *in vitro* (Schoneich et al. 1992), although these reactions are of uncertain significance *in vivo*.

1.7.3.2 Animal studies

Previous animal studies of the use of NAC during myocardial ischaemia followed by reperfusion are summarised in Table 1.11 in 1.6.1. Ferrari *et al* (Ferrari et al. 1991a) have demonstrated a dose dependant increase in myocardial intracellular GSH concentration in isolated hearts perfused with NAC, which was not seen with the addition of other thiols such as captopril and glutathione to the perfusate. They suggest that NAC, at least in part, protects the myocardium from oxidative stress by increasing intracellular GSH concentration, which would otherwise decrease during the ischaemic period (Ceconi et al. 1988), and that this replenished GSH maintains the endogenous glutathione-dependent antioxidant systems. As the increase in free radical activity occurs almost immediately after reperfusion, it is logical that NAC would be of greatest benefit if introduced either before or during the ischaemic period, potentially maintaining myocardial antioxidant defences and acting as an antioxidant in its own right during reperfusion.

From the animal models reported, it can be concluded that NAC appears to ameliorate various manifestations of reperfusion injury. However, consistent with the hypothesis of Ceconi (Ceconi et al. 1988)and Ferrari (Ferrari et al. 1991a), a significant antioxidant effect by NAC was only seen if NAC infusion began prior to and continued during reperfusion.

1.7.3.3 Studies in humans

If NAC must be present within the ischaemic myocardium prior to the onset of reperfusion, various difficulties arise in the clinical setting. Certainly, during cardiac surgery, complete control as regards cardioplegia and reperfusion allows for the potentially optimal preparation of the myocardium for a defence against the ensuing oxidative stress. However, in the management of acute myocardial infarction with reperfusion, the onset of ischaemia and infarction generally occurs

prior to presentation for treatment. The occluded infarct-related artery prevents adequate perfusion of the most ischaemic myocardium with NAC. Therefore, in the absence of retroperfusion strategies, NAC theoretically is only likely to have access to the myocardium at risk of oxidative stress at the time of reperfusion, although this may be slower, staggered or intermittent as compared the sudden reperfusion of animal models. Therefore, with this limitation in mind, any therapeutic manoeuvre involving NAC as an antioxidant would require a rapid intravenous loading of NAC prior to, and during reperfusion therapy to maximise myocardial concentrations at the onset of reperfusion. Furthermore, prolonged infusion for at least 24 hours is likely to be necessary, in order to limit the demonstrated prolonged oxidative stress after reperfusion for acute myocardial infarction (Grech et al. 1993).

To date there are no completed studies assessing the antioxidant effect of NAC in humans for the management of myocardial ischaemia and reperfusion. As discussed in 1.6.2, only two case studies of NAC infusion either during cardioplegia for cardiac surgery (Ferrari et al. 1991b) or for the management of acute myocardial infarction treated with thrombolytics and early PTCA (Sochman and Peregrin, 1992) have been reported. Therefore, no evidence is available in humans as regards the effectiveness of NAC as an antioxidant.

1.8 Aims of the current investigation

The primary null hypotheses to be tested are:

(1) N-acetylcysteine has no effect on the extent of myocardial oxidative stress, metabolism, function or viability in the setting of myocardial ischaemia and reperfusion.

(2) There are no significant interactions between NAC and glyceryl trinitrate as regards hypothesis (1).

To test these hypotheses, three models of myocardial ischaemia followed by reperfusion were utilised:

(1) Isolated Krebs-perfused rat heart.

(2) Human pacing-induced myocardial ischaemia, utilising coronary sinus catheterisation to assess trans-coronary gradients of biochemical markers for myocardial ischaemia, oxidative stress and cardiac haemodynamics.

(3) Human evolving acute myocardial infarction treated with thrombolysis, assessing safety, rate of reperfusion, myocardial salvage, residual myocardial function and oxidative stress.

NAC, GTN and a combination of both NAC and GTN were administered to determine:

(1) The effect of NAC on extent of oxidative stress in these models.

(2) The effect of NAC on the extent of the haemodynamic change in these models.

(3) The differences between rat and human models as regards the correlation between extent of myocardial ischaemia and oxidative stress, and the differential effect of NAC.

(4) The interaction of NAC and GTN as regards the extent of myocardial oxidative stress, metabolism, ischaemia and / or salvage, and cardiac haemodynamics in the human models.

A secondary hypothesis also to be tested in the model of evolving acute myocardial infarction treated with thrombolysis was that the intensity of myocardial ischaemia could not be modulated pharmacologically prior to reperfusion of the infarct-related artery. In particular, streptokinase infusion did not affect the severity of myocardial ischaemia prior to reperfusion, and that the occurrence or severity of hypotensive responses to streptokinase infusion did not affect myocardial ischaemia.

To test this hypothesis the objective was to examine the temporal and quantitative correlations

between the extent of myocardial ischaemia as assessed by the rate of change of S-T deviation on the 12 lead ECG and peripheral systolic blood pressure prior to, during and after intravenous streptokinase infusion for evolving acute myocardial infarction in humans.

Chapter 2 gives a review of the methodologies available to assess oxidative stress in biological systems, giving a rationale for the choice of biochemical markers utilised in the models of myocardial ischaemia in this study. A summary of the development of the methodology for the assay of these biochemical markers follows, including an examination of the normal range of plasma malondialdehyde in humans.

Chapter 3 describes the development and utilisation of an isolated Krebs-perfused rat heart model of myocardial ischaemia and reperfusion, with retrograde coronary perfusion against a left ventricular workload, measuring perfusion pressure, heart rate, left ventricular systolic and diastolic pressures. The development of a protocol examining the conditions required for the myocardial release of malondialdehyde into the coronary effluent in association with a variety of ischaemic and oxidative stresses is described. Subsequently, examination of the effect of NAC on haemodynamic recovery during reperfusion after myocardial ischaemia is performed.

Chapter 4 describes the development and utilisation of a model of human pacing-induced myocardial ischaemia. Full detail of methodology is given, including a description of preliminary experiments to determine the accuracy of the biochemical parameters measured and the optimal conditions required to assess the extent of oxidative stress in this setting. Results and discussion of an assessment of the extent of oxidative stress and its correlation to the extent of myocardial ischaemia are given. This is followed by a description and discussion of a study determining the

differential effects on myocardial oxidative stress, metabolism and haemodynamics of NAC, GTN and the combination of NAC / GTN in this model of human myocardial ischaemia.

Chapter 5 presents the methodology, results and discussion of a study examining the secondary hypothesis of this thesis. In the setting of evolving acute myocardial infarction in humans, the effect of streptokinase of the extent of myocardial ischaemia and an examination of the relationship to peripheral systolic blood pressure is examined.

Chapter 6 presents the methodology, results and discussion of a study of the safety, biochemical, haemodynamic and clinical effects of NAC in combination with GTN and streptokinase for the treatment of human evolving acute myocardial infarction. The primary assessment is of the effect of NAC on biochemical parameters of oxidative stress in peripheral venous plasma. Other haemodynamic and clinical assessments are made and presented, although the predictive power of the study does not allow for accurate interpretation of these other parameters.

A general discussion in Chapter 7 makes comparisons between the different models utilised and summarises the effects of NAC and its interaction with GTN in these models. Potential further areas suggested by the results of these studies are discussed. Finally, in the light of the results of these studies, potential clinical implications regarding the utility of antioxidants in the management of myocardial ischaemia are examined.

Chapter 2:

Assay Development

2.1 Biochemical markers of oxidative stress *in vivo*

Oxidative stress in a biological tissue is generally considered to be caused by the increased presence of free radicals or oxidants and/or the depletion of antioxidant mechanisms within the intra-cellular and extra-cellular environment as discussed in 1.4 and 1.6. For *in vivo* and organ perfusion models, two approaches to the assessment of oxidative stress are generally utilised. The first and most common approach involves either the direct detection of free radicals or measurement, either qualitatively or quantitatively, of the products of their inevitable reaction with tissue components. The second approach is to assess the overall redox 'status' of the tissue or organ, by measuring the relative concentrations of antioxidant molecules and enzymes, or the ratio of substrate to product of oxidising reactions with endogenous antioxidants. This section presents an overview of the generally available biochemical markers of oxidative stress in models applicable to those utilised in these current studies. These markers and their method of assay are summarised in Tables 2.1 and 2.2.

2.1.1 Direct detection of radical species *in vivo*

Because radical species have extremely short half-lives, direct qualitative and quantitative determination of radical species is difficult *in vivo*. Free radicals can be measured directly utilising electron spin resonance (ESR) spectrometry. This method detects the magnetic moment exerted by the unpaired electron (Rosen and Rauckman, 1984). However, it is relatively insensitive requiring steady state concentrations of free radicals in the micromolar range, which is generally higher than those generated by biological systems (Holley and Cheeseman, 1993; Rosen and Rauckman, 1984).

A more practical, but still semiquantitative method, is to analyse samples through the technique

TABLE 2.1 Commonly used biochemical indicators of free radicals and their products in plasma and tissues

	Class of Indicator	Indicator	Method of Assay	Comment
I	Free Radical	direct detection	Electron spin resonance	Insensitive and requires a steady state production of free radicals
		spin traps	electron spin resonance	Spin traps toxic to humans. *Ex vivo* traps do not measure the original radical species
II	Product of free radical reactions			
A	Lipid peroxidation	lipid hydroperoxides	1.spectrophotometry 2.enzymatic reaction with an oxygen probe 3.HPLC	1. non-specific assay. 2. very specific and sensitive. 3. time consuming but sensitive.
		conjugated dienes	spectrophotometry	not specific for lipid peroxidation *in vivo*.
		volatile hydrocarbons	collect exhaled breath & measure with GC	Useful for serial sampling. Isolating chamber cumbersome. May be non-specific for lipid peroxidation in humans.
		4-hydroxynenonal	1. HPLC 2. GC-MS	Usually measured when considering the mutagenicity and cell toxicity of lipid peroxidation
		malondialdehyde	1. TBA test with a) fluorimetry b) HPLC 2. direct HPLC 3. GC	1. Sensitive but nonspecific assay Needs careful clean-up and/or separation of interfering substances 2. Specific, but most assays lack sensitivity 3. Assays not readily feasible in complex biological mediums.
		Schiff bases & lipofuscin	fluorimetry	Accumulate in tissues. Not measured in plasma. Used as an indicator of aging.
B	Protein modification	carbonyl group formation	1.spectrophotometry 2.monoclonal antibodies	1. assesses tissue effects of lipid peroxidation 2. good sensitivity
C	DNA modification	1. thymine glycol 2. 8-hydroxy-guanine	1. HPLC 2. GC	Measure content in urine. Predominantly used to assess carcinogenesis.

TABLE 2.2 Commonly measured antioxidants and markers of redox status.

Class of Antioxidant Mechanism	Antioxidant	Method of Assay	Comment
Vitamin	Ascorbic acid	HPLC	Plasma indicator. Interacts extensively with α-tocopherol
	α-Tocopherol	HPLC	Measures extent of oxidative stress in lipid particles and membranes
	Ubiquinol	HPLC	Present in lipid particles and membranes. Oxidised in preference to α-tocopherol
	β-Carotene	HPLC	Low levels in the myocardium
Enzymes	Superoxide dismutase	1. Indirect (incomplete list) a) Reduction of cytochrome C b) Reduction of nitroblue tetrazolium c) Chemiluminescence of luminol 2. Direct (incomplete list) a) Rapid freeze EPR b) Pulse radiolysis	Generally used in pure *in vitro* systems or tissue fractions.
	Catalase	1. Determination of H_2O_2 removal a) Titrimetric methods b) Spectrophotometry c) Photometry d) Fluorimetry 2. Determination of O_2 production a) Oxygen probe 3. Immunoprecipitation with anti-catalase	Generally measured in erythrocytes and tissues with high concentration of catalase. Low concentration in myocardium.
	GSH peroxidases	Measurement of GSH or NADPH removal by polarography	Measures tissue activity of the enzyme.
Nonenzymatic Antioxidants	Uric acid, Allantoin & Parabanic acid	HPLC	Plasma indicator of free radical activity
	Glutathione	1. Enzymatic reaction 2. HPLC a) electrochemical detection b) with derivitisation	1. Assays vary in specificity. Some assays only measure total glutathione. 2. Sensitive and specific Sensitive indicator of redox status in plasma and tissues

of spin trapping. Spin traps react rapidly with free radicals to form radical-trap adducts which are more stable and have longer half-lives and can therefore reach a steady state in the detectable range for ESR. These spin traps are directly added to the medium under investigation. It is essential that the spin trap can diffuse to the location of free radical generation such as into the cell or perfused organ of interest. Unfortunately, all commercially available spin traps are toxic to humans. Thus in human studies, the spin trap is added as soon as possible *ex vivo* to the sample. This *ex vivo* technique is more likely to measure radicals that have been generated from a chain reaction initiated by the original radical species (Holley and Cheeseman, 1993).

Commonly used spin traps are N-*tert*-butyl-α-phenylnitrone (PBN) and 5',5'-dimethylpyroline-*N*-oxide (DMPO) of the nitrone group of compounds. The nitroso group of compounds are also utilised as spin traps (Green and Hill, 1984; Janzen, 1984). DMPO will form adducts with both hydroxyl and hydroperoxyl radicals, but in general the nitrones are non-specific, trapping carbon-, hydrogen-, oxygen-, nitrogen-, and halogen-centred radicals. Only the nitrones are capable of detecting oxygen-centred radicals like superoxide and hydroxyl radical at room temperature. They covalently bind to the free radical to produce a nitroxide (N-O•) spin adduct, which gives a characteristic spectrum. Occasionally, from the resultant spectrum, the identity of the original radical can be identified (Rosen and Rauckman, 1984).

2.1.2 Measurement of products of free radical reactions

Because of the limitations associated with the direct detection of free radicals, non-radical and stable products of free radical reactions with biomolecules are the usual biochemical "indicators" of free radical activity. Understandably, there are many problems related to the specificity, sensitivity and validity of these indirect and often non-specific indicators. As a consequence, there are many available methodologies. Often, investigators in this area recommend that any study

involving oxidative chemistry should utilise at least two methods, to allow cross-checking of results (Slater, 1984; Holley and Cheeseman, 1993).

2.1.2.1 Detection of hydroxyl radical

In addition to the direct detection of the hydroxyl radical by electron spin resonance, this radical species can also be measured by the non-radical products of its reaction with aromatic compounds, salicylic acid and phenylalanine (2,3-dihydroxybenzoate, 2,5-dihydrobenzoate and *o*-and *m*-tyrosines respectively). These competitive non-enzymatic reactions give a semi-quantitative measurement of hydroxyl radical generation by cells, organelles and perfused organs (Holley and Cheeseman, 1993).

2.1.2.2 Assessment of the extent of lipid peroxidation

Since lipid peroxidation is a prominent part of the free radical chemistry of biological tissues, including the heart, the measurement of products of lipid peroxidation are commonly used. Lipid peroxidation should affect both the lipid membranes of organelles and the plasma membrane allowing the detection of lipid peroxides or their byproducts to be measurable in both tissue homogenates and plasma. An important aspect of the measurement of lipid peroxidation is to prevent artefactual production of the measured parameter during or after sampling. Lipid peroxidation is an ongoing chain reaction once initiated by free radical oxidation. Also, autoxidation of lipid can occur. Generally, lipid peroxidation and further breakdown of by-products can be slowed by freezing. Enzymatic reactions that affect the level of by-products are eliminated by the addition of acid or organic solvents. Other alternatives are to add antioxidants or metal-chelating agents to the sample to prevent further radical-induced oxidation (Holley and Cheeseman, 1993).

2.1.2.2.1 *Lipid hydroperoxides*

Lipid hydroperoxides themselves are very labile molecules rapidly decomposing by enzymatic and non-enzymatic pathways to a variety of products including volatile hydrocarbons, aldehydes and carbon dioxide. Therefore, addition of antioxidants and processing of all samples at 4°C is essential. This can limit the versatility of the assay, especially if it is applied to a clinical experimental setting. There are several methodologies available of varying technical difficulty, specificity and sensitivity. Some are outlined briefly here. Total plasma hydroperoxide can be measured by its reaction with a haem compound and the oxidation of the precursor of methylene blue, which is subsequently measured spectrophotometrically. However, this is a very nonspecific assay and does not always correlate well with other measures of lipid peroxidation. A sensitive and specific assay measures (via an oxygen electrode) the production of oxygen which is produced when lipid hydroperoxides activate the enzyme prostaglandin endoperoxide synthase and thereby initiate the cyclooxygenase reaction. There are also more specific and sensitive (but technically difficult and expensive) methods involving reaction to luminol or isoluminol, separation and quantitation with either gas chromatography-mass spectrometry (GC-MS) or high performance liquid chromatography (HPLC) and chemiluminescence detection (Holley and Cheeseman, 1993).

2.1.2.2.2 *Conjugated dienes*

On the formation of a lipid hydroperoxide, the diene moiety rearranges into conjugation, which has a characteristic UV absorption at 234 nm. Four isomeric conjugated diene hydroperoxides can be formed in this way (Pryor and Castle, 1984). This indicator of lipid peroxidation is a very useful and relatively simple test in isolated tissue preparations, but there are many interfering substances in blood such as haem proteins which have similar UV absorption spectra. These can be eliminated by the extraction of the conjugated dienes into organic solvents. Furthermore, PUFA and a variety of other breakdown products absorb UV at about 210 nm. Because the relative concentrations of

conjugated dienes are low in human plasma, overlap of the absorption spectra of PUFA results in poor assay sensitivity. However, of greater importance, the specificity of the presence of conjugated dienes as a marker of oxidative stress is low in human plasma. Up to 90% of plasma conjugated dienes are non-oxygen containing dienes of linoleic acid which are of either dietary origin or formed via metabolism of gut bacteria, rather than lipid peroxidation by free radicals. Thus absolute concentrations of such dienes are a poor indicator of oxidative stress in human blood (Holley and Cheeseman, 1993; Halliwell and Gutteridge, 1990).

2.1.2.2.3 *Volatile hydrocarbons*

A noninvasive method of measuring the extent of lipid peroxidation in animals is the measurement of exhaled hydrocarbons, in particular ethane, ethylene and pentane. These volatile hydrocarbons are breakdown products of lipid hydroperoxides. This method allows repeated and prolonged measurements to be made in the one animal, without the problems associated with autoxidation or artefactual change after sampling of specimens. The concentration of hydrocarbons exhaled is small, but the sensitivity of the assay is quite high. The inspired air must not be contaminated with pollutant hydrocarbons such as from car exhaust or cigarettes. Therefore, the animal or human studied must be placed in a sealed chamber, inspiring only purified air for at least 90 minutes to eliminate previously inhaled pollutants. The equilibrium period in an isolation chamber thus limits this method's versatility in clinical trials. Samples of expired air are taken and the hydrocarbon of interest is adsorbed and concentrated prior to measurement by gas chromatography (Holley and Cheeseman, 1993). The size of the isolation chamber is dependent upon the size of the animal being studied, a cumbersome limitation in the case of human studies. The exact tissue source of the hydrocarbon cannot be determined by this method. In fact, it does not always correlate with the extent of oxidative stress because ethane is produced from other sources such as gastrointestinal bacteria. This can be eliminated in animal studies by feeding the animals on a fat-

free diet for 36 hours prior to study, but is understandably difficult in human studies (Lawrence and Cohen, 1984).

2.1.2.2.4 *Conjugated Schiff bases and lipofuscin*

A variety of products of lipid peroxidation such as malondialdehyde readily react with amino acids, proteins, amino phospholipids, nucleic acids and other biological compounds to form fluorescent conjugates, which are called Schiff bases (Dillard and Tappel, 1984). Also, malondialdehyde can become polymerised to produce fluorescent pigments (Esterbauer et al. 1991; Holley and Cheeseman, 1993). These can then be measured after extraction into chloroform with fluorometric spectrophotometry (Dillard and Tappel, 1984). These products are very similar to lipofuscin or age pigments. Lipofuscin accumulates predominantly in metabolically active postmitotic cells of several tissues. They are a heterogeneous collection of chromophores within lysosomal residual bodies, considered to contain remnants of damaged cellular membranes that were phagocytosed (Stark et al. 1984). Because the formation of fluorescent products occurs at the very end of the lipid peroxidation process, and accumulate in cells with time, they lack specificity for a particular acute event of oxidative stress in a tissue (Holley and Cheeseman, 1993), and appear to be more a marker of aging. (Dillard and Tappel, 1984) Also, they are predominantly intracellular molecules, not readily detectable in blood or urine.

2.1.2.2.5 *Aldehydes*

There are many stable products of lipid peroxidation including n-alkanals, hydroxyalkanals and aldehydes. A variety of sensitive HPLC and GC-MS methods have recently been developed, with potential to be highly sensitive biochemical markers of lipid peroxidation (Holley et al. 1993; Esterbauer and Zollner, 1989). However, the aldehyde products of lipid peroxidation currently remain the more frequently utilised biochemical indicators of this free radical initiated process. Of

these, two aldehydes frequently utilised are 4-hydroxynonenal (HNE) and malondialdehyde (MDA). HNE, being one of the more important aldehyde products involved in the cytotoxic and mutagenic effects of lipid peroxidation, is usually measured in the context of this range of potential effects. HNE is assayed using HPLC with UV detection or via GC-MS (Holley and Cheeseman, 1993; Esterbauer et al. 1991).

2.1.2.2.6 *Malondialdehyde*

MDA is assayed in a wide variety of situations associated with lipid peroxidation and oxidative stress. The determination of MDA was historically one of the first estimates of lipid peroxidation and continues to be frequently used, despite continuing controversy as to optimal methodology and adequate specificity of the many methods available. Part of the reason for the confusion relates predominantly to the nonspecific nature of the most commonly used method, the thiobarbituric acid (TBA) test. In effect not all TBA tests measure the same biomolecule. Therefore many workers in this area choose to call the biochemical markers of lipid peroxidation derived from the TBA test, "thiobarbituric acid reactive substances" (TBARS). In view of questionable specificity, the assays utilising TBARS have been suggested as essentially screening tests for lipid peroxidation (Esterbauer et al. 1991).

The methodologies can be divided into two groups. Firstly there are a variety of TBA tests, where MDA or MDA-like substances covalently bind to TBA to produce a coloured and fluorescent adduct. Secondly, there are chromatographic methods, such as thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) or gas chromatography (GC) with or without derivatisation (Esterbauer et al. 1991).

2.1.2.2.6.1 *The thiobarbituric acid test*

In the basic TBA test, one molecule of MDA covalently binds to two molecules of TBA in the presence of low pH and heat, producing a chromogen which can be quantified by UV absorption (λ = 532 nm) or fluorescence spectroscopy (excitation λ = 530nm and emission λ = 547 nm). The nonspecificity of this test as a measure of free MDA arises due to several factors:

a) Bound MDA, especially that bound to protein can become "free" MDA during the heating stage of the reaction with TBA (Esterbauer et al. 1991; Draper and Hadley, 1990).

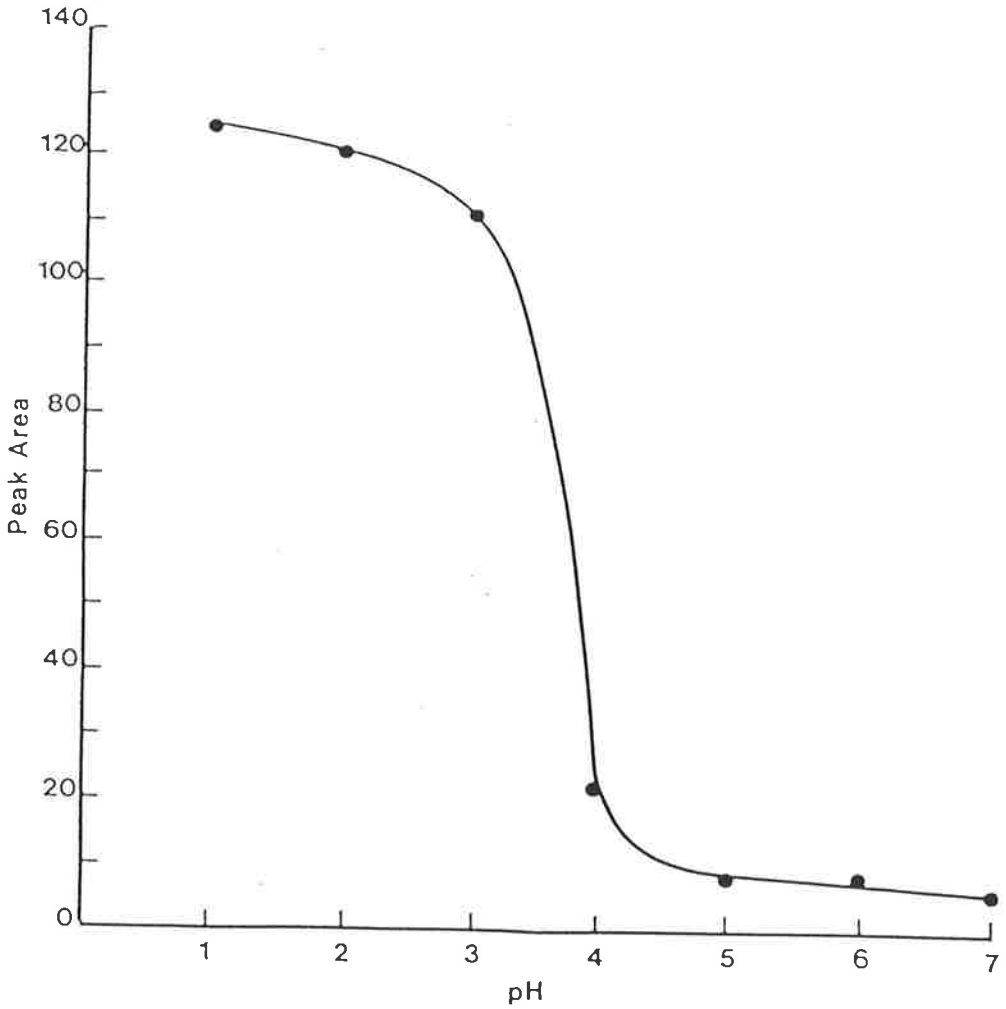
b) Autoxidation of lipid may occur as a result of poor handling of the samples, prolonged storage, or presence of transition metal ions in the sample or reagents, initiating a Fenton-like reaction (Duthie et al. 1992; Bird and Draper, 1984).

c) Interfering substances, such as haem or pigments such as in coloured sample containers, may have a colour in close range to the pink of the TBA-MDA adduct. Their peaks increase the background UV absorption or fluorescence (Esterbauer et al. 1991).

d) Other substances in the sample either become MDA or bind to TBA as "MDA-like" substances, with the same or similar UV and fluorescent properties as the wanted MDA-TBA adduct. These substances include other oxidised lipids, aldehydes, sucrose and amino acids, urea and biliverdin (Esterbauer et al. 1991; Wong et al. 1987).

In the methods involving the assay of plasma or serum, variation in assay methodology involves the use of whole plasma or different sample pretreatments allowing for different components of plasma to be assessed. Also, differing acid concentrations and heating temperatures and duration are utilised. In general TBA is added in excess to the reaction. It is probably not the type of acid used in the TBA test that is important, but the final pH achieved that determines the extent of TBA-MDA adduct formation. A pH of 2 to 3 appears to be ideal (see Figure 2.1). Acidic conditions are essential for TBA-MDA adduct formation, but the preparation of TBA in strong acid may inhibit colour formation. Furthermore, Bird and Draper (Bird and Draper, 1984)

FIGURE 2.1 The effect of pH on TBA-MDA complex formation. Free MDA was reacted with TBA at 100°C for 30 minutes. (Bird and Draper, 1984)



speculated that acid addition may cause MDA to be released from protein. Sample preparation is also important and may contribute to considerable variations in results with different methodologies. Heparinised plasma samples do not store well and are excessively affected by the presence of transition metal ions in the plasma or reagents. The use of potassium EDTA as the anticoagulant for the plasma acts as a chelator of these metal ions and may help limit autoxidation during sampling, storage and assay (Duthie et al. 1992). Other antioxidants that may be used to prevent autoxidation of the sample are butylated hydroxianisole (BHA) or butylated hydroxytoluene (BHT) (Draper and Hadley, 1990). Possibly the most important variation between assays is the pretreatment of the plasma samples. Some TBA assays use untreated plasma. This is truly a measure of TBARS, because as well measuring free and protein bound MDA, there will be interfering substances such as other aldehydes and lipids that will contribute to the final result, even if chelating agents are added to minimise autoxidation.

In the commonly used method by Yagi (Yagi, 1984), plasma is first treated with phosphotungstic acid which precipitates out protein and lipid. The precipitate is assayed with TBA and the resulting TBA-MDA adduct extracted into butanol, prior to fluorescence measurement. Therefore, this assay is not really a measure of free MDA, but also of protein bound MDA and probably other lipids which are autoxidised during the heating step (Esterbauer et al. 1991). In a method used for blood, tissue and peroxidised microsomes, trichloroacetic acid precipitates out protein and most other lipids prior to heating with TBA and has been found to represent free MDA in peroxidised microsomes by comparing to a direct assay of MDA with HPLC (Esterbauer et al. 1991). Therefore, when using plasma and aiming to measure free MDA with a TBA test, the removal of protein and lipid prior to heating with TBA appears to be one of the more important manoeuvres. This still does not remove all other substances that potentially interfere, but their overall effect is quite small. Of course, utilising the same medium (plasma) for the standard curve helps to alleviate

the problem further, but does not eliminate it. This is predominantly because blank plasma has some fluorescence due to a combination of the inherent fluorescence of TBA, native MDA in the plasma being utilised, as well as other interfering substances. The effect of TBA itself can be eliminated by subtracting an aqueous blank from all plasma 'unknowns' or subtracting the plasma blank from all other standards, thus forcing the standard curve's plasma blank through the origin. This manoeuvre cannot distinguish the small effect of other interfering substances in plasma, which may still have a small effect and lead to a falsely high "MDA" concentration.

2.1.2.2.6.2 *HPLC methods*

The HPLC assays for MDA in different mediums can be divided into three categories: precolumn derivitisation, postcolumn derivitisation and direct determination without derivitisation (Esterbauer et al. 1991). In plasma the methodologies available are more limited. Wong *et al* (Wong et al. 1987) and Wade *et al* (Wade et al. 1985) form an TBA adduct with MDA, then separate out the adduct with HPLC. This removes interfering TBA-MDA-like products, but does not address the issue of free versus bound MDA and the production of MDA during the heating period in acid. Therefore, these methods are still likely to overestimate the plasma free MDA concentration. The most accurate, but often insensitive methods for free MDA measurement in plasma utilising HPLC are the direct determinations, without derivitisation. There are several types of columns used, with UV detection at 245 nm in acid solutions and 267 nm in neutral solutions (Esterbauer et al. 1991). The methods by Behrens *et al* (Behrens and Madere, 1991) and Largilliere *et al* (Largilliere and Melancon, 1988) lack sensitivity with the lower limit of detection 1 - 1.5 µmol/L and 0.48 µmol/L respectively. The Largilliere group concluded that MDA does not exist in the free form in plasma. This statement was later refuted by the Lazzarino and Giardina group (Lazzarino et al. 1991; Giardina et al. 1993). With their direct method the mean plasma concentration of free MDA in normal human subjects is 0.051 µmol/L, being far lower than the lower limit of detection of the

other direct methods.

2.1.2.2.6.3 *Gas chromatography*

Finally, there are several GC and GC-MS methods that require the MDA to be derivatised into a volatile product. For example, MDA can be derivitised into an alcohol when reduced by borane trimethylamine after ether extraction of MDA (Des Rosiers et al. 1993). Other derivatising agents include methylhydrazine, 2-hydrazino-benzthiazole and penta-fluorophenylhydrazine (Esterbauer et al. 1991). They are usually used to measure free MDA in fats and oils, rather than biological tissues such as blood (Draper and Hadley, 1990).

2.1.2.3 Measurement of protein modifications

Rather than measuring the effect of free radical attack on lipids as the biochemical indicator of oxidative stress, proteins which undergo amino acid modification, causing cross-linking, changes in conformation and function could be measured. In general any assay used must be very sensitive, as these modified proteins are readily removed by proteases rather than accumulating to easily detectable concentrations (Holley and Cheeseman, 1993). Spectrophotometric measurement of carbonyl group formation that occurs with oxidation by free radicals, such as the hydroxyl radical, on amino acid side groups is fairly sensitive (Levine et al. 1990). There are also a variety of monoclonal antibodies to carbonyl modified proteins that can be utilised to assess the dynamics of these biomolecules in tissues (Holley and Cheeseman, 1993).

2.1.2.4 Measurement of DNA modifications

When DNA bases are oxidised by free radicals many products are possible. Two of these are thymine glycol and 8-hydroxy-guanine. These are eliminated by repair enzymes and excreted in the urine either unchanged or as thymidine glycol and 8-hydroxydeoxyguanosine. Oxidative

damage to DNA occurs constantly due to physiological free radical sources and external irradiation. Assay in urine is carried out by either HPLC with electrochemical detection or GC-MS. This biochemical marker cannot isolate the source of the products to a particular organ, and there is a more imprecise time correlation between oxidative stress and excretion of urine as compared to plasma. In general, these parameters tend to be used in studies of carcinogenesis and its association with free radical DNA damage (Holley and Cheeseman, 1993).

2.1.3 Measurement of redox status and endogenous antioxidants

The previous discussion has focussed on the biomolecular products of free radical reactions in biological systems. Another approach to the assessment of oxidative stress is to measure the effect on endogenous antioxidant mechanisms. In plasma, commonly measured antioxidants include the vitamins, α-tocopherol, ascorbic acid, ubiquinol and β-carotene, glutathione redox status and breakdown products of uric acid.

2.1.3.1 Antioxidant vitamins

Plasma α-tocopherol, ascorbic acid, ubiquinol and β-carotene concentrations are generally determined using HPLC with UV or electrochemical detection. Ascorbate has a UV absorption of 266 nm, thus can be seen on the same chromatograph as underivatised free malondialdehyde in neutral conditions (Lazzarino et al. 1991), whereas α-tocopherol UV absorption occurs at approximately 290 nm (Hill and Burk, 1984; Jandak et al. 1989). All of the vitamins may be detected in one assay with electrochemical detection. The vitamins of interest are extracted into hexane, dried and resuspended in ethanol, then injected onto the HPLC column with a mobile phase of methanol and ethanol and an applied potential of +0.6 V (Lang et al. 1986). The plasma concentrations of these vitamins have been extensively utilised in epidemiological studies associating antioxidant "status" with cancer and atherosclerosis. β-carotene which is present in

such low concentrations in the heart, is a poor biochemical indicator of oxidative stress in this organ.

The preferential order of depletion of the various vitamins in plasma or isolated LDL particles appears to be ascorbate, ubiquinol then α-tocopherol (Stocker et al. 1991; Ingold et al. 1993). Ascorbate as a ratio of its product after exposure to an oxidant, dihydroascorbate, has been demonstrated to be a sensitive biochemical indicator of oxidative stress *in vivo* (Frei et al. 1990). A potential confounder during prolonged serial sampling is that the plasma concentrations of the vitamins may be influenced by dietary intake. Therefore, in serial sampling, especially if protracted, analysis of several vitamins, and/or the ratio of ascorbate and dihydroascorbate may give a better indication of the extent of oxidative stress than either alone.

2.1.3.2 Products of uric acid

Because allantoin and parabanic acid are only produced in plasma when uric acid is nonenzymatically oxidised, these products of the interaction between uric acid and radical species is a useful plasma marker of *in vivo* redox status. There are several assays utilising HPLC to separate out uric acid and its products using UV detection (Hicks et al. 1993b; Hochstein et al. 1984). These assays have not been widely utilised in plasma, possibly due to technical difficulties with interfering peaks on chromatography. Therefore their application and usage are still not fully elucidated and validated. However they offer the potential for a useful biochemical marker of oxidative stress in plasma.

2.1.3.3 Glutathione

Because of its importance as a reducing agent of reactive oxygen species, glutathione is understandably a commonly utilised marker of oxidative stress. The redox state of glutathione,

expressed as the ratio of GSH to GSSG is an highly sensitive marker of this in the intracellular compartment. As GSSG is actively transported out of cells when in high concentration during situations of oxidative stress, the change in ratio of GSH to GSSG in plasma reflects these intracellular changes (Ishikawa and Sies, 1984). Also, the concentration of GSH in plasma is proportional to the intracellular concentration (Meister and Anderson, 1983). This interaction between intracellular and extracellular glutathione redox status allows for a convenient biochemical marker of oxidative stress *in vivo*.

However, because the concentration of glutathione in plasma is low, there are many technical difficulties as regards assay sensitivity. As with other methodologies, extreme care is also required to prevent degradation of GSH and GSSG *ex vivo*. Therefore, the use of preservatives such as dithiothreitol, a thiol that is preferentially oxidised over GSH can be added to the plasma sample (Johansson and Lenngren, 1988). Also, rapid (within 10 minutes) acidification, deproteination and freezing slows any change in GSH:GSSG. Importantly *ex vivo*, not all GSH degrades to GSSG, but to other disulphide molecules, thereby decreasing GSH:GSSG (Meister and Anderson, 1983; Johansson and Lenngren, 1988).

There are a variety of enzymatic methods for the determination of GSH and GSSG. For example, glyoxylase may be used to determine GSH, and GSSG reductase is used to measure GSSG. Other enzymes used in assays of GSH determination include malepyruvate isomerase, GSH S-transferase and formaldehyde dehydrogenase, with subsequent spectrophotometric measurement (Meister and Anderson, 1983).

HPLC, usually with electrochemical detection, offers an highly sensitive assay of both GSH and GSSG. An electrochemical detector can be used to monitor current generated against a fixed

potential across a graphite or gold-mercury amalgam electrode by the reaction of the electrochemically active thiols. The current generated is directly proportional to concentration and is subsequently converted to a voltage, producing the chromatogram. With dual electrodes, both oxidised and reduced forms can be detected simultaneously. Separation of the various polar thiols through the column usually requires ion-pairing agents, such as heptanosulfonic acid in the mobile phase (Allison and Shoup, 1983). Attempts to improve sensitivity without electrochemical detection by pre- and post-column derivitisation have been described. Precolumn derivitisation with fluorescent agents such as N-(9-acridinyl)maleimide have been utilised, but specificity decreased due to the binding of this agent to other products. 5,5'-Dithiobis(2-nitrobenzoic acid) has also been used for precolumn derivitisation, but the detection at UV 280 nm was associated with many interfering peaks (Meister and Anderson, 1983, Svardal et al. 1990).

2.2 Rationale for the choice of biochemical markers of oxidative stress utilised in this thesis

The models utilised to assess the extent of oxidative stress and its modification by the thiol-containing agent N-acetylcysteine are predominantly human *in vivo* studies. Plasma was therefore the only easily accessible and time-sensitive medium available for serial sampling. Tissue, and therefore intracellular measurements of oxidative stress were not possible. Similarly, to parallel the human studies, the isolated rat heart preparation used the coronary effluent for serial measurement of biochemical markers of oxidative stress. It was therefore necessary to choose plasma parameters that have been validated as reflecting myocardial tissue oxidative stress. Also, especially in regards to the human *in vivo* studies, we chose to use two parameters, as recommended by other workers in this area (Slater, 1984). This allowed for cross-checking of these indirect determinations, minimising the potential for mis-interpretation of potentially non-

specific results. Furthermore, the choice of both a biochemical breakdown product of free radical reactions in biological tissues, and a measure of the antioxidant response of that tissue is theoretically attractive.

Malondialdehyde, the aldehyde product of lipid peroxidation, being commonly used in previous studies, was chosen as one of the biochemical parameters. There are adequate amounts of lipid in the heart, and a rise in plasma concentration has been shown to occur during myocardial ischaemia and reperfusion of the human heart *in vivo* (Giardina et al. 1993; Davies et al. 1993; Oldroyd et al. 1990; Roberts et al. 1990; De Scheerder et al. 1991). As discussed above, assay methodology for free MDA is variable. An important part of this thesis was to develop a modification of the TBA fluorometric test that allowed for a simple measure of this biomolecule.

The second biochemical marker chosen, reflecting the cellular antioxidant response to oxidative stress, was the redox status of glutathione, measuring both oxidative and reduced forms. Glutathione, as discussed above, is one of the most important antioxidant defense mechanisms in the myocardium, reacting both enzymatically and nonenzymatically with reactive oxygen species (Das and Maulik, 1994; Ferrari et al. 1991b). Like MDA, the plasma determination of GSH:GSSG has been utilised in previous studies (Ferrari et al. 1990; Ferrari et al. 1992; Ferrari et al. 1991a) assessing oxidative damage to the heart. Furthermore, the effect of N-acetylcysteine as a precursor of glutathione can be quantitatively assessed in the setting of oxidative stress (Ceconi et al. 1988).

2.3 Fluorometric quantitation of free malondialdehyde in plasma and Krebs buffer

2.3.1 Development and modification of the thiobarbituric acid test in plasma

2.3.1.1 Materials

Materials required included 10 mL clear polypropylene conical tubes with a clear or white screw cap (Disposable Products, Adelaide, South Australia), 1.5 mL capped polypropylene tubes (Dispolab, Kartell, Italy), 4.5 mL disposable cuvettes (Dispolab, Kartell, Italy), vortex mixer, refrigerated centrifuge, microcentrifuge, hot plate, and luminescence spectrometer (Perkin Elmer LS 50B luminescence spectrometer). The reagents are 2-thiobarbituric acid (Sigma, St Louis, Mo, USA), HPLC grade perchloric acid (BDH Lab. Supplies. Poole, BH 15 ITD, UK), HPLC grade orthophosphoric acid (Ajax Chemicals, Sydney, Australia), HPLC grade chloroform and methanol (BDH Laboratory Supplies, Poole, England), potassium ethylenediamine tetracetic acid (EDTA) (Sigma) and 1,1,3,3-tetraethoxypropane (TEP) (Sigma). All water was glass distilled.

Stock MDA was prepared by acid hydrolysis of TEP which becomes free MDA in a 1:1 stoichiometric ratio. A stock solution of 10 mmol/L TEP in 1% orthophosphoric acid was stirred for 1 hour. The stock MDA remains stable at room temperature for at least 6 weeks. TBA was dissolved in water to a dilution of 42 mmol/L, utilising a sonicator and stored at room temperature for 2 weeks. 2.3 mol/L perchloric acid was stored at 4°C and added to plasma at this temperature. EDTA was dissolved as a 4.5% g:v solution in water and stored at 4°C until use.

2.3.1.2 Methods

Blood was sampled and placed in a clear screw-top tube with 200 µL 4.5% EDTA per 5ml blood. This was immediately placed in ice and could be stored for up to 2 hours in this manner without appreciable change in MDA concentration. The blood was then spun at 3,000 rpm for 15 minutes at 4°C and the plasma aspirated. This was immediately frozen and stored at -70°C until assay. At no time should the plasma sample have been allowed to thaw to room temperature prior to acid precipitation of protein. After the addition of perchloric acid and the resultant protein

precipitation, the supernatant was relatively stable and further steps in assay preparation could occur at room temperature.

To prepare the plasma for the TBA reaction, 160 µL 2.3 mol/L ice cold perchloric acid was added to 800 µL plasma and vortex mixed, centrifuged at 13,000 rpm for 5 minutes and the supernatant aspirated. 500 µL chloroform was added and vortex mixed for 30 seconds and centrifuged at 13,000 rpm for 5 minutes. 200 µL of the supernatant (top layer) was added to 750 µL 1% orthophosphoric acid, 250 µL 42 mmol/L TBA and 300 µL water. The pH of the resultant mixture was 1.8 - 2.0. The tubes were capped and the caps pricked with a needle to relieve pressure from the steam generated during boiling. The tubes were placed in water-filled beakers and boiled for 1 hour. After boiling the sample tubes were cooled in ice. 3 mL 70:30 v:v chloroform:methanol was added and the tube vortexed for 1 minute followed by centrifugation at 3,000 rpm for 5 minutes. The top layer was aspirated into a cuvette and its fluorescence intensity read, at room temperature, at excitation λ 530 nm, slit width 5 nm, emission λ 547 nm, slit width 10 nm utilising a Perkin Elmer LS 50B luminescence spectrometer (figure 2.2). All samples and standards were carried out in triplicate.

A standard curve was constructed from plasma and remained linear within the range of 0.05µmol/L to 5 µmol/L. To account for endogenous free MDA within the plasma, the blank plasma was subtracted from all standards and the linear regression line forced through the origin. Similarly, to remove the background fluorescence of unbound TBA, an aqueous blank was boiled separately from all other samples (to prevent contamination during the boiling stage) and subtracted from all patient samples. The quality controls were 0.2 µmol/L and 0.5 µmol/L MDA spiked plasma samples stored in 800 µL alliquots at -70°C.

FIGURE 2.2 A Excitation fluorescence scan of TBA-MDA adduct. Emission λ = 547 nm.

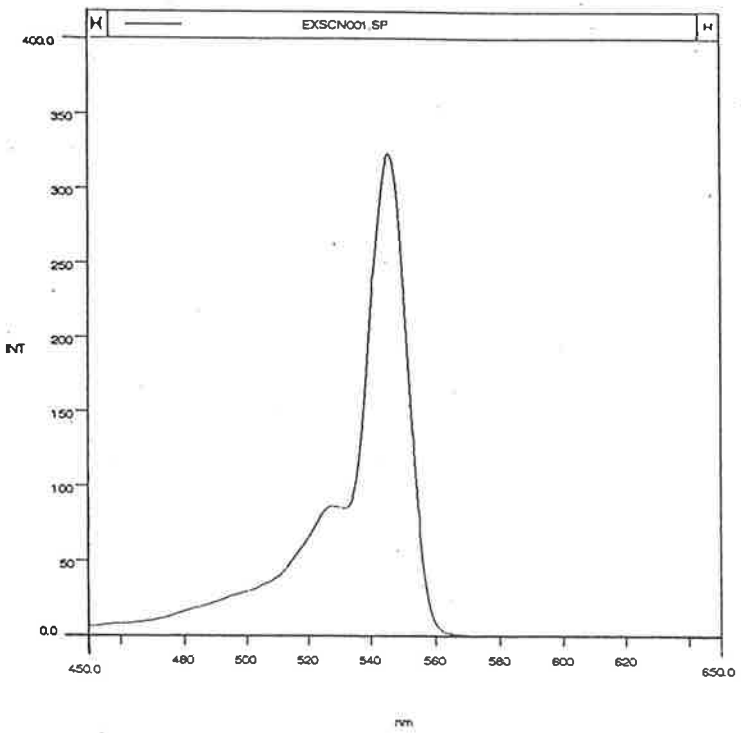
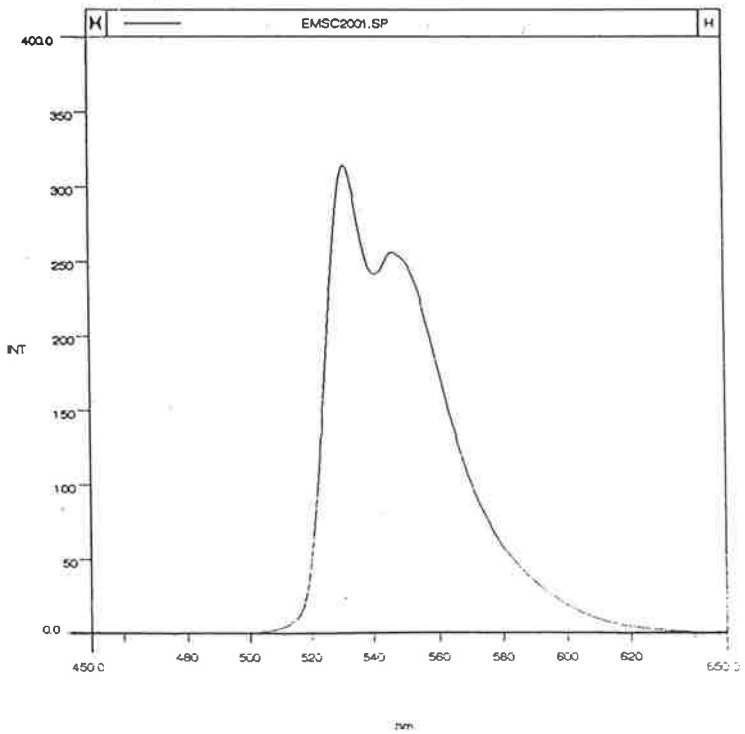


FIGURE 2.2 B Emission fluorescence scan of TBA-MDA adduct. Excitation λ = 530 nm.



2.3.1.3 Results

The intra-assay coefficient of variation for triplicate samples for 6 separate estimations was 8.8 ± 5.9% for 0.05µmol/L and 2.2 ± 0.7% for 2 µmol/L MDA standards. The aqueous blank had an overall average fluorescence intensity of 6.228 ± 3.341 in these 6 standard curves, with an intra-assay coefficient of variation of 7.0 ± 2.5%. Inter-assay coefficient of variation utilising the slope of the standard curve was 6.4% (Figure 2.3), the 0.2 µmol/L quality control plasma sample 7.4%, and the 0.5 µmol/L quality control plasma sample 5.0% (n=6) (Figure 2.4). There was no significant change in the quality control concentrations over a 6 month period of storage. The lower limit of detection utilising 200 µL plasma in the assay was approximately 0.04 µmol/L. Sensitivity could be improved by the addition of a larger amount of plasma per sample for reaction with TBA, which is in considerable excess in this assay. Recovery of MDA was only 20% at plasma concentrations less than 0.1 µmol/L, but 80% at higher concentrations (see Figure2.5).

2.3.1.4 Determination of the purity of the TBA-MDA adduct

An important modification of this TBA test was to improve its specificity for plasma free MDA. This was achieved by the removal of protein, a source of protein-bound MDA and lipids, a source of autoxidation during boiling. Perchloric acid was considered to be a very efficient precipitator of plasma protein (Johansson and Lenngren, 1988; Giardina et al. 1993). Several experimental procedures were utilised to determine the "purity" of the subsequent TBA-MDA adduct. Utilising a modification of the HPLC method for separation of the TBA-MDA adduct developed by Wong *et al* (Wong et al. 1987) an assessment was made of the effectiveness of perchloric acid precipitation. Briefly, a Millipore Waters model 510 pump with WISP 712 autosampler attached to a 5 µm, 15 cm C18 Novapak column and guard-pak (Waters-Millipore Corp, Milford, Ma, USA) was utilised. The mobile phase consisted of 73% 50mmol/L KH_2PO_4 in water, pH 6.8 and 27% HPLC grade methanol pumped at a rate of 1 - 1.5 ml/min. Detection of the TBA-MDA

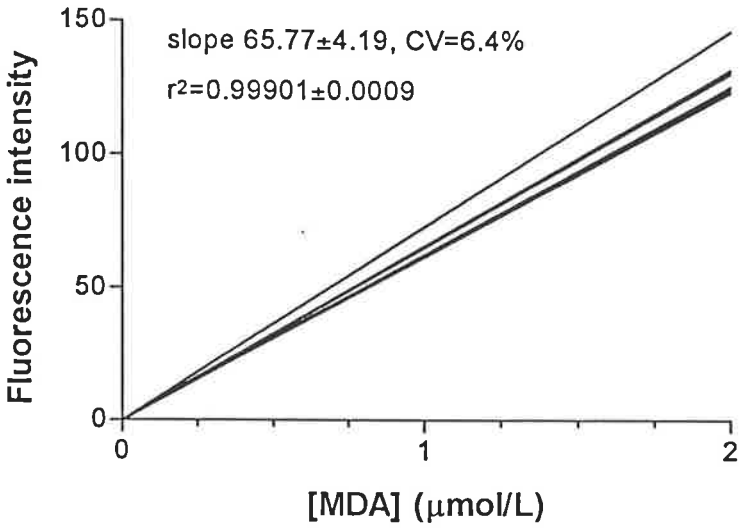


FIGURE 2.3 Individual standard curves (n=6) of modified TBA test in plasma

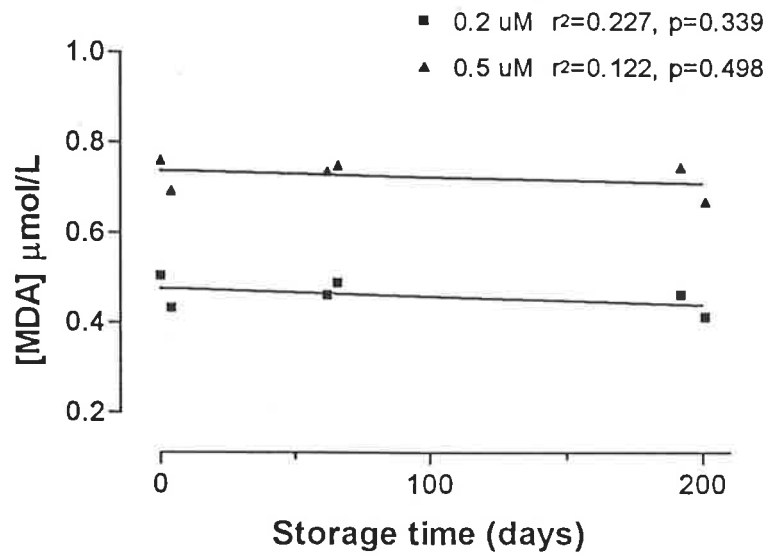


FIGURE 2.4 Quality control plasma samples

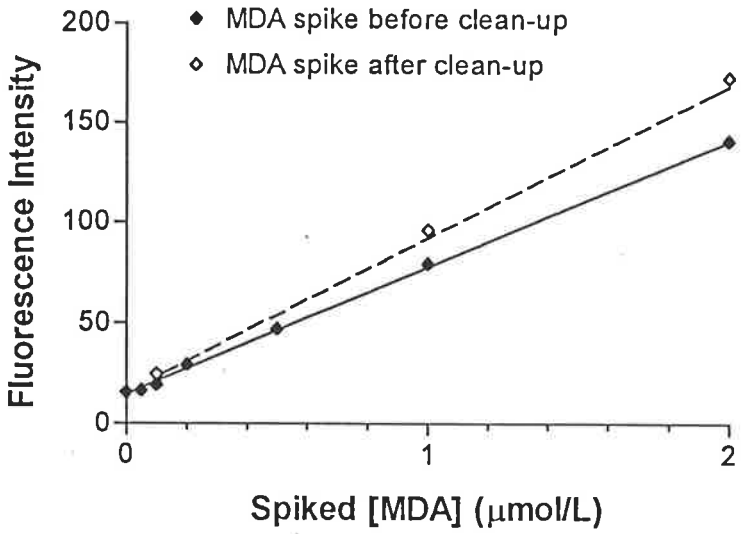


FIGURE 2.5 Recovery of MDA with clean-up

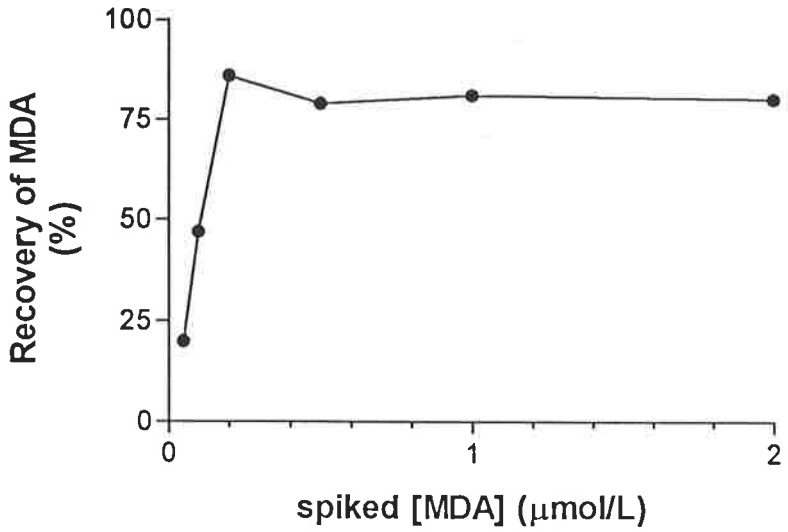


FIGURE 2.6 Percentage recovery MDA with clean-up

adduct was initially with an UV spectrophotometric detector with λ 532nm and sensitivity 0.005 Å. The chromatographic tracings were recorded onto a chart recorder (Rikadenki Kogyo Co., Tokyo, Japan) with a chart speed of 0.5 cm/min. The plasma was precipitated with perchloric acid, as described above, before the TBA reaction was carried out. The TBA-MDA adduct after boiling was either extracted into butanol, or methanol/water after mixing with chloroform/methanol. 0.5ml of the top layer was dried down in a speed vacuum container, then reconstituted in 100 µL mobile phase and 20 µL injected onto the column.

Representative chromatograms are shown in Figure 2.7 to Figure 2.9. Extraction into methanol was considered to be superior to butanol, as there were fewer endogenous peaks on the chromatogram without significant change in the size of the MDA-TBA peak. With UV detection a large solvent front was present. On changing to a fluorescence detector (Perkin Elmer) with excitation λ 520 nm, emission λ 552 nm the solvent front was eliminated, leaving only a single peak. Therefore, the conclusion from this chromatogram was that with this sample preparation and fluorescence settings, only TBA-MDA fluoresced. Other MDA-like substances that bound to TBA with similar fluorescence had been eliminated. Furthermore, the simpler fluorometric technique to read the fluorescence intensity could be utilised without the need to perform HPLC separation first.

2.3.1.5 Prevention of autoxidation

However, the problem of autoxidation and the formation of MDA during boiling remained. This problem was highlighted by a large inter-assay coefficient of variation of up to 44% for 2 µmol/L MDA quality control sample when only protein precipitation was performed. This suggested that other compounds, possibly plasma lipids, were present and yielded MDA during the assay procedure. The use of chloroform extraction prior to the addition of TBA was aimed to remove

FIGURE 2.7 Representative HPLC chromatogram of plasma spiked with MDA, protein precipitated with perchloric acid and butanol extraction after TBA test. UV detection.

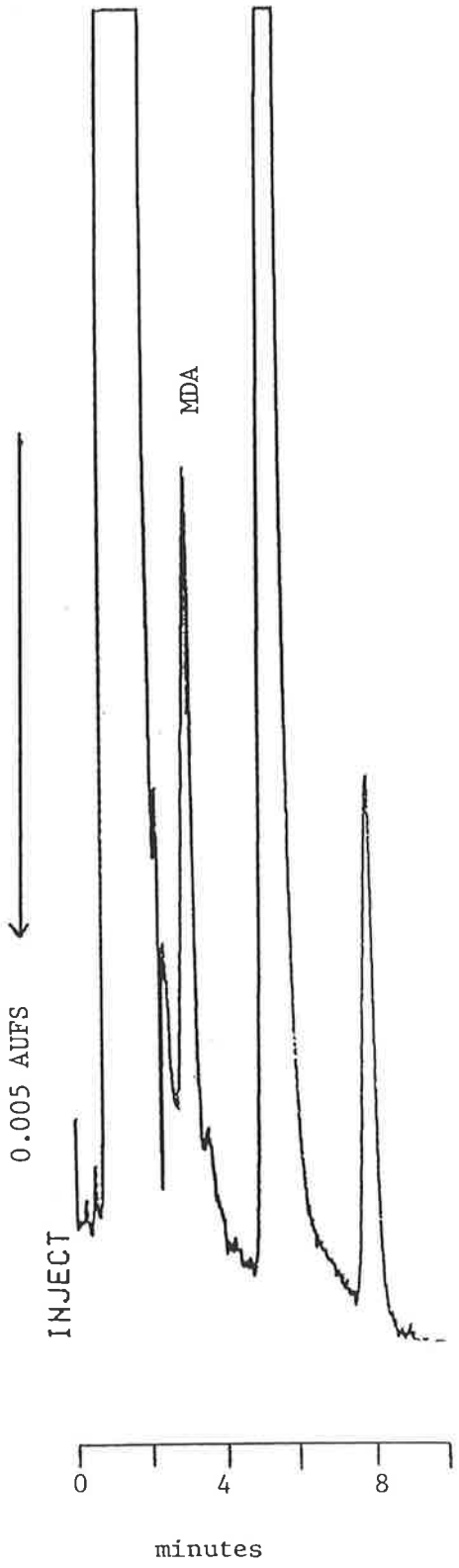


FIGURE 2.8 Representative HPLC chromatogram of plasma spiked with MDA, protein precipitated with perchloric acid and chloroform/methanol extraction after TBA test. UV detection.

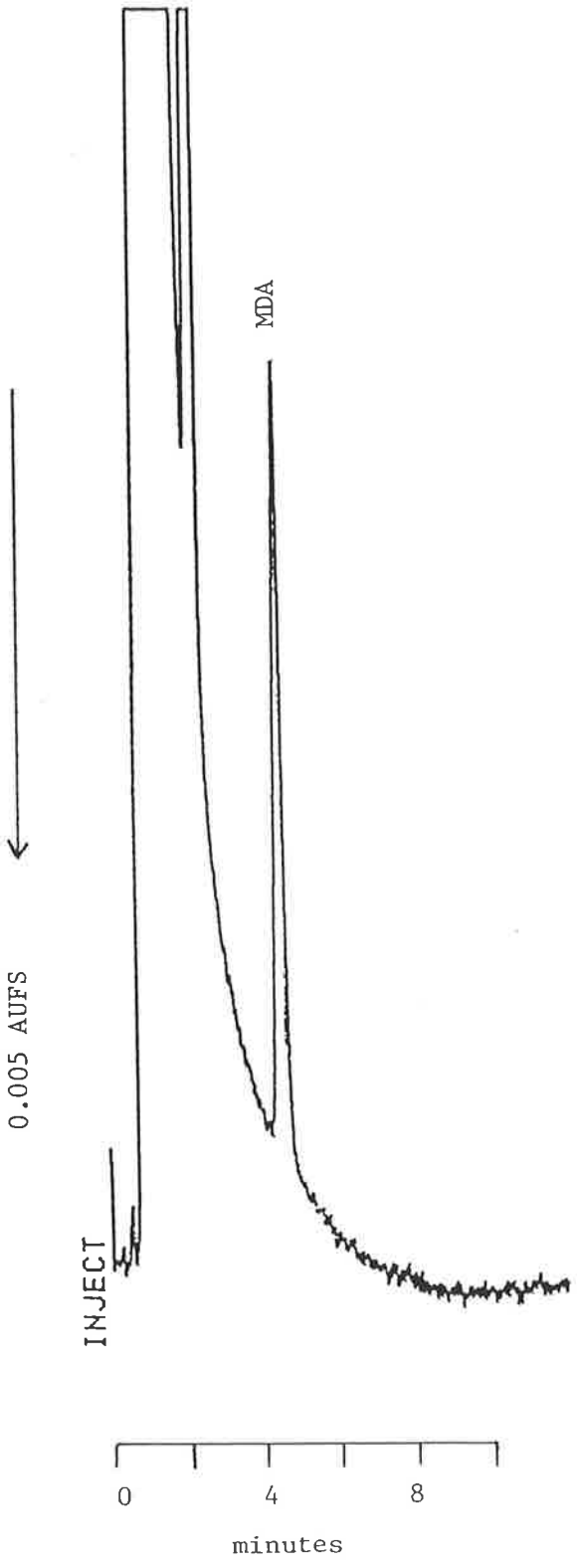
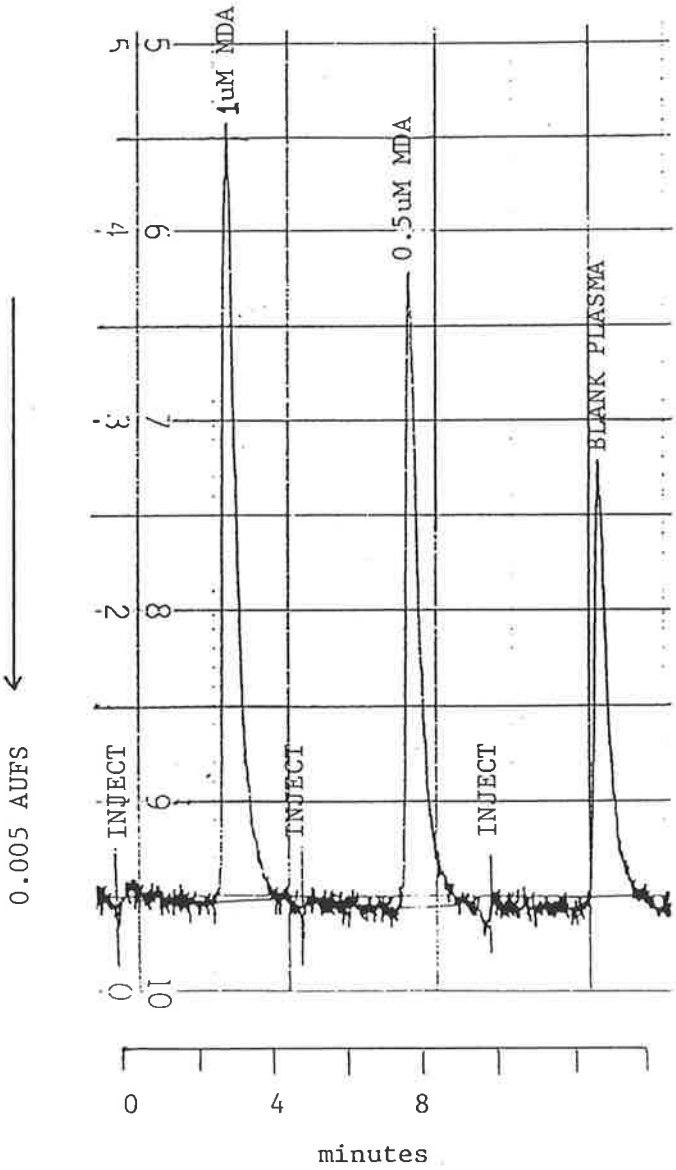


FIGURE 2.9 Representative HPLC chromatograms of plasma spiked with MDA, protein precipitated with perchloric acid and chloroform/methanol extraction after TBA test. Fluorescence detection.



these lipids and leave free MDA in the aqueous layer. With this manoeuvre, the problem of inter-assay variability was eliminated. Similarly, the second chloroform/methanol extraction improved the intra-assay coefficient of variation, probably via removal of MDA-like substances.

2.3.1.6 Other interfering substances

A further confirmation that only MDA was binding to TBA in this assay was shown by the close correlation between the aqueous and cleaned plasma standard curves. In figure 2.10, the effect of protein in the TBA test is seen. Not only did the presence of protein lead to a measurement of bound MDA as seen by the large blank fluorescence, but also caused the slope of the standard curve to be steeper. With the elimination of protein and lipid there was very little difference between the aqueous blank and cleaned plasma blank. The difference could be attributed to the presence of endogenous free MDA and other interfering substances, such as bilirubin and sucrose in plasma. The subtraction of the plasma blank from the standard curve eliminated the fluorescence effect of endogenous MDA. Furthermore, subtraction of the aqueous blank from all patient samples eliminated the fluorescence effect of TBA itself. This left only the effect of the other interfering substances, which can be deduced from Figure 2.11 to have only a small effect on the overall determination of free MDA in plasma. However, the differing slopes highlighted the need to use the same medium for the standard curve as the sample. Recovery of MDA was only 20% at plasma concentrations less than 0.1 µmol/L, but 80% thereafter. (see figure2.6).

Attention was also given to other potentially interfering factors. Firstly, other pink or yellow coloured compounds may fluoresce at the same or similar wavelengths as the TBA-MDA adduct, causing a falsely high result. The use of a pink coloured lid on the blood sampling tube was

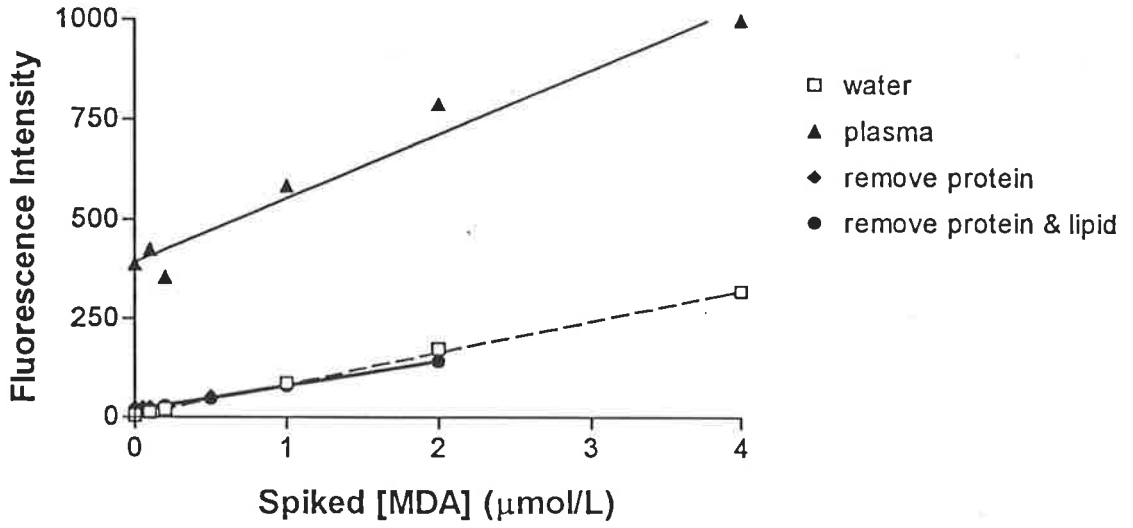


FIGURE 2.10 Comparison of standard curves at different stages of clean-up with water standards

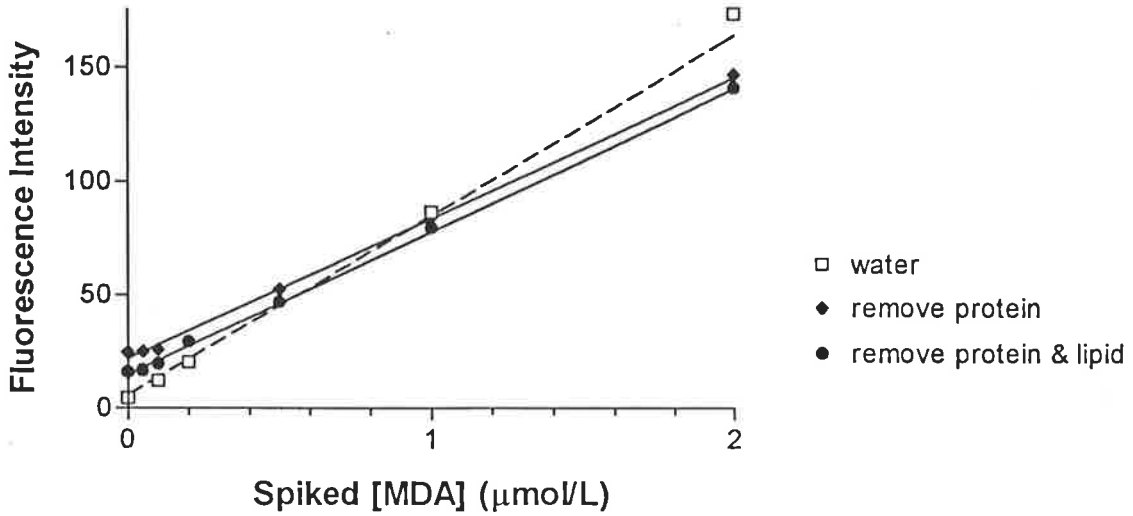


FIGURE 2.11 Effect of plasma clean-up on standard curve as compared to water standards.

associated with a significant rise of the "MDA" concentration (0.09 ± 0.02 μmol/L to 0.17 ± 0.04 μmol/L, n=3), despite plasma being transferred from the tube after centrifugation. Obviously, the colour rapidly leached into the plasma. It was also noted that if a yellow-capped tube was used in the boiling process, the mean aqueous blank fluorescence intensity rose from 10.72 ± 0.491 to 53.41 ± 5.080, due to marked interference from the leached yellow colour into the assay fluid. There was no difference in the fluorescence readings of samples assayed in clear polypropylene compared to glass.

Interference by haemoglobin due to blood haemolysis only became significant if the plasma was heavily haemolysed. Furthermore, TBA itself has inherent fluorescence. This was shown by the presence of a small fluorescence reading in an aqueous blank. TBA was in excess in this assay. However, a 20% decrease in TBA concentration from 42 mmol/L to 34 mmol/L was associated with approximately a 10% decrease in the slope of the standard curve.

2.3.1.7 Storage of plasma samples

The storage and handling of the plasma was very important. One previous study has shown poor reproducibility of MDA concentration with storage by freezing (Duthie et al. 1992). The former study used sodium heparin as the anticoagulant, while this assay used EDTA for the dual purpose of anticoagulation and iron chelation, to prevent Fenton reaction-induced lipid peroxidation *ex vivo*. Re-use of previously thawed plasma was generally associated with a highly variable rise in MDA plasma concentration indicative of *ex vivo* lipid peroxidation. Therefore, only fresh or once frozen plasma samples were considered reliable. Overall, the long-term storage of plasma with EDTA at -70°C appeared to be associated with no change in free plasma MDA concentration over a period of 200 days as indicated by the quality control samples in Figure2.4. Also, it was apparent that once the plasma sample was thawed once after freezing, it had to be used immediately,

because standing the sample at room temperature during the assay procedure was associated with a rise in MDA concentration. However, whole blood could stand at 4°C for up to 6 hours without a significant rise in MDA concentration. Blood immediately centrifuged and assayed had an MDA concentration of 0.10 ± 0.02 µmol/L, and storing the whole blood at 4°C for 6-8 hours prior to centrifugation and assay was associated with an MDA concentration of 0.11 ± 0.01 µmol/L (n=2). This allowed for serial samples to be taken, stored on ice and centrifuged 1-2 hours later at the end of the research protocol without significant artefactual autoxidation interfering with the final results. Once MDA is in an acid environment it is stable for 6 weeks (Esterbauer et al. 1991). Also, an acid pH inhibits any enzymatic action and usually further lipid peroxidation.

2.3.2 Determination of plasma malondialdehyde concentration in humans

2.3.2.1 Normal human population

Consistent with the large variation in the methodology for the plasma determination of free and bound malondialdehyde by the TBA test, the normal plasma range for each is also highly variable. Esterbauer (Esterbauer et al. 1991) in his review summarised many of the reported "normal ranges". In the TBA tests where plasma protein was included in the TBA reaction, the range in normal human plasma is between 0.32 to 47.2 µmol/L. In the method by Wong *et al* (Wong et al. 1987; Knight et al. 1987), whole plasma is used in the TBA test, protein precipitated following this and the TBA-MDA adduct separated on HPLC: the mean normal plasma MDA concentration was 0.60 (95% confidence interval 0.18 - 1.02) µmol/L for men (n=230) and 0.54 (95%CI 0.14 - 0.94) µmol/L in women (n=148). This large variation emphasises the methodological and assay interpretation problems in this field. Probably the best methods to assess the 'true' free MDA concentration in plasma is to consider the normal human plasma ranges utilising the direct determination of MDA by HPLC. The method by Wade *et al* (Wade et al. 1985) determined a mean plasma MDA concentration in 6 normal humans of 4.57 ± 0.33 µmol/L, whereas, later

published methods found free MDA to be undetectable (Largilliere and Melancon, 1988; Behrens and Madere, 1991). Since then, a more sensitive method found the normal range in 10 healthy humans to be 0.051 ± 0.013 µmol/L (Giardina et al. 1993), which was below the lower limit of detection of the previous 2 methods.

To determine the healthy human plasma range in an Australian population utilising the modified TBA test as described in 2.3.1, 20 volunteers were venesected 5 mL blood into a clear tube with 200 µL 4.5% EDTA. The blood was centrifuged 3000 rpm for 15 minutes at 4°C and the plasma frozen to -20°C until assay within 48 hours. There were 11 males, and the mean age of the group was 42 ± 13 years (range 24 - 65 years). All were healthy and on no medication. The mean plasma MDA determination was 0.16 ± 0.03 µmol/L (range 0.11 - 0.20) in men and 0.17 ± 0.04 µmol/L (range 0.12 - 0.24) in women (see figure 2.12). There were no differences in MDA concentration as regards age or gender, although the number of subjects was small.

2.3.2.2 Stable cardiac disease

Plasma MDA concentration has been shown to be elevated in several cardiac conditions, including cardiac failure (McMurray et al. 1993; Belch et al. 1991) and stable angina pectoris (McMurray et al. 1992). This data suggest that the myocardium is under oxidative stress, even in these chronic stable conditions. Furthermore, plasma TBARS concentration have been shown to be elevated in the acute ischaemic syndrome of unstable angina pectoris (McMurray et al. 1992; Dubois Rande et al. 1994). Therefore, some preliminary data were gathered to determine peripheral venous plasma MDA concentrations in stable angina pectoris and cardiac failure. Plasma samples were collected and assayed as described in 2.3.1 and 2.3.2.1.

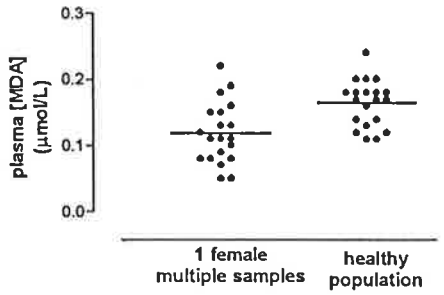


FIGURE 2.12 A Peripheral plasma MDA concentrations in healthy human subjects (n=20) and multiple sampling in 1 individual (n=20).

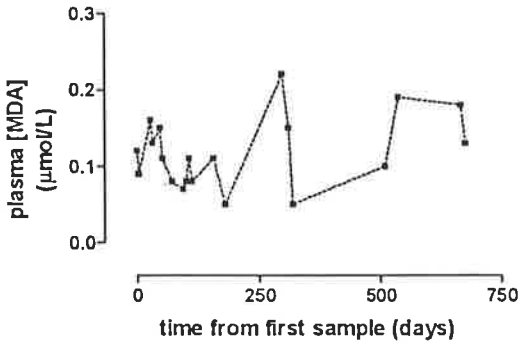


FIGURE 2.12 B Plasma MDA concentrations in 1 healthy individual with repeated sampling over time (n=20)

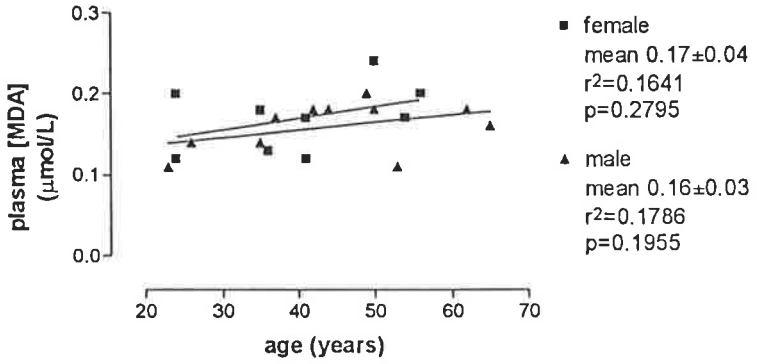


FIGURE 2.12 C Plasma MDA concentrations in healthy female and male human subjects of varying age

Results are shown in Table2.1. The sample numbers are too small for definitive statistical analysis, but show no obvious differences to the normal healthy population.

2.3.3 Malondialdehyde determination in Krebs buffer

2.3.3.1 Modifications of TBA test methodology

When coronary effluent from the isolated rat heart was the medium for MDA determination, samples were immediately frozen and stored at -20°C until assay within 2 weeks. Perchloric acid protein precipitation was only performed when plasma had been added to the perfusate. If polymorphonuclear cells were added to the perfusate, the effluent was spun at 13,000 rpm for 5 minutes and the cell free effluent utilised without further clean-up. All other samples were spun at 6,000 rpm for 5 minutes prior to the TBA test to precipitate out any extraneous particulate matter from the effluent. To improve assay sensitivity, 1000 µL of effluent was added to 750 µL 1% orthophosphoric acid and 250 µL TBA. Samples were assayed in unison, although the standard curve was determined with standards in triplicate. After 1 hour of boiling and cooling in ice, the samples' fluorescence intensities were read without further chloroform/methanol extraction. The standard curve was constructed using the coronary effluent, with the effluent blank subtracted from all standards and the linear regression curve forced through the origin. The relevant perfusate blank was subtracted from each rat coronary effluent sample.

2.3.3.2 Results

The standard curve was linear between 5 nmol/L and 200 nmol/L (Figure 2.13). The intra-assay coefficient of variation was 8.4 ± 5.3% for 5 nmol/L and 2.4 ± 1.2% for 200 nmol/L standards analysed from 7 standard curves. The inter-assay coefficient of variation was 8.1% assessing the variation of slope of the standard curve. Due to the addition 1 mL of sample to the TBA assay,

TABLE 2.1 Peripheral venous plasma MDA concentration in stable cardiac disease, compared with normal subjects. No significant differences were seen between groups.

Group	**No. in sample**	**mean age years±SD**	**plasma [MDA] (μmol/L±SD)**
normal	20	42 ± 13	0.16 ± 0.03
stable angina pectoris	6	68 ± 8	0.15 ± 0.04
cardiac failure	4	61 ± 13	0.18 ± 0.13

Legend: [MDA], concentration of malondialdehyde; No., number; μmol/L, micromoles/litre; SD, standard deviation.

the lower limit of detection of MDA was approximately 4 - 5 nmol/L.

2.3.3.3 Discussion

The coronary effluent from the isolated rat heart perfused with Krebs solution was cleaner, with less interfering substances than plasma. Therefore, clean-up of the sample prior to assay was unnecessary apart from the removal of particulate matter or cells by centrifugation. In fact this assay was more accurate for free MDA than plasma. Interfering substances could be fairly accurately accounted for by subtracting the inherent fluorescence of Krebs solution from all samples. The effect of interference from Krebs solution was apparent from the lower fluorescence intensity of an aqueous blank. The interfering substances in Krebs solution or coronary effluent had a small, variably decreasing effect on fluorescence intensity, as simultaneous aqueous, coronary effluent and Krebs solution standard curves converged at the high standards (figure 2.14).

2.4 HPLC quantitation of N-acetylcysteine, reduced and oxidised glutathione in plasma

2.4.1 Methods

Reagents included GSH, GSSG, NAC, d,l-penicillamine and dithiothreitol (Sigma Chemical Co. St Louis, MO, USA), heptanesulfonic acid (Alltech Assoc. Deerfield, IL, USA), chloroacetic acid, HPLC grade perchloric acid, methanol, and ethyl acetate (BDH Lab. Supplies. Poole, BH 15 ITD, UK), disodium EDTA (Ajax Chemicals (Auburn, NSW, Australia) and NaOH (BDH Analar. Kilsyth, Vic, Australia). Double glass-distilled water was used throughout. Stock solutions of 1 mmol/L GSH, 1 mmol/L GSSG, 5 mmol/L NAC and 1 mmol/L penicillamine in 2.7 mmol/L EDTA where stored at 4°C and replaced monthly. Standard solutions in mobile phase or water

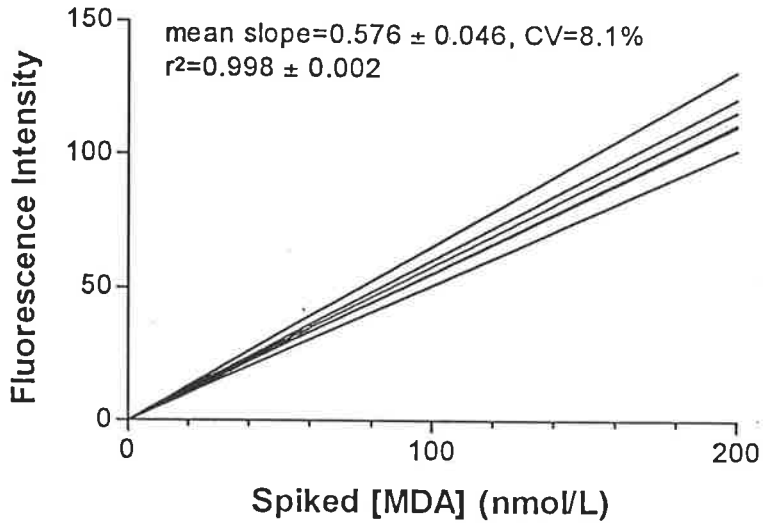


FIGURE 2.13 Standard curves with blank subtracted (n=7) for coronary effluent from Krebs-perfused isolated rat hearts

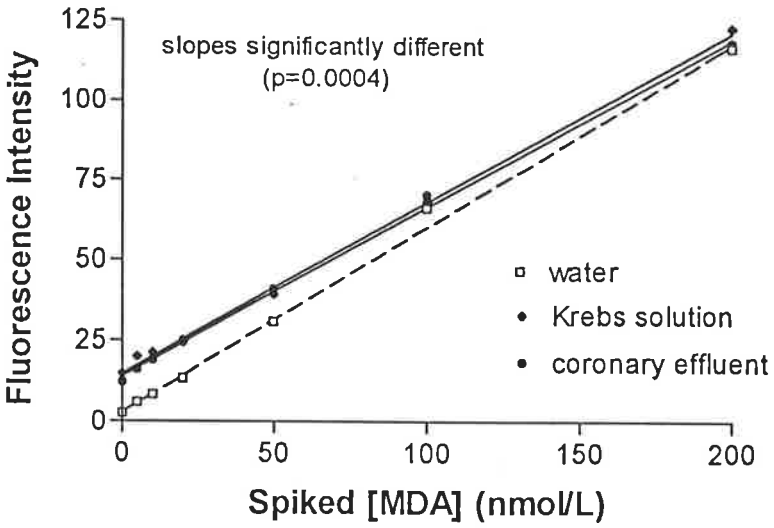


FIGURE 2.14 Comparison of MDA standard curves in water, Krebs solution and coronary effluent of Krebs-perfused isolated rat heart

were prepared from the stock solutions daily.

Plasma was obtained as in 2.3.1, but protein was immediately precipitated from 2 mL plasma alliquots with 400 µL of 2.3 mol/L perchloric acid containing 7 mmol/L dithiothreitol (Johansson and Lenngren, 1988). The supernatant was immediately frozen to -20°C and stored at -70°C until assay.

Prior to HPLC assay, 0.1 mL of 100 µmol/L penicillamine was added to 0.5 mL supernatant as an internal standard and excess dithiothreitol removed by the addition of 10 mL ethyl acetate. The mixture was vortex mixed for 2 minutes, then centrifuged at 4°C at 1,800 rpm for 10 minutes. The upper layer containing ethyl acetate/dithiothreitol was aspirated off and discarded, and stream nitrogen used to evaporate traces of ethyl acetate from the aqueous sample. The sample was again centrifuged at 15,000 rpm for 2 minutes. Ten µL of the cleaned supernatant was injected onto the column.

Separation of NAC, GSH and GSSG by HPLC was performed utilising a modification of a previously described method (Richie, Jr. and Lang, 1987). A Millipore Waters model 510 pump with a WISP 712 autosampler was used. The column was a 220 x 4.6 mm, 5 µm C_{18} Brownlee with a 3 cm x 4.6mm, 5 µm C_{18} Brownlee pre-column (Applied Biosystems), with a mobile phase consisting of 96% 50 mmol/L chloroacetic acid, 4% methanol and 3 mmol/L sodium heptanesulphonate, adjusted to pH 3 with fresh concentrated NaOH. Separation occurred at ambient temperature with flow rate of 1 mL/min resulting in a back pressure of 2500 psi. The mobile phase was continuously recirculated and changed weekly. An electrochemical detector (Coulochem II, model 5200, ESA Inc, Bedford, MA,USA), equipped with a dual high sensitivity analytical cell containing two porous graphite electrodes (E_1 and E_2) in series, was used to provide

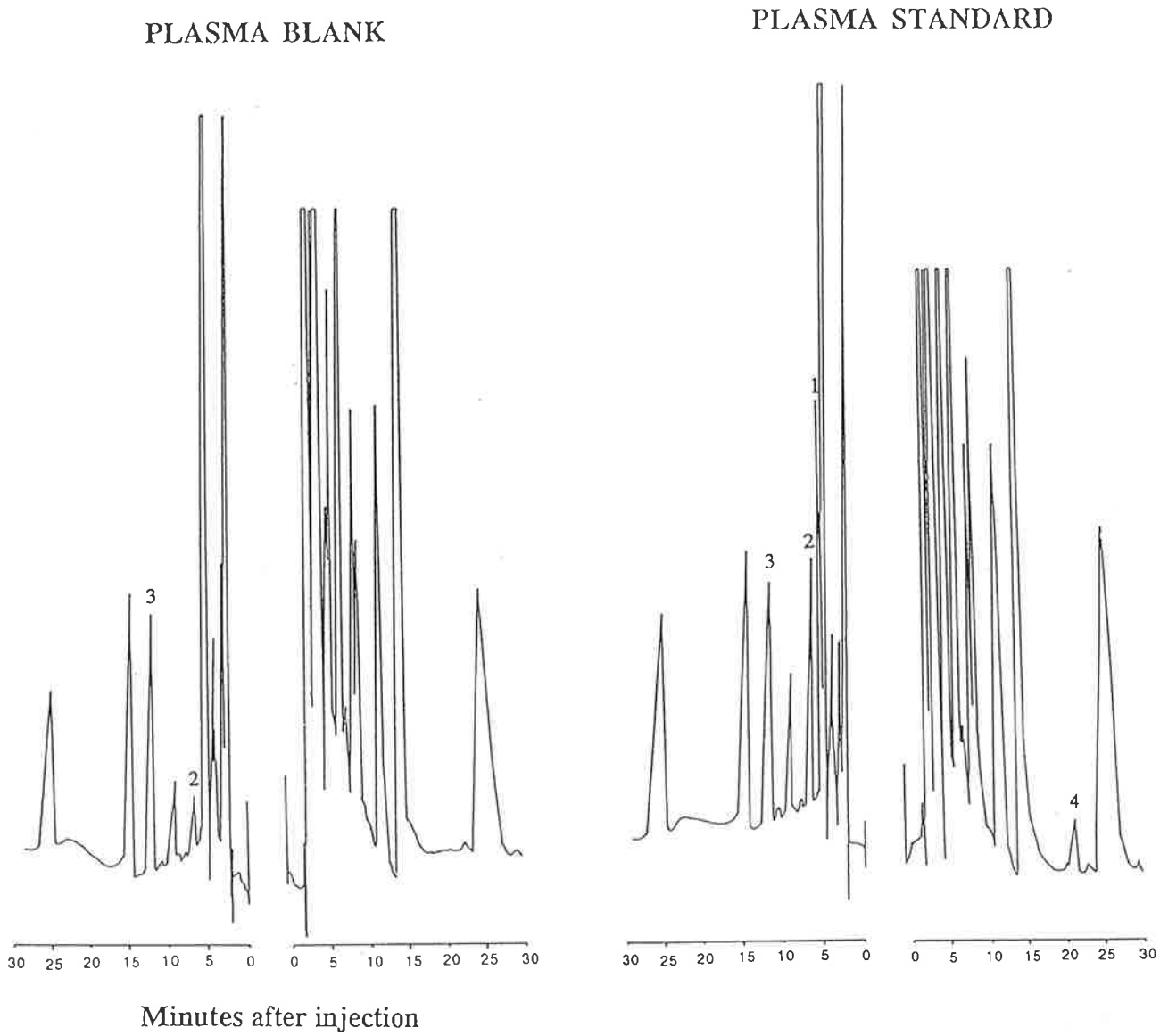
potential for the generation of current from the species of interest (Krien et al. 1992). A single large porous graphite electrode with reference electrode acted as a guard cell (model 5020). It was placed between the pump and injector, oxidising or reducing electroactive materials in the mobile phase to reduce background current. The applied electrode potential for E_1 was +0.75 V and detected NAC, GSH and penicillamine. GSSG was detected with E_2 at a potential of +0.9V, whereas the guard cell potential was held at +0.95 V. Both electrodes were set at a range of 2 µA, 100 mV output and the resultant signal recorded with a dual pen chart recorder (Rikadenki Kogyo Co., Tokyo, Japan) using detection outputs of 100 mV and 10 mV for E_1 and E_2 respectively.

2.4.2 Results

Typical retention times for NAC, GSH, GSSG and penicillamine were 5.8, 7, 25 and 14 minutes respectively (see Figure 2.15). The resultant profiles were quantified on the basis of peak height ratios of each substance to penicillamine for both plasma standards and unknowns. Thresholds (three times baseline noise) for the detection in plasma of GSSG and NAC were 0.05 µmol/L and 12.5 µmol/L respectively. Due to the presence of endogenous GSH in plasma, the limit of detection for this thiol was determined in non-extracted solution, and found to be 0.19 µmol/L. For purposes of calculation of a GSH to GSSG ratio, a value of 0.04 µmol/L was arbitrarily assigned to all GSSG plasma concentrations < 0.05 µmol/L.

The plasma standard curve for GSH, GSSG and NAC was linear in the concentration range utilised. Recovery of the measured thiols in 5 samples were 98.7 ± 1.1 (standard error of mean) %, 97.8 ± 1.7% and 14 ± 0.4% for GSH, GSSG and NAC respectively. Intra-assay coefficient of variation for 5 samples was 1.5% for 5 µmol/L GSH, 1.5% for 0.4 µmol/L GSSG and 3.6% for 100 µmol/L NAC. Inter-assay coefficient of variation for 4 different sample runs were 5.6% for 2.5 µmol/L GSH, 7.4% for 0.2 µmol/L GSSG and 1.6% for 100 µmol/L NAC.

FIGURE 2.15 Representative chromatograms of a plasma blank and plasma standard spiked with NAC 10 µmol/L, GSH 5 µmol/L, penicillamine 16.7 µmol/L and GSSG 0.4 µmol/L. (1) NAC, (2) GSH, (3), penicillamine at E_1 and (4) GSSG at E_2.



2.4.3 Discussion

This methodology appeared to give adequate sensitivity and specificity for the purposes of the human studies carried out within this thesis. The lower limit of detection using the coulometric detector was better than that achieved in previous assays utilising an amperometric detector (Richie, Jr. and Lang, 1987; Allison and Shoup, 1983). An amperometric detector has the mobile phase and sample flowing by the electrode surface. As a consequence only 5-15% of the electrochemically active substance reacts with the electrode, while most of the sample passes the electrode without reacting. In contrast, the coulometric detector is designed so that the eluent flows through a porous graphite electrode. This allows almost all of the electrochemically active species to react with the electrode, improving sensitivity without increased noise in the system. This important improvement made the assessment of glutathione redox status in plasma feasible.

Recovery of GSH and GSSG was almost 100%, indicating no autoxidation during the assay period. The low recovery of NAC was due to losses during ethyl acetate extraction. However, this did not effect accurate assessment of NAC concentration as plasma concentrations in the patient samples remained well above the lower limit of detection.

In previous similar assays (Richie, Jr. and Lang, 1987; Meister and Anderson, 1983) perchloric acid was considered to alter the GSH:GSSG ratio and interfere in the chromatographic peaks of interest. This was not the case here, with excellent recoveries of GSH and GSSG and no interfering peaks noted. The major interfering substances were dithiothreitol and EDTA. The ethyl acetate extraction was effective in eliminating most of these substances prior to injection, thereby limiting the size of the interfering peaks.

The disadvantage of using this method is that it is very labour intensive. Obsessive attention must

be given to the quality of the chromatography by fine adjustment the pH and methanol content of the mobile phase. Furthermore, regular cleaning of the graphite electrodes with 6 mol/L nitric acid, followed by water and 100% methanol of the whole system, with subsequent re-calibration of the electrodes, is necessary to remove electrode surface coating with lipophilic and organic particles which cause a decrease in signal generation over time. This limits the number of samples that can be processed. Therefore, careful choice and timing of samples during any research protocol was important to clearly delineate any trends associated with glutathione metabolism in the subjects studied.

2.5 Determination of plasma lactate

Plasma lactate determination was performed in The Clinical Chemistry Department of The Queen Elizabeth Hospital, utilising a colourmetric method developed within the department (Buttery et al, 1985). Intrassay and interassay coefficient of variation were 2.8% and 9.3% respectively.

Chapter 3:

Isolated Perfused Rat Heart Model of Myocardial Ischaemia and Reperfusion: Correlation with Lipid Peroxidation and Haemodynamic Effects of N-Acetylcysteine.

3.1 Haemodynamic effects and release of malondialdehyde from the isolated perfused rat heart associated with oxidative stress

3.1.1 Background

The demonstration of increased free radicals within the myocardium during reperfusion after myocardial ischaemia has been well documented in many species (Maupoil and Rochette, 1988; Bolli et al. 1988a; Garlick et al. 1987; Grech et al. 1993; Ambrosio et al. 1993). Furthermore, depletion of myocardial antioxidants has been demonstrated during this setting of myocardial ischaemia and reperfusion (Ceconi et al. 1988). Lipid peroxidation is widely believed to be a consequence of free radical oxidation of PUFAs, with subsequent production of a wide variety of stable products such as MDA (see1.4), which may then act as biochemical markers of this increased free radical activity. The Langendorff-perfused isolated heart model has frequently been utilised, with biochemical effects of oxidative stress assessed by evaluating changes in either the myocardial tissue or coronary effluent. Furthermore, haemodynamic consequences of oxidative stress may be measured in the isolated heart by the insertion of a fluid filled balloon in the left ventricle which would also ensure that the ventricle contracts against a load (Ceconi et al. 1988; Tavazzi et al. 1992b). Utilising this model in this chapter, a better understanding of the determinants of oxidative stress and reperfusion injury in the rat have been sought.

3.1.2 Aims

The aims of this series of investigations were to:

(1) Determine the extent of the lipid peroxidation product, MDA released by rat myocardium during different forms of oxidative stress.

(2) Determine the extent of the lipid peroxidation product, MDA release by rat myocardium

during reperfusion after myocardial ischaemia.

(3) Determine the correlation between lipid peroxidation and extent of myocardial left ventricular dysfunction associated with myocardial ischaemia and reperfusion and/or oxidative stress in the rat heart.

3.1.3 Methods

3.1.3.1 Dissection and catheterisation of the rat heart

Male or female Sprague-Dawley rats weighing 390 ± 130 g were anaesthetised with 0.15 ml/100g of 60 mg/mL intraperitoneal injection of sodium pentobarbitol (Ceva Chemicals, Hornsby, Australia). The heart was rapidly excised through a sternal excision and placed in ice cold Krebs solution. This consisted of 119 mmol/L NaCl, 5.36 mmol/L KCL, 1.20 mmol/L KH_2PO_4, 25.0 mmol/L $NaHCO_3$, 5.56 mmol/L glucose, 2.50 mmol/L $CaCl_2$, 0.95 mmol/L $MgCl_2$, 0.01 mmol/L EDTA (di-sodium salt). The perfusate was bubbled with 95% O_2 / 5% CO_2, resulting in a final pH of 7.4. The ascending aorta was opened and canulated with a 5 cm length of 2 mm polypropylene tubing. The cannula was pushed into the left ventricle (LV), securely tied around the ascending aorta, then the cannula pulled back until the tip was in the aorta. An incision was made in the right ventricle to assist drainage of coronary effluent. A fluid filled 7 F Swan-Ganz single lumen catheter (Baxters, Irvine, California USA) was inserted via an incision in the left atrium, through the mitral valve to the left ventricle. The balloon was inflated with water to a volume of 0.2 to 0.4 mL, depending on the size of the heart, left ventricular diastolic pressure and to avoid damped left ventricular pressures. The heart was then flushed via the aortic cannula with ice cold Krebs solution prior to perfusion with Krebs solution at 37°C via the Langendorff mode (Langendorff, 1895) at a constant flow of 10 mL/min utilising a Minipuls 3 rotary pump (Gilson, Villiers Le Bel, France), a 37°C recirculating water bath warming the Krebs solution reservoir and heating coil

with bubble trap beyond the pump. Both left ventricular (via the lumen of the Swan Ganz catheter) and perfusion pressures were recorded via fluid filled tubes to two pressure transducers attached to a chart recorder (Rikadenki Kogyo Co., Tokyo, Japan). Heart rate was derived from the left ventricular trace.

3.1.3.2 Protocol for the induction of myocardial hypoxia or global ischaemia followed by reperfusion

After dissection of the rat heart and perfusion in the Langendorff mode, the heart was allowed to stabilise for at least 30 minutes, adjusting the left ventricular balloon volume if necessary to try to minimise left ventricular diastolic pressure. If the heart developed sustained ventricular fibrillation, the LV was flushed with 2 to 10 mLs ice cold Krebs solution via the Swan Ganz catheter to restore sinus rhythm. LV systolic, diastolic and developed pressures (LV systolic - LV diastolic pressure) and perfusion pressure (PP) were continuously recorded throughout the experiment. Heart rate was measured at least every 5 minutes . Coronary effluent was sampled at least every five minutes during the equilibration period and immediately frozen to -20°C. This effluent was later assayed for MDA concentration as previously described in 2.2.1.

Myocardial hypoxia was achieved by perfusing the heart with Krebs solution at 37°C bubbled with 95% N_2 / 5% CO_2. Myocardial ischaemia was produced by either decreasing coronary flow rate to 2.1 mL/min or stopping coronary flow, keeping the heart at 37°C. The period of hypoxia or ischaemia varied according to the experimental protocol utilised.

In one set of experiments an additional metabolic stress of 10 minutes of hypoglycaemia was introduced by infusing the heart for the 10 minutes prior to stopping the coronary flow for 30 minutes with Krebs solution containing no glucose. The heart was then reperfused with normal

Krebs solution for 30 minutes.

Following the myocardial stress, coronary flow was restored at 10 mL/min and the heart reperfused for at least 30 minutes. If after greater than 10 minutes of reperfusion the heart remained in sustained ventricular fibrillation, ice cold Krebs solution was flushed into the left ventricle in an attempt to restore sinus rhythm. This was performed at least one minute before coronary effluent sampling for measurement of MDA concentration. Sampling occurred at frequent intervals over the first five minutes of reperfusion, then five to ten minutely thereafter.

3.1.3.3 Protocol for infusion of human neutrophils combined with global ischaemia followed by reperfusion

The Langendorff perfused rat heart was allowed to equilibrate with Krebs solution for at least 25 minutes as previously described in 3.1.3.1 and 3.1.3.2.

Human neutrophils were isolated by a method previously described by Parente *et al* (Parente et al. 1989). Neutrophil count was determined by a Coulter counter (Coulter Electronics, Harpenden Herts, England). The neutrophils were prepared on the same day as preparation and stored at room temperature until infusion. As the neutrophil count/mL was high there was a tendency for the cells to aggregate. This was minimised by constant gentle stirring of the cell suspension on a magnetic stirrer.

In the first experiment, rat plasma was obtained by withdrawing blood from a rat heart via needle puncture after sternal incision into a 10 mL syringe containing 2 mL 4.5% EDTA. The total of 10 mL blood/EDTA was then immediately spun for 15 minutes at 3000 rpm to obtain plasma. Five percent rat plasma and a final concentration of 500,000 cells/mL neutrophils were mixed just prior

to use and infused, firstly as a test dose for one minute, 25 minutes into the equilibrium period. LV function was temporarily depressed by this infusion but recovered to baseline over approximately 5 minutes after restoration of normal Krebs solution, which was infused for another 12 to 15 minutes. The neutrophil/plasma/Krebs suspension was again infused for ½ minute prior to cessation of coronary flow for 30 minutes. At reperfusion, the neutrophil/plasma/Krebs suspension was infused a further 10 minutes followed by normal Krebs solution for 30 minutes. Activation of neutrophils was determined by comparison of the ability of the neutrophil/plasma/Krebs suspension to reduce cytochrome C in the absence and presence of 4 nmol/L fMLP prior to infusion through the heart and as the coronary effluent of a non-stressed and stressed heart.

In the next set of experiments, human neutrophils were activated by 2μmol/L PMA five minutes prior to diluting the suspension with Krebs solution to a final neutrophil concentration of 400,000 to 500,000 cells /mL and infusing the equilibrated rat heart for one minute prior to 30 minutes of cessation of coronary flow. More neutrophils were activated then diluted five minutes prior to reperfusion of the heart with this PMA/neutrophil/Krebs suspension for 9 to 10 minutes followed by normal Krebs for a further 50 minutes.

3.1.3.4 Protocol for the infusion of an oxygen-derived free radical flux

Two oxygen-derived free radical fluxes were used. In each set of experiments of three rats each, 300 μmol/L xanthine (Sigma, St Louis, Mo, USA) was added to the Krebs perfusate. Xanthine was prepared as a 30 mmol/L solution in 100 mmol/L NaOH. Ten mL of this solution was then added to the Krebs with a final pH remaining at 7.4. The rat heart was perfused with normal Krebs perfusate for a 30 minute equilibrium period.

In the first set of experiments, a superoxide flux was produced by adding xanthine oxidase (Sigma,

St Luois, Mo, USA) (final dilution of 2mU/mL) to the Krebs/xanthine solution and perfused through the heart for 30 minutes before returning to normal Krebs for a further 30 minutes. The size of the flux was not measured. Coronary effluent was sampled intermittently throughout the experiment for assessment of MDA and creatine kinase concentration.

In the second set, an hydroxyl radical flux was produced. 100 µmol/L ferric chloride dissolved in glass distilled water with 100 µmol/L EDTA was added to the Krebs perfusate. After 30 minutes equilibrium, 300 µmol/L xanthine and 2mU/mL xanthine oxidase was added to the perfusate. Coronary effluent was sampled intermittently throughout for MDA, but not creatine kinase.

3.1.4 Statistical Analysis

For the purpose of comparison between groups, area under the curve (AUC) of the coronary effluent MDA concentration versus time was utilised. In the experiments involving myocardial ischaemia and reperfusion, the baseline for this AUC was the defined as the mean of the 3 baseline points and the point at 30 minutes reperfusion in order to normalise different baseline MDA concentrations between experiments. In the experiments involving a radical species flux, a separate AUC was calculated for the 30 minute equilibrium period and the 30 minutes free radical infusion period, with the baseline at Y = 0. Left ventricular systolic function was estimated by the measurement of left ventricular developed pressure (LV systolic pressure - LV diastolic pressure, LVDP). All values were calculated as a percentage of the mean baseline value. Left ventricular diastolic function was estimated by the measurement of LV diastolic pressure as absolute values (LVDias). Comparisons between groups were made by calculating the change in LVDias from the mean baseline value at 30 minutes reperfusion. All results were expressed as mean and standard deviation (SD), although all graphs utilised mean and standard error of mean. Comparisons

between groups were made with the non-paired t test. Two tailed statistical significance was defined as $p < 0.05$.

3.1.5 Results

3.1.5.1 Cardiac haemodynamics during myocardial ischaemia and reperfusion

The effects of anoxia, ischaemia alone, ischaemia in combination with a metabolic stress or ischaemia in combination with a neutrophil infusion on cardiac haemodynamics are summarised in Table 3.1 and Figures 3.1 to 3.8. Generally, anoxia resulted in a slower development of LV dysfunction than total global ischaemia. During anoxia, LVDias gradually rose and continued to increase after reoxygenation. In contrast, LVDP did not alter significantly during the anoxic period but rapidly decreased on reoxygenation. There was a trend towards a direct correlation between the severity of myocardial ischaemia and subsequent left ventricular systolic and diastolic dysfunction. Also, LV diastolic dysfunction tended to be more severe than systolic. Perfusion pressure failed to return to baseline, probably as a result of myocardial oedema secondary to increased coronary vascular resistance related to myocardial cellular damage, the osmotic effects on the myocardium of Krebs solution over time, and increased small vessel endothelial dysfunction.

When total global ischaemia was utilised as the myocardial stress, a 5 minute duration of ischaemia resulted in a 90% recovery in LVDP, with complete recovery of increased LVDias and no significant change in PP. However, at 15 minutes ischaemia continuing LV systolic and diastolic dysfunction were apparent after 30 minutes reperfusion. At 60 minutes ischaemia, there was no recovery of systolic function and high LVDias, suggesting extensive myocardial necrosis with ischaemic contracture secondary to the protracted ischaemic insult.

TABLE 3.1 Summary of haemodynamic effects and release of MDA by the isolated rat heart after a variety of myocardial stresses. (results mean ± SD)

Experimental protocol	No. of hearts	MDA release (AUC)	Haemodynamic Parameters After 30 minutes Reperfusion		
			Recovery of LVDP (% baseline)	δLVDias (mmHg)	Perfusion Pressure (% baseline)
30 min anoxia	2	427 ± 164	75 ± 9	15 ± 14	131 ± 29
15 min total global ischaemia	3	232 ± 106	69 ± 36	25 ± 14	145 ± 49
30 min total global ischaemia	12	154 ± 53	40 ± 18	25 ± 14	109 ± 14
60 min total global ischaemia	2	138 ± 50	0 ± 0	55 ± 37	129 ± 38
10 min no glucose + 30 min total global ischaemia	6	316 ± 63*	12 ± 18†	42 ± 23‡‡	144 ± 27††
PMN/plasma + 30 min total global ischaemia	2	64 ± 17	42 ± 45	18 ± 14	132 ± 10
Activated PMN + 30 min total global ischaemia	6	215 ± 70‡	16 ± 12¥	45 ± 25#	186 ± 72**

Legend: AUC, area under the curve; δLVDias, change in left ventricular diastolic pressure from baseline; LVDP, left ventricular developed pressure; MDA, malondialdehyde; min, minutes; mmHg, millimetres of mercury; No., number; PMN, polymorphonuclear neutrophils; U, units. (*p<0.0001, ‡p=0.054, †p=0.007, ¥p=0.01, ‡‡p=0.07, #p=0.0.048, ††p=0.006, **p=0.002 versus 30 minutes total global ischaemia)

Depletion of glucose had a profound effect on cardiac haemodynamics with elevation of LVDias ($p = 0.07$ versus 30 minutes total global ischaemia) and decreased percentage recovery of LVDP ($p = 0.007$ versus 30 minutes total global ischaemia).

The infusion of human neutrophils/rat plasma did not cause increasing LV dysfunction as compared to the same period of ischaemia without this infusion. The results may have been influenced by a loss of neutrophil effect secondary to several factors including mild haemolysis of the rat plasma during preparation and aggregation of neutrophils. The PMN cells were responsive to fMLP stimulation as seen by superoxide reduction of cytochrome C on UV spectrophotometry (DU 650 Spectrophotometer, Beckman, USA) prior to infusion into the rat. During reperfusion, the neutrophil/rat plasma/Krebs coronary effluent demonstrated no measurable spontaneous superoxide production or response to fMLP. However, there was extensive aggregation and precipitation of the neutrophils during the assessment of cytochrome c reduction. With this loss of an even suspension of neutrophils in the cuvette, adequate UV analysis was not possible, and prevented a true assessment of the status of the neutrophils.

The infusion of activated PMN in combination with 30 minutes total global ischaemia was associated with profound LV dysfunction during the reperfusion period. The effects were significantly more marked than those occurring in the absence of PMN (%LVDP, $p = 0.01$, δLVDias, $p = 0.048$, %PP, $p = 0.002$ versus 30 minutes total global ischaemia). Furthermore, the technical limitations of PMN aggregation were predominantly overcome.

3.1.5.2 Myocardial MDA release during myocardial ischaemia and reperfusion.

At equilibrium there was either no or a small flux of MDA from the myocardium. During anoxia, low flow ischaemia or depletion of glucose (Figures 3.1, 3.2, 3.6), the MDA flux decreased often

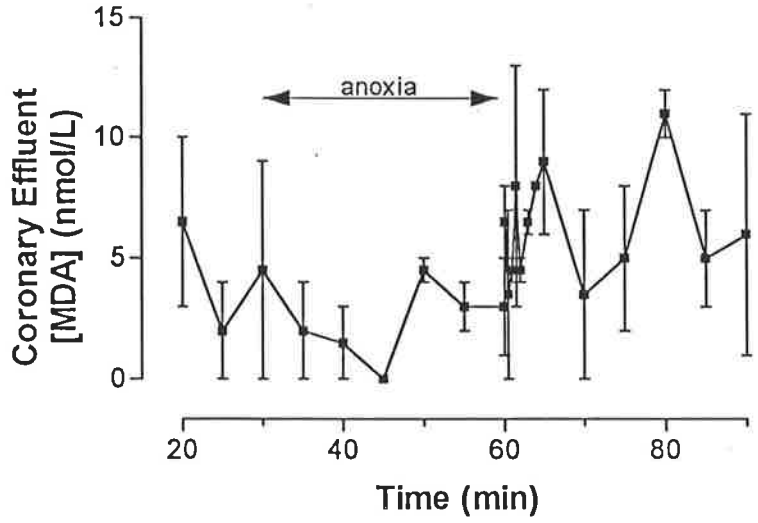


FIGURE 3.1 A Coronary effluent MDA concentration. 30 minutes anoxia. (n=2)

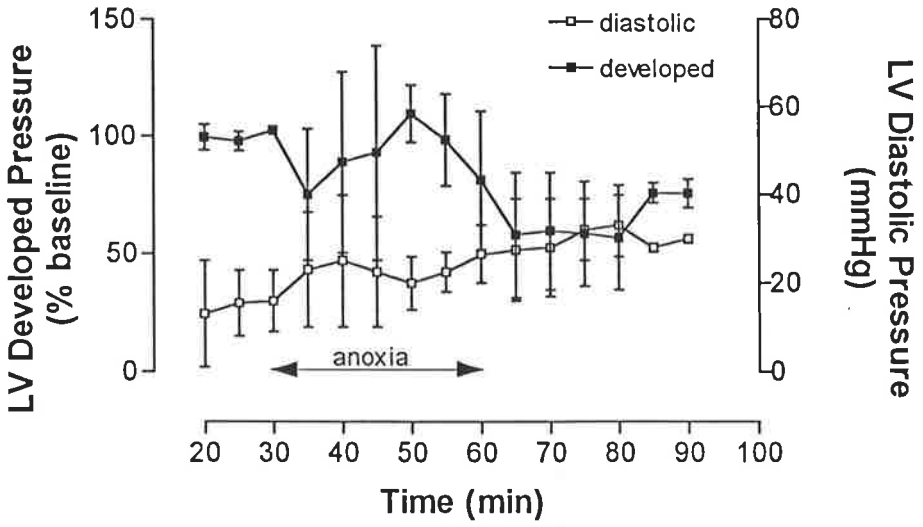


FIGURE 3.1 B Left ventricular pressures. 30 minutes anoxia (n=2)

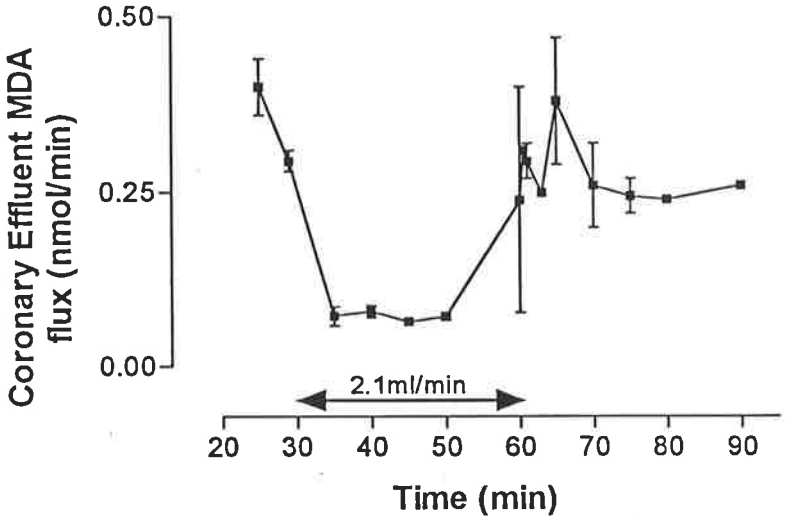


FIGURE 3.2 Coronary effluent MDA concentration. 30 minutes 80% reduction coronary flow. (n=2)

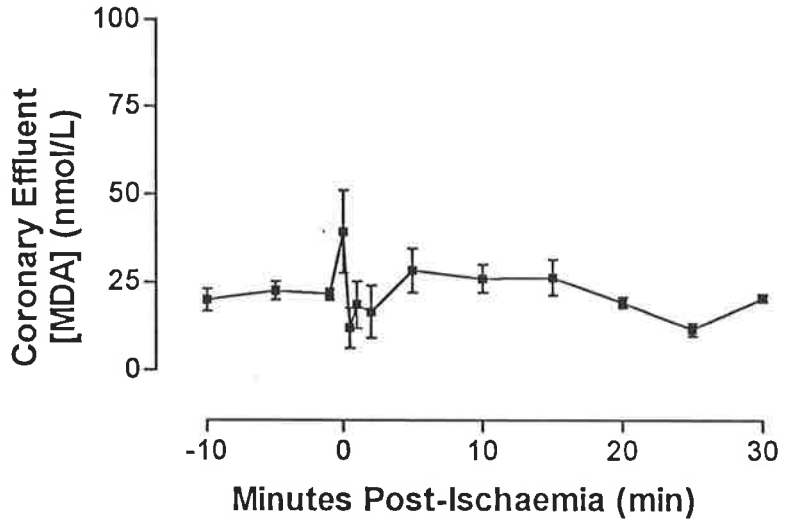


FIGURE 3.3 A Coronary effluent MDA concentration. 15 minutes total global ischaemia. (n=3)

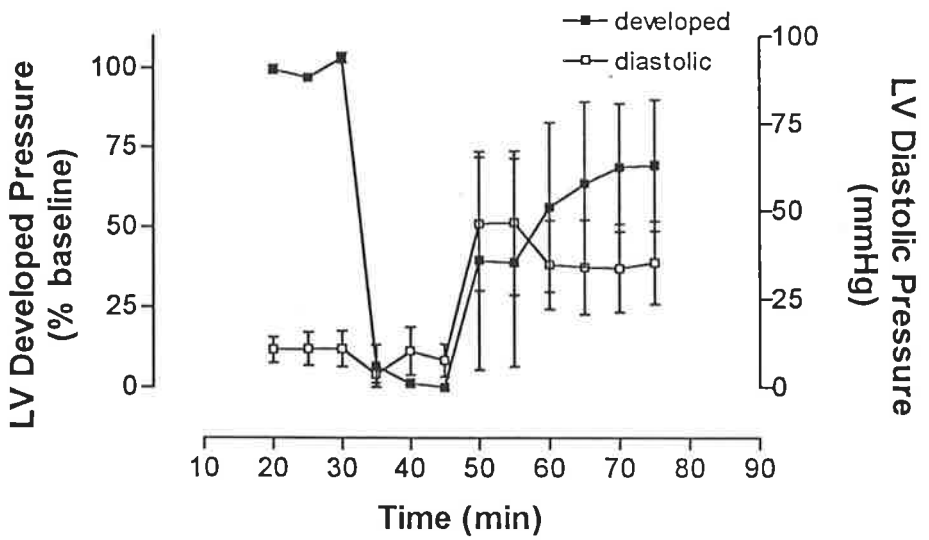


FIGURE 3.3 B Left ventricular pressures. 15 minutes total global ischaemia (n=3).

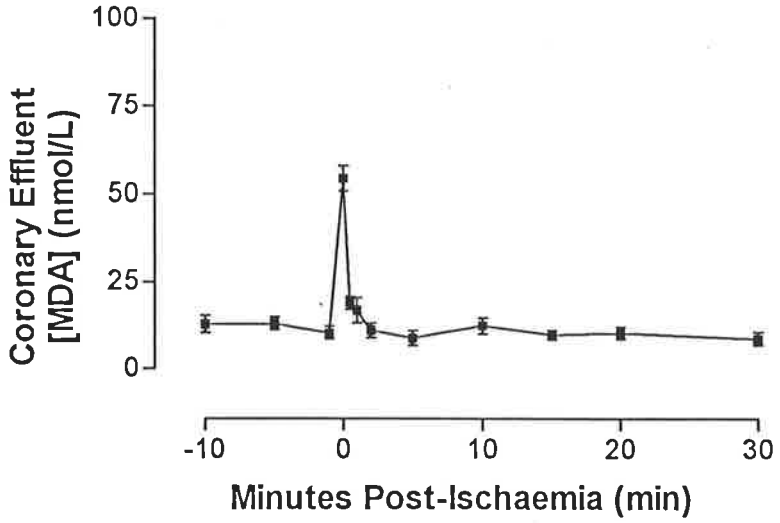


FIGURE 3.4 A Coronary effluent MDA concentration. 30 minutes total global ischaemia. (n=12)

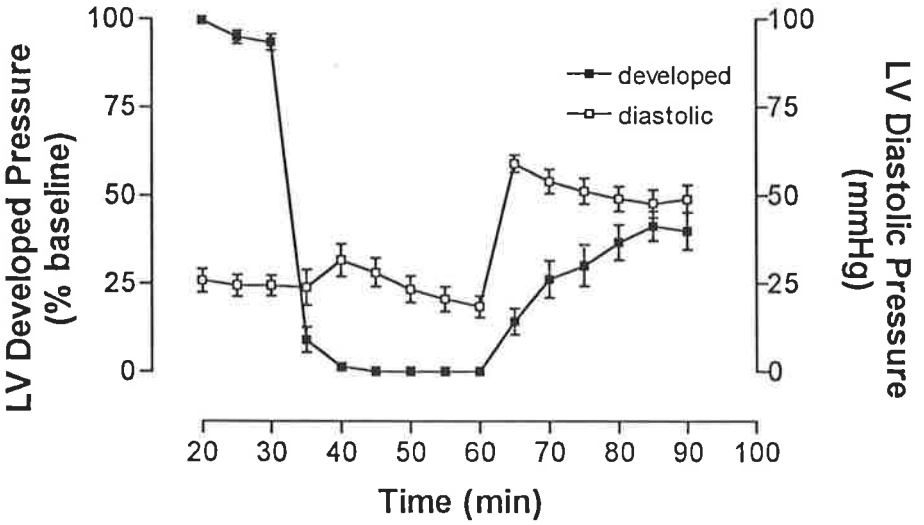


FIGURE 3.4 B Left ventricular pressures. 30 minutes total global ischaemia (n=12).

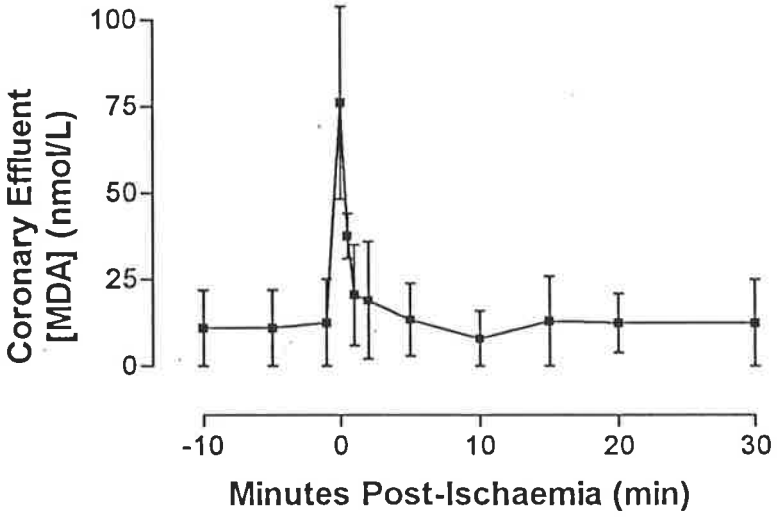


FIGURE 3.5 A Coronary effluent MDA concentration. 60 minutes total global ischaemia. (n=2)

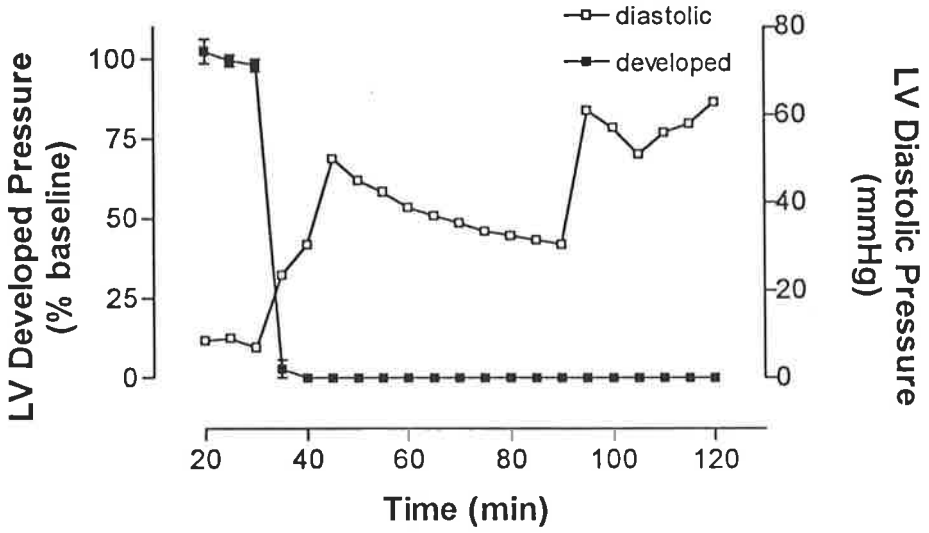


FIGURE 3.5 B Left ventricular pressures. 60 minutes total global ischaemia. (n=2)

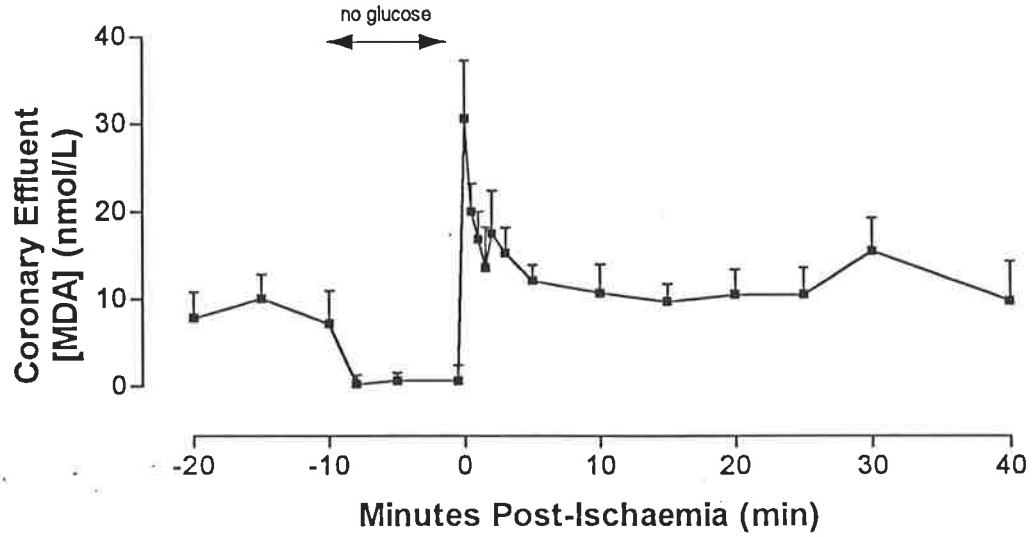


FIGURE 3.6 A Coronary effluent MDA concentration. 10 minutes glucose-free Krebs perfusate prior to 30 minutes total global ischaemia. (n=6)

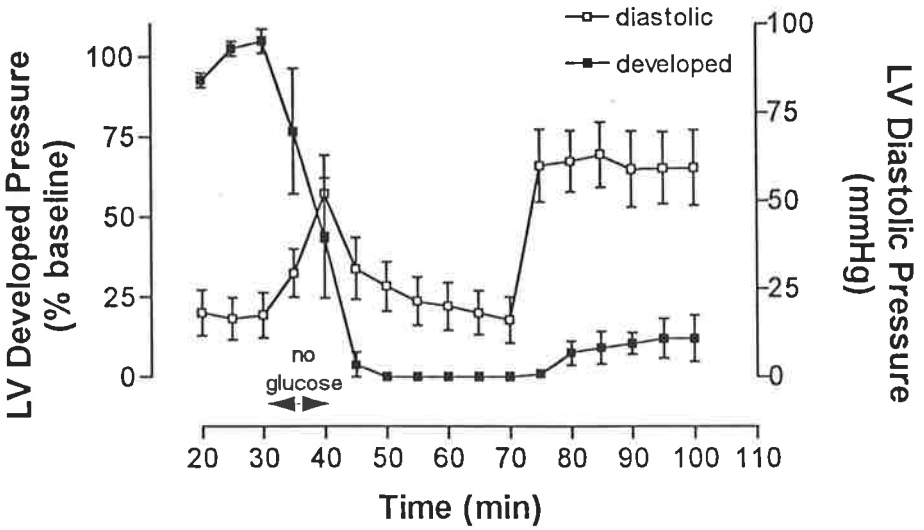


FIGURE 3.6 B Left ventricular pressure. 10 minutes glucose-free Krebs perfusate prior to 30 minutes total global ischaemia. (n=6)

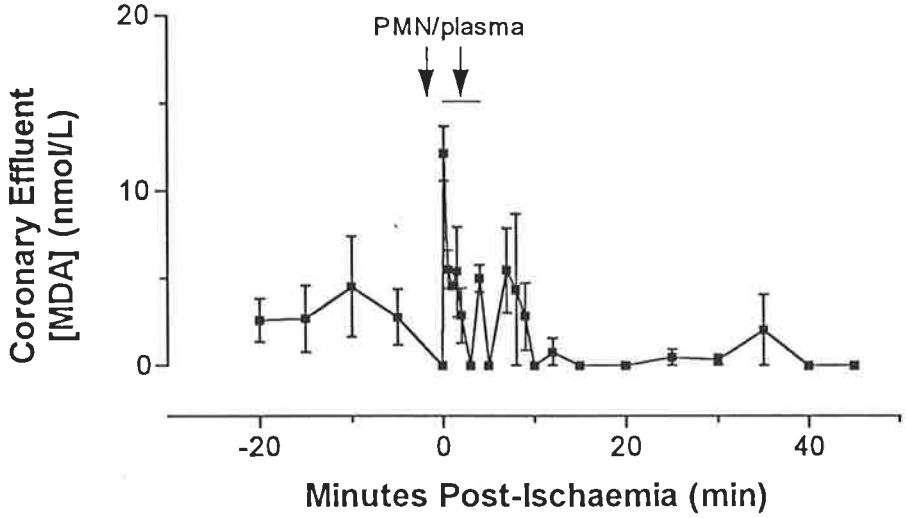


FIGURE 3.7 A Coronary effluent MDA concentration. Human polymorph/rat plasma infusion prior to and following 30 minutes total global ischaemia. (n=2)

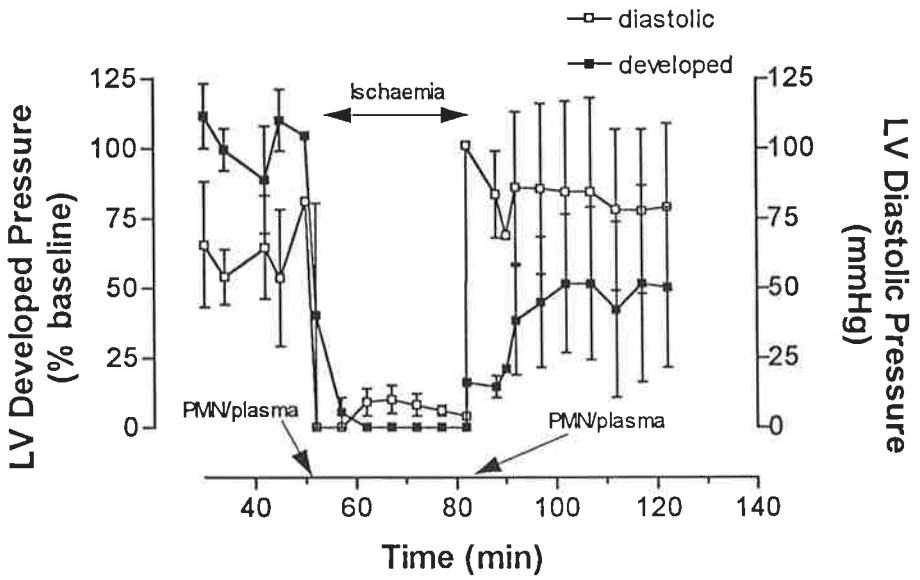


FIGURE 4.7 B Left ventricular pressures. Human polymorph/rat plasma infusion prior to and following 30 minutes total global ischaemia. (n=2)

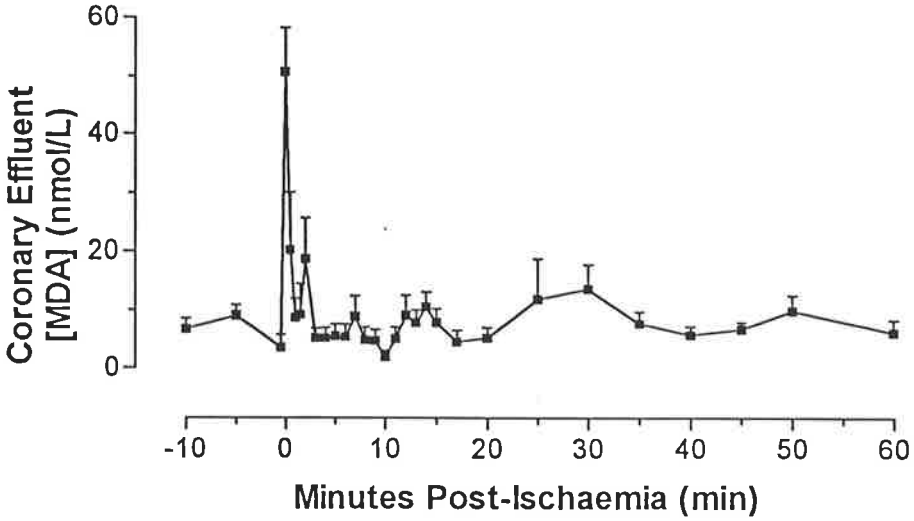


FIGURE 3.8 A Coronary effluent MDA concentration. PMA-activated human polymorph infusion prior to and Following 30 minutes total global ischaemia. (n=6)

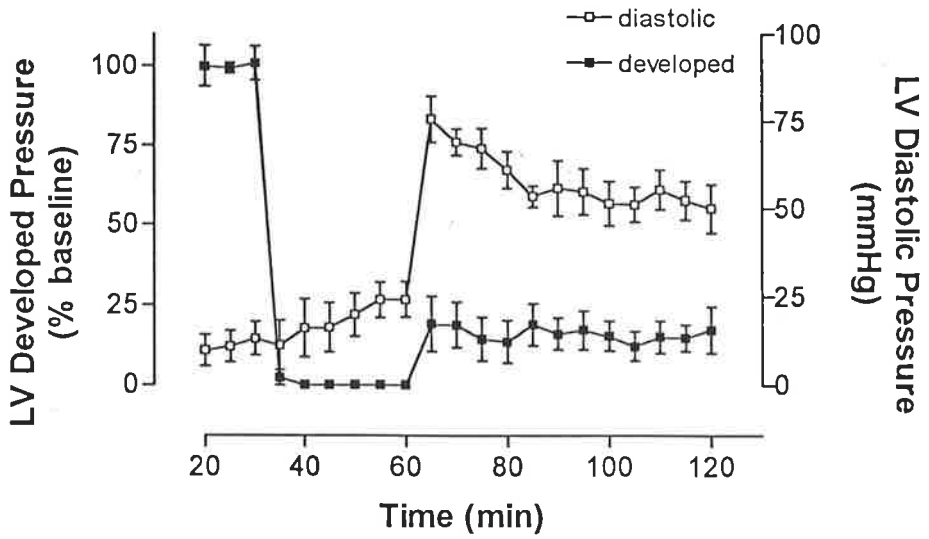


FIGURE 3.8 B Left ventricular pressure. PMA-activated human polymorph infusion prior to and following 30 minutes total global ischaemia (n=6).

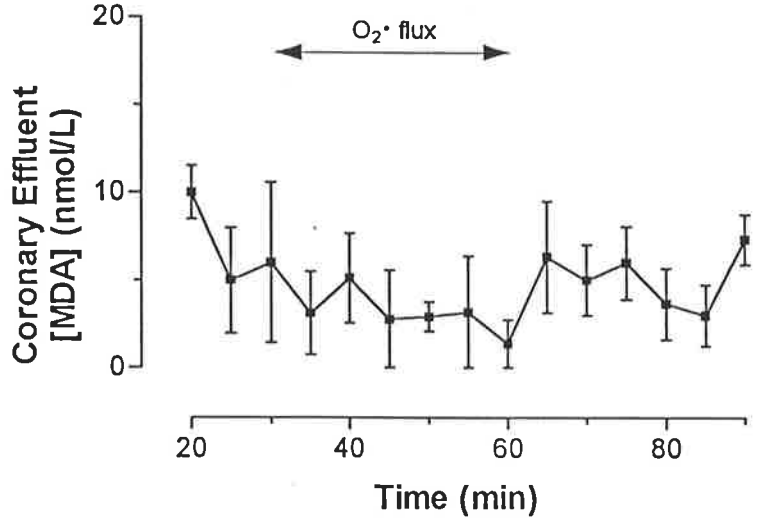


FIGURE 3.9 A Coronary effluent MDA concentration. 30 minute perfusion with a superoxide flux. (n=3)

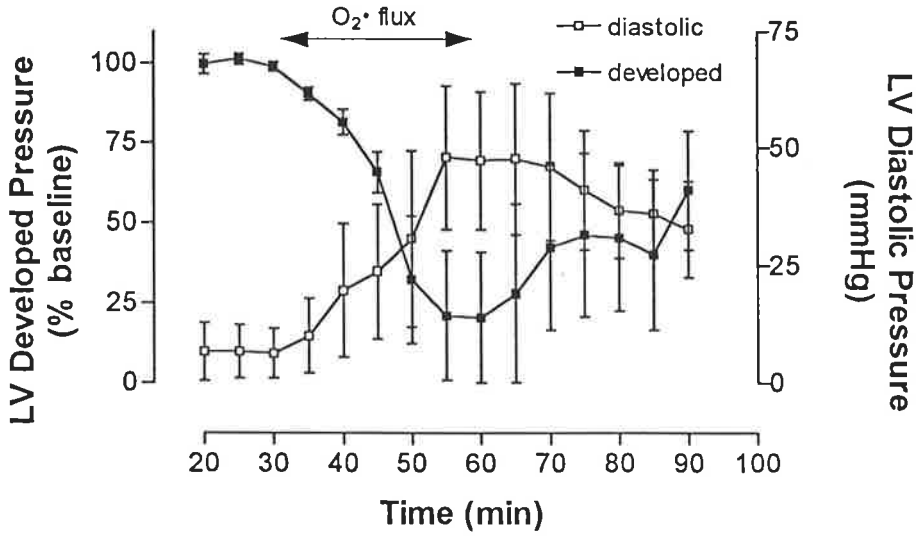


FIGURE 3.9 B Left ventricular pressures. 30 minute superoxide flux. (n=3)

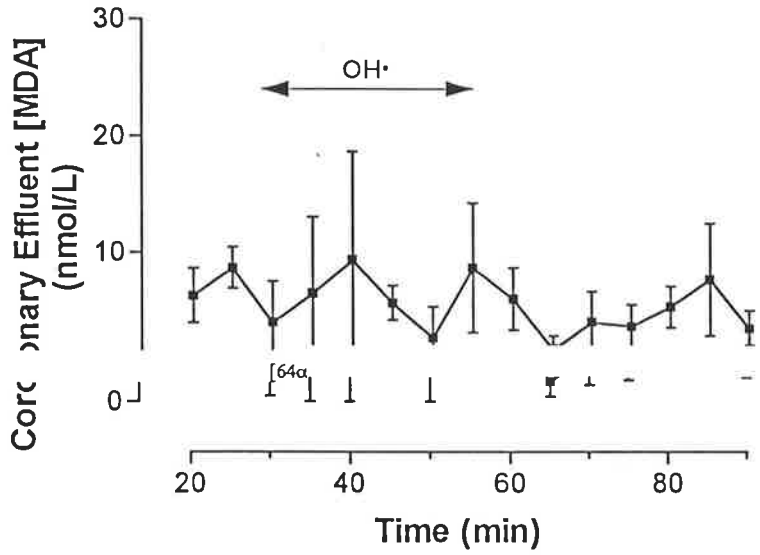


FIGURE 4.10 A Coronary effluent MDA concentration. 30 minute infusion of hydroxyl radical flux. (n=3)

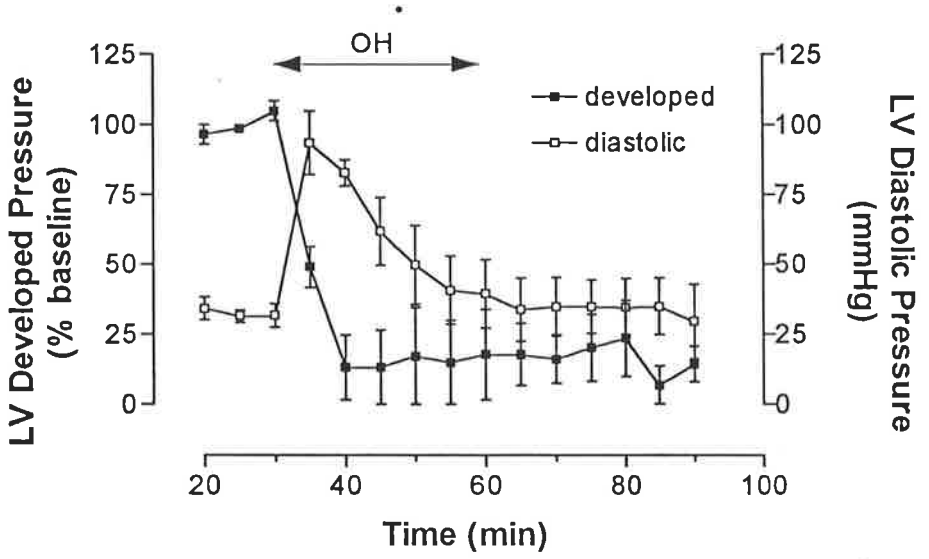


FIGURE 4.10 B Left ventricular pressures. 30 minute infusion hydroxyl radical flux (n=3).

to below the limit of detection of the assay (< 1 nmol/L, and designated a zero value). If total global ischaemia was utilised, a washout of accumulated myocardial MDA occurred within the first 1 minute of reperfusion, accounting for the large peak at this time. However, there was no sustained flux of MDA in the reperfusion period.

Inflicting more oxidant stress on the heart over and above total global ischaemia produced a larger release of MDA from the myocardium. In particular, 10 minutes of hypoglycaemia prior to ischaemia was associated with a significantly increased release of MDA (316 ± 63 versus 154 ± 53, $p < 0.001$). The infusion of activated PMN prior to and after reperfusion was also associated with a similar trend but did not quite reach statistical significance (215 ± 70 versus 154 ± 53, $p = 0.054$). This increased release of myocardial MDA during reperfusion was associated with increased left ventricular dysfunction. Despite these results demonstrating the capability of the isolated Krebs-perfused rat heart to release MDA, the signal remained relatively small and of very short duration.

3.1.5.3 Effects of infusion of an oxygen-derived radical species flux

These results are tabulated in Table 3.2 and Figures 3.9 and 3.10. In the case of the superoxide infusion, there was a marked decrease in LVDP and increase in LVDias, which partially recovered after reperfusion of normal Krebs solution. The hydroxyl radical flux was associated with more rapid deterioration of LVDP than the superoxide flux, a transient rise in LVDias, a large sustained increase in perfusion pressure and little recovery after reperfusion with normal Krebs solution.

Myocardial release of creatine kinase was simultaneously measured during the infusion of superoxide. Only 1 of the 3 hearts released creatine kinase, occurring after reperfusion with normal Krebs solution. Therefore, LV dysfunction occurred in the absence of significant necrosis in 2

hearts, and LV dysfunction preceded myocardial necrosis in the third.

During superoxide infusion there was a decrease in myocardial MDA release. However, there was no change in the myocardial release of MDA during hydroxyl radical infusion.

3.1.6 Discussion

The Langendorff isolated heart preparation removes the potential confounding problems of oxidative stress in organs other than the heart during myocardial ischaemia and extra-cardiac sources of biochemical markers of ischaemia and oxidative stress. It may permit a separation of the various components of the mechanisms involved in reperfusion injury and oxidative stress. With the isolation of different mechanisms, their individual contribution towards myocardial injury may be examined both qualitatively and quantitatively.

However, there are several limitations associated with the Langendorff technique of isolated heart perfusion that need to be remembered. In an isolated preparation, it is impossible to mimic completely the loading and perfusion conditions which apply *in vivo*. Furthermore, if the organ is not perfused with blood, the important but complex interaction between blood elements and the mechanisms involved in myocardial oxidative stress and reperfusion injury *in vivo* may not be imitated *in vitro*.

One of the main purposes of this series of experiments was to determine the conditions that were required for the rat myocardium to release the lipid peroxide product MDA. This putative marker of oxidative stress could then have been utilised to assess the effect of pharmacological interventions aimed at reducing oxidative stress. These experiments demonstrated that the isolated rat heart released a small amount of MDA under normal conditions which did not result in a

TABLE 3.2 Summary of haemodynamic effects and release of MDA by the isolated rat heart after infusion of oxygen-derived radical species.

Radical Species	No of hearts	MDA release (AUC)		Haemodynamics after 30 minute Infusion		
		baseline	flux	LVDP (% baseline)	δLVDias (mmHg)	Perfusion pressure (% baseline)
Superoxide	3	247±86	81±50	20±35	41±17	144±111
Hydroxyl Radical	3	208±42	254±147	18±28	7±17	375±223

Legend: AUC, area under the curve; LVDP, left ventricular developed pressure; δLVDias, change in left ventricular diastolic pressure from baseline; MDA, malondialdehyde; mmHg, millimetres of mercury; No., number.

sustained, elevated release after a variety of manoeuvres previously shown to induce oxidative stress (Maupoil and Rochette, 1988; Garlick et al. 1987; Maupoil et al. 1990), but which impaired LV performance to a variable but marked extent. Only after significant preceding combined ischaemic and metabolic or oxidative stress did there appear to be any significant lipid peroxidation as measured by myocardial MDA release. The absence of sustained elevated MDA release after ischaemia or free radical flux alone, which were capable of inducing significant LV dysfunction suggested that in this rat heart model there is little lipid peroxidation and this is an insensitive and/or late biochemical marker of oxidative stress.

Because no other biochemical markers of oxidative stress were utilised, it is unknown whether another marker (for example the redox status of glutathione) may have yielded more sensitive results that correlated more closely with the extent of oxidative stress in this species (Ji et al. 1993). In humans, peripheral plasma MDA concentration changes were less marked than the changes in redox status of glutathione in the *in vivo* study described in chapter 6. However, Verbunt *et al* (Verbunt et al. 1995) found no increase in intracellular GSSG concentration or increased active transport of GSSG in isolated buffer-perfused rat hearts subjected to 30 minutes of hypothermic total global ischaemia and 30 minutes reperfusion. They suggested that reactive oxygen species generated upon post-ischaemic reperfusion (Garlick et al. 1987; Ambrosio et al. 1993) did not lead to oxidative stress in isolated rat hearts.

The insensitivity of lipid peroxidation as a biochemical marker of oxidative stress in the rat heart has also been noted in some other studies (Brasch et al. 1989; Maupoil et al. 1990; Ballagi Pordany et al. 1991). For example, Maupoil *et al* (Maupoil and Rochette, 1988; Maupoil et al. 1990) found no increase in myocardial tissue TBARS concentration after 10 minutes of total global ischaemia followed by 10 minutes reperfusion, despite a significant increase in myocardial

tissue intensity of radical species as measured by electron spin resonance. They suggested that lipid peroxidation was a late product of oxidative stress and the duration of ischaemia used was not prolonged enough to induce such changes.

On the other hand, a longer duration of ischaemia was utilised by Cordis *et al* (Cordis et al. 1993), who demonstrated an increase in the coronary effluent MDA concentration from 49 ± 16 nmol/L at baseline to 109 ± 33 nmol/L after 30 minutes total global ischaemia followed by 30 minutes reperfusion in Krebs + 3% bovine serum albumin perfused isolated rat hearts. Furthermore, Tavazzi *et al* (Tavazzi et al. 1992a) demonstrated a significant and progressive increase in myocardial tissue MDA concentration both at 30 minutes total global ischaemia and after 30 minutes reperfusion utilising a direct HPLC measurement of MDA. It is not possible to account for failure to observe such profound changes in myocardial MDA reported by the above investigators. However, the data within the current study are in concordance with Brasch (Brasch et al. 1989) and Maupoil(Maupoil et al. 1990) that MDA efflux from the rat heart is at best a late occurrence after severe oxidant stress.

Several studies have confirmed a correlation between duration of oxidative stress and lipid peroxidation. In an isolated canine sarcolemmal and microsomal membrane preparation (Kramer et al. 1984) the production of MDA was directly correlated to decreasing Na^+,K^+-ATPase and reductase activity over time in response to a radical species flux. Parinandi *et al* (Parinandi et al. 1990) also demonstrated increasing lipid peroxide products in a similar isolated rat myocardial membrane model exposed to a radical flux. A direct correlation between the duration of oxidative stress and myocardial MDA release was demonstrated by Bagchi *et al* (Bagchi et al. 1990) in isolated rat hearts. Increasing duration of the infusion of superoxide, hydroxyl radical, hypochlorous acid or fMLP-activated rabbit PMN was associated with progressively increasing

myocardial MDA release. After 30 minutes there was a 10%, 25%, 17% and 22% increase in coronary effluent MDA concentration respectively. Microscopic examination of these hearts demonstrated similar histological changes to those seen after myocardial ischaemia and reperfusion. The induction of myocardial intracellular lipid peroxidation and LV dysfunction by hydrogen peroxide was also demonstrated by Onodera *et al* (Onodera et al. 1992). Conversely, Ballagi Pordany *et al* (Ballagi Pordany et al. 1991) only demonstrated an increase in myocardial tissue TBARS but not MDA after infusion of high concentration (12 mmol/L) hydrogen peroxide, and no increase in either TBARS or MDA with infusion of 6 mmol/L hydrogen peroxide. Therefore they suggested that previous positive results as regards MDA formation may be attributed to methodological artefacts.

The current studies showed evidence consistent with a correlation between the extent of the ischaemic insult or oxidative stress and myocardial release of MDA. However, opposite to the study of Bagchi *et al* (Bagchi et al. 1990), infusion of superoxide was associated with a decrease in coronary effluent MDA concentration. Of interest, baseline concentrations of MDA in the coronary effluent were 5 to 10 times lower in the current study than those described by Bagchi *et al* (47 ± 7 nmol/L) (Bagchi et al. 1990) or Cordis *et al* (49 ± 16 nmol/L) (Cordis et al. 1993). Whether these differences simply imply calibration differences, or the measurement of substances other than MDA in these previous studies, as suggested by Ballagi Pordany *et al* (Ballagi Pordany et al. 1991) remains unclear.

Jansenn *et al* (Janssen et al. 1993) noted in an isolated Tyrode's buffer-perfused rat heart model that there were no significant changes in either tissue or coronary effluent concentrations of MDA after 20 minutes of total global ischaemia followed by 30 minutes reperfusion. However, a 20 minute infusion of cumen hydroperoxide was associated with a 3.6 times increase in tissue MDA

concentration. These results imply that MDA was only produced under extreme conditions in the rat heart. Although the radical-generating systems produce a much larger oxidative stress than can be induced by myocardial ischaemia/reperfusion, they also failed to increase myocardial MDA release in the current study. Hence it may be possible that the protocols utilised were less extreme than those in other studies. This estimation of the exact "burden" of oxidative stress that was inflicted on the heart with a particular stressor was not easily apparent and not closely related to haemodynamic recovery. For example, in the current studies 30 minutes of total global ischaemia with prior hypoglycaemia was associated with a small non-sustained release of MDA which was not apparent with 60 minutes total global ischaemia alone, despite a significantly worse haemodynamic recovery in the latter.

Shandelya *et al* (Shandelya et al. 1993) demonstrated that in the isolated Krebs-perfused rat heart exposed to 20 minutes total global ischaemia followed by reperfusion, the increased free radical activity (measured by ESR) only persisted for 100 seconds after the onset of reperfusion. This is consistent with the current studies. In this bloodless rat heart model the extent of oxidative stress, irrespective of severity, may never have been sustained for long enough to induce sufficient and sustained lipid peroxidation, and thus be predominantly obscured by the washout of MDA at the onset of reperfusion. Shandelya (Shandelya et al. 1993) went on to demonstrate that the addition of neutrophils and complement (within plasma) in the perfusate resulted in neutrophil activation during myocardial ischaemia and reperfusion, and was associated with a more prolonged increase in radical species intensity during reperfusion. Semb *et al* (Semb et al. 1989) also noted that activated PMNs without ischaemia also increased myocardial chemiluminescence and decreased LVDP. These findings are concordant with the trend towards an increased release in myocardial MDA and decreased recovery of cardiac haemodynamics, with the addition of activated PMN to 30 minutes total global ischaemia in the current studies.

Hegstad *et al* (Hegstad et al. 1994b) measured conjugated diene formation in myocardial tissue PUFAs in isolated Krebs-perfused rat hearts subjected to 60 minutes total global ischaemia followed by 10 minutes reperfusion. They demonstrated an increase in myocardial cellular lipid peroxide concentration after 60 minutes of ischaemia with no incremental increase after a short period of reperfusion. This is the opposite to that demonstrated in the current study. Although coronary effluent rather than tissue MDA concentrations were utilised, MDA release decreased during ischaemia, hypoxia, metabolic stress or infusion of a radical flux.

The requirement for severe oxidant stress before the release of appreciable amounts of myocardial MDA during only the early reperfusion period, suggested that lipid peroxidation was a poor biochemical marker of oxidative stress in the isolated rat heart. However, as free radical activity was not directly measured in these experiments, the possibility that lipid peroxidation did in fact act as a sensitive reflection of the extent of free radical activity in this model, and that oxygen-derived free radicals play a more subtle role in the induction of LV dysfunction associated with myocardial ischaemia/reperfusion cannot be completely dismissed.

3.2 Assessment of the effects of N-acetylcysteine on cardiac haemodynamics and lipid peroxidation during total global ischaemia followed by reperfusion in the isolated rat heart.

3.2.1 Background

The model of Langendorff-perfused isolated rat heart developed for this thesis was further utilised to assess the effect of NAC on the amelioration of reperfusion injury. Although coronary effluent MDA concentration was unlikely to be a useful marker of oxidative stress as discussed in 3.1, the

assessment of the recovery of LV function at reperfusion after myocardial ischaemia could be used as an indicator of a change in the functional response to oxidative stress. In order to have an adequate signal, 30 minutes of total global ischaemia followed by 30 minutes of reperfusion was utilised as this was associated with a 40 ± 18 % recovery in LVDP. The concentration of NAC utilised in this study was similar to that achieved in humans receiving an intravenous infusion of 15g/24 hours (see 5.1).

3.2.2 Aims

The aims of this study were to

(1) Examine the cardiac haemodynamic effects and release of myocardial MDA of NAC in the isolated Langendorff-perfused rat heart during 30 minutes total global ischaemia and reperfusion.

(2) Examine the relative effect of the utilisation of NAC during both myocardial ischaemia and reperfusion or during the reperfusion period alone.

3.2.3 Method

For this series of experiments, the hearts were perfused for a 30 minute equilibrium period, a 30 minute period of cessation of coronary flow, then 30 minutes of reperfusion. as described in 3.1.3.1 and 3.1.3.2. There were 12 rats in the control group and six rats in each treatment group. The control group was perfused with normal Krebs, the first treatment group with 200 µmol/L N-acetylcysteine (Glaxo, Melbourne, Australia) in the Krebs perfusate throughout the experiment (pH of gassed Krebs/NAC = 7.45) and the second treatment group with normal Krebs solution during equilibrium and 200 µmol/L N-acetylcysteine in the Krebs perfusate at reperfusion. The 200 µmol/L NAC was added to the Krebs solution and stored at 4°C until use within 24 hours.

The Krebs solution contained EDTA as mentioned previously which potentially prevented significant autoxidation of NAC in oxygenated Krebs (Ceconi et al. 1988). For each experiment the Krebs solution was oxygenated and warmed to 37°C 20 to 30 minutes prior to perfusion of the heart.

All results were expressed as mean and standard deviation, although standard error of mean was utilised in the graphs. Myocardial MDA release was expressed as the area under the curve of coronary effluent MDA concentration versus time, with the baseline starting at the mean of the 3 baseline points and ending with the one 30 minute reperfusion point. Haemodynamics were calculated and expressed as in 4.1. ANOVA was used for comparisons between groups after 30 minutes reperfusion.

3.2.4 Results

All results are summarised in Table 3.3 and Figure 3.11. As regards the myocardial release of MDA during myocardial ischaemia and reperfusion, there was no difference between the groups. Furthermore, there was no significant differences between groups as regards recovery of systolic function, amelioration of LV diastolic dysfunction and reduction in coronary artery resistance.

The storage of NAC in Krebs solution containing EDTA was associated with autoxidation of NAC. After storage at 4°C in Krebs solution for 24 hours and 60 hours there was a 30% and 50% reduction in NAC concentration respectively, as measured by HPLC (see 2.4). The autoxidation of NAC in oxygenated Krebs solution at 37°C was more rapid, with 17% and 42% loss at 1 and 2 hours respectively

TABLE 3.3 Summary of the effect on haemodynamic function and coronary effluent MDA concentration of 200 µmol/l NAC infusion in isolated rat hearts subjected to 30 minutes total global ischaemia and 30 minutes reperfusion.

Treatment Group	No. of hearts	MDA Release (AUC)	Haemodynamics at 30 minutes Reperfusion		
			LVDP (% baseline)	δLVDias (mmHg)	Perfusion Pressure (% baseline)
control	12	154±53	40±18	25±15	109±14
NAC throughout	6	181±54	32±22	20±15	135±65
NAC at reperfusion	6	160±98	44±21	32±32	150±27
ANOVA		p, NS	p, NS	p, NS	p, NS

Legend: ANOVA, analysis of variance; AUC, area under the curve; LVDP, left ventricular developed pressure; δLVDias, change in left ventricular diastolic pressure from baseline; MDA, malondialdehyde; mmHg, millimetres of mercury; NAC, N-acetylcysteine; No., number; NS, not significant; p, probability.

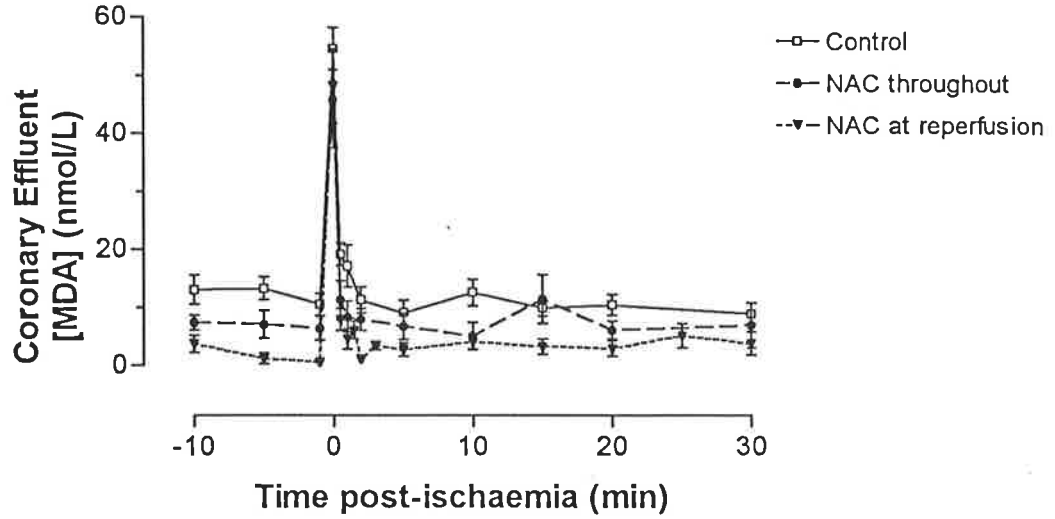


FIGURE 3.11 A Coronary effluent MDA concentration. Effect of 30 minutes total global ischaemia with no drug (n=12) or 200µmol/L NAC throughout Experiment (n=6) or at reperfusion (n=6).

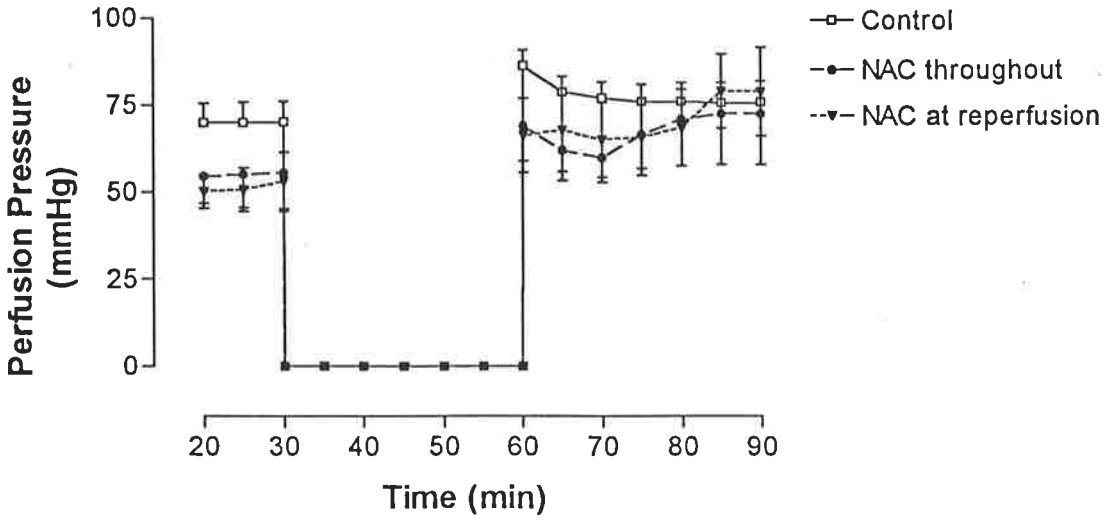


FIGURE 3.11 B Perfusion pressure. effect of 30 minutes total global ischaemia with no drug (n=12) or 200µmol/L NAC throughout experiment (n=6) or at reperfusion (n=6).

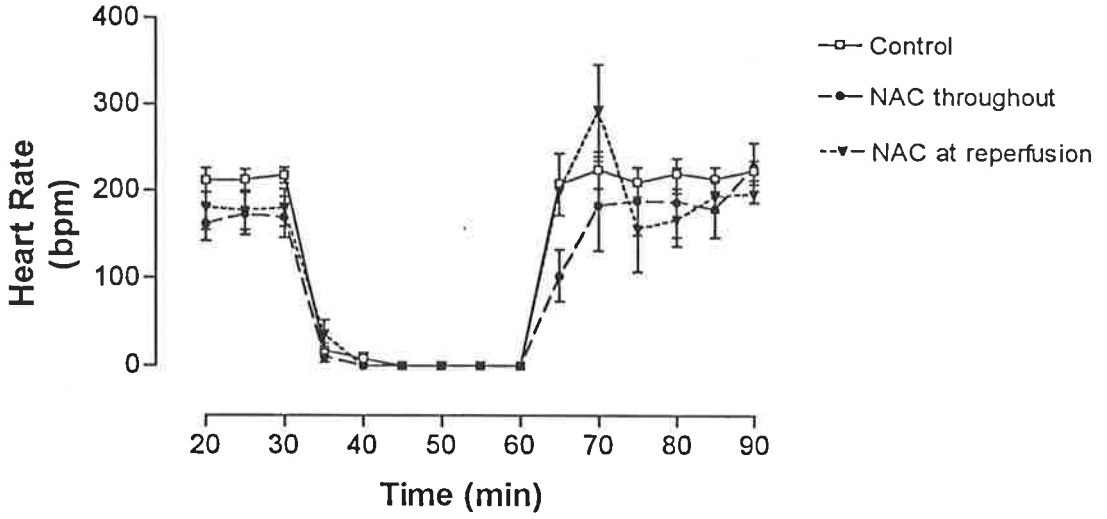


FIGURE 3.11 C Effect on heart rate of 30 minutes total global ischaemia with no drug (n=12) or 200µmol/L NAC throughout experiment (n=6) or at reperfusion (n=6).

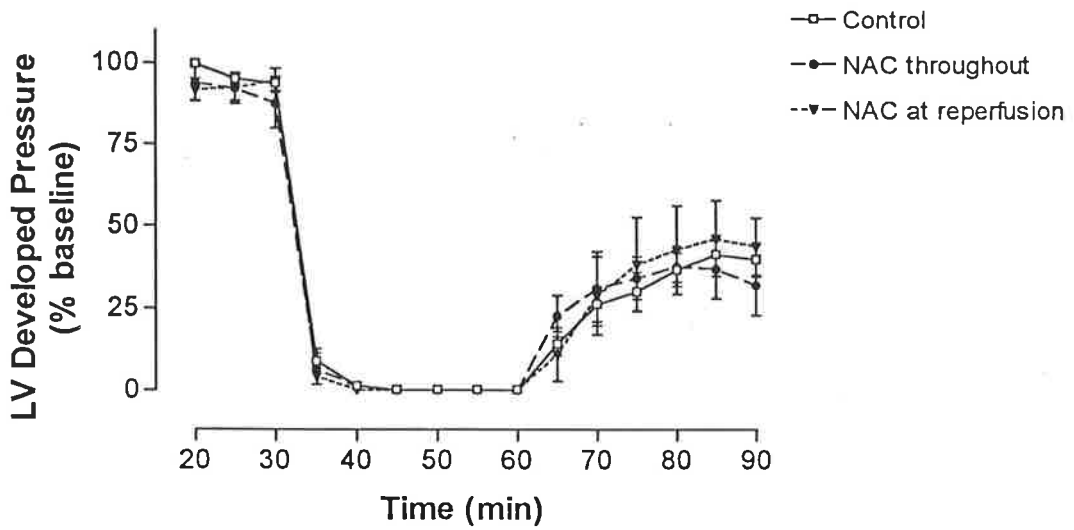


FIGURE 3.11 D Percentage recovery of left ventricular developed pressure. Effect of 30 minutes total global ischaemia with no drug (n=12) or 200µmol/L NAC throughout experiment (n=6) or at reperfusion (n=6).

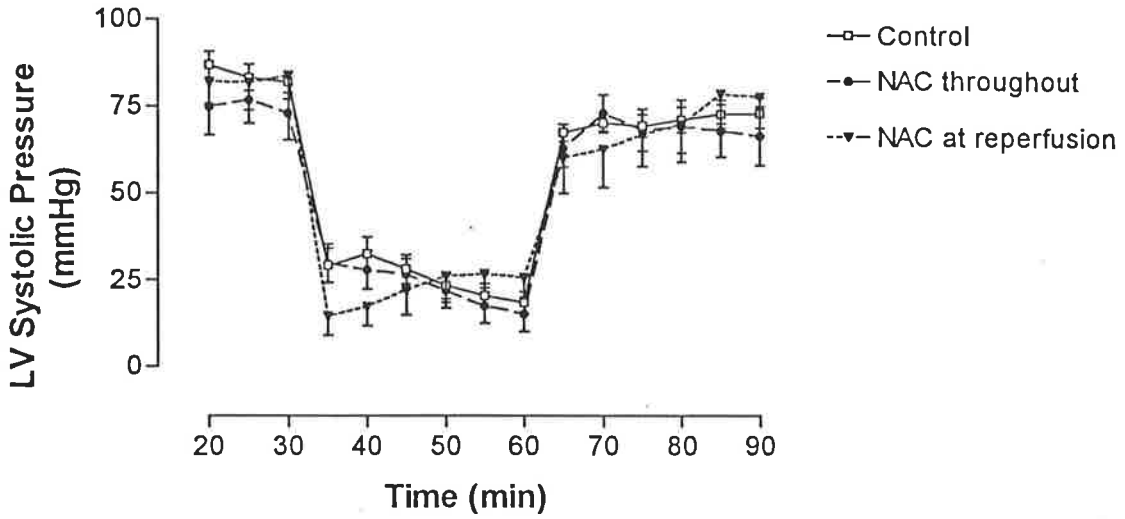


FIGURE 3.11 E Left ventricular systolic pressure. Effect of 30 minutes total global ischaemia with no drug (n=12) or 200µmol/L NAC throughout experiment (n=6) or at reperfusion (n=6).

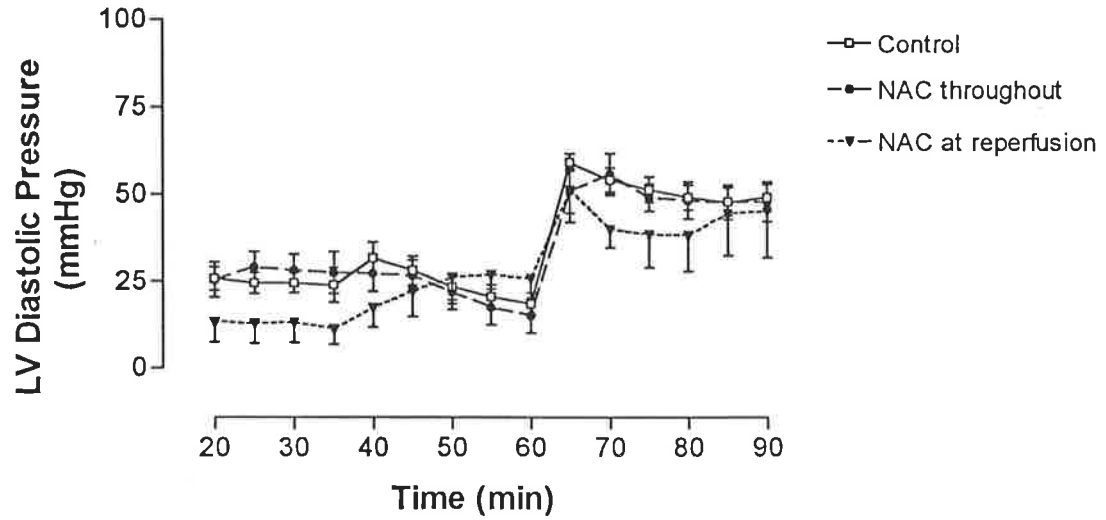


FIGURE 3.11 F Left ventricular diastolic pressure. Effect of 30 minutes total global ischaemia with no drug (n=12) or 200µmol/L NAC throughout experiment (n=6) or at reperfusion (n=6).

3.2.5 Discussion

These results showed that 200 µmol/L NAC did not effect cardiac haemodynamics before the onset of myocardial ischaemia, or when utilised during ischaemia and/or reperfusion. Consistent with the results of 3.1, myocardial release of MDA was unaffected by the infusion of NAC, as this was previously shown to be an insensitive indicator of changes in oxidative stress.

The lack of effect of NAC may potentially have been related to autoxidation of NAC in the oxygenated Krebs solution (Ceconi et al. 1988). Certainly there was no statistically significant difference between results of experiments carried out with fresh as compared stored NAC/Krebs solution. Furthermore, even if autoxidation during the experimental period decreased the available NAC concentration to 100 µmol/L (the NAC/Krebs solution was gassed at 37°C from 30 minutes prior to the onset of the experiment), this lower concentration was still comparable to that achieved during human intravenous infusions (see chapter 6).

There are only three previous published studies of the antioxidant effect of NAC on myocardial ischaemia and reperfusion in the isolated rat heart (Qiu et al. 1990; Tang et al. 1991; Menasche et al. 1992). Qui *et al* (Qiu et al. 1990)carried out a series of studies in the isolated rat heart. In a Langendorff-mode 30 minutes of regional ischaemia followed by 40 minutes reperfusion an 8 µmol/L infusion of NAC throughout the experimental period as compared no NAC was associated with 68 ± 5% (SEM) recovery of LVDP and 42 ± 4% respectively ($p<0.05$), but no difference in LVEDP at 40 minutes reperfusion. There was no difference between groups as regards the leakage of creatine kinase during reperfusion. In a working heart mode of 20 minutes global ischaemia and 35 minutes reperfusion, 8 µmol/L NAC infusion throughout the experiment was also associated with a significantly better recovery of aortic flow, coronary flow, aortic developed pressure and cardiac output as compared no NAC. Tang *et al* (Tang et al. 1991) administered 3

µmol/min NAC (the current study utilised 2 µmol/min) beginning 10 minutes prior to a 20 minute ligation of the left anterior descending artery followed by 30 minutes reperfusion in a working rat heart model. The recovery of the rate-pressure product was significantly better in NAC treated hearts as compared to saline treated hearts (98.3 ± 4.5% vs 73.6 ± 3.8%, $p < 0.01$). Menasché *et al* (Menasche et al. 1992) examined the effect of the addition of 0.04 mol/L NAC to cardioplegic solution in isolated Langendorff Krebs-perfused rat hearts subjected to 3 hours of cold cardioplegia followed by 1 hour of reperfusion. The cardioplegic solution was infused at 30 minute intervals during cardioplegia. After 1 hour reperfusion, the addition of NAC to the cardioplegic solution as compared to controls was associated with an LVDP of 103 ± 2.5 versus 92.9 ± 3.1 mmHg ($p<0.05$), significantly better recovery of LV dP/dt and almost no increase in LVDias. These studies, with varying protocols and concentrations of NAC all demonstrated a beneficial haemodynamic effect from the use of NAC during myocardial ischaemia and reperfusion. It is unclear why these results differ so considerably from those of the current study.

Similar protective effects on LV function of NAC perfused throughout the ischaemic and reperfusion periods have been noted in dog (Forman et al. 1988) and rabbit (Ceconi et al. 1988) models. However, this protective effect was not demonstrable if NAC was only infused during the reperfusion period (Ceconi et al. 1988). Furthermore, there have been both positive (Sochman et al. 1990; Ceconi et al. 1988) and negative (Forman et al. 1988; Kingma and Rouleau, 1989; Qiu et al. 1990) results as regards limitation of infarct size. This parameter was not assessed in the current study.

The negative results of the current study do not necessarily imply that NAC will have no effect on the limitation of myocardial dysfunction in other species or other *in vivo* and *in vitro* models of myocardial ischaemia and reperfusion. Furthermore, it could be postulated that NAC may be more

effective if administered in conjunction with GTN, thereby potentiating the anti-ischaemic and cytoprotective effects of exogenous nitrates (Cooke and Tsao, 1993; Lefer et al. 1993) as well as having potential antioxidant properties *per se*. This putative potentiation of the cytoprotective effects of NAC has not been specifically studied in the literature, but is consistent with the finding that the sulphydryl-containing organic nitrate SPM-5185 infused at reperfusion after 1 hour occlusion of the left anterior descending artery was associated with decreased infarct size and LV dysfunction after 4.5 hours reperfusion in an open-chest dog model.

Nevertheless, the overall conclusions to be drawn from the current experiments are that the ischaemia/reperfused rat heart model, despite the bulk of previous data, cannot be shown either to release appreciable, sustained amounts of MDA during reperfusion, nor to exhibit salvage of myocardial function in the presence of NAC. These results, while at odds with most previous published data in this model are not unique and suggest that the utility of this model for conventional *in vitro* studies of ischaemia/reperfusion requires re-examination.

Chapter 4:

Human *in vivo* Studies of Pacing-Induced Myocardial Ischaemia and Recovery.

4.1 Introduction

Most of the studies involving the modulation of oxidative stress with antioxidants (as discussed in 1.6) have involved animal models, which may not mimic the human situation either metabolically or as regards the induction of oxidative stress. Usually the myocardial stress that is utilised is global and severe and in humans *in vivo* would often not be compatible with survival. Commonly, an epicardial coronary artery is occluded and subsequently reperfused (Bolli et al. 1988a), or in isolated organ systems, global ischaemia is induced by stopping all coronary flow (Garlick et al. 1987). In these studies, most oxidative stress appears to occur at the time of reperfusion (Bolli et al. 1988a; Garlick et al. 1987), although depletion of endogenous antioxidants has been demonstrated during the ischaemic period (Ferrari et al. 1992; Ceconi et al. 1988).

As the extent and duration of myocardial ischaemia appears to be directly proportional to the extent of oxidative stress (Ferrari et al. 1990) (predominantly in the reperfusion period), mild ischaemia is likely to be associated with only subtle biochemical changes associated with mild oxidative stress. The measurement of transcoronary gradients of biochemical markers of oxidative stress, ischaemia and metabolism is a technique that may be used to quantify these small changes. Several studies have used this technique to demonstrate oxidative stress during PTCA (Coghlan et al. 1991; Blann et al. 1993; Oldroyd et al. 1992; De Scheerder et al. 1991; Roberts et al. 1990) and during tachycardia-induced myocardial ischaemia in humans *in vivo* (Oldroyd et al. 1990), which represent quite mild stresses. Coghlan *et al* (Coghlan et al. 1991) demonstrated that reperfusion after coronary occlusion with PTCA, but not the preceding ischaemic period was associated with release into the coronary sinus blood of radical species detected by *ex vivo* addition of the spin trap N-*tert*-butyl-α-phenylnitrone. However, the extent of radical species release was correlated to coronary sinus lactate concentration suggesting a relationship between the extent of oxidative stress and the intensity and duration of preceding ischaemia. However,

Oldroyd *et al* (Oldroyd et al. 1992) did not find any correlation between coronary sinus lactate concentration and lipid hydroperoxide concentration, suggesting that the extent of preceding ischaemia was not the only determinant of extent of oxidative stress.

This chapter describes a series of studies utilising coronary sinus catheterisation *in vivo* to determine transcoronary gradients of myocardial metabolic products. Myocardial stress was induced by pacing tachycardia in patients with significant left coronary artery disease. Firstly, studies to confirm previous reports, as regards measurement of the induction of oxidative stress with myocardial ischaemia without occlusion of an epicardial coronary artery were performed. Whether recovery from myocardial ischaemia represents relative "reperfusion" is unclear, but may be a parallel of global hypoperfusion-induced ischaemia and reperfusion, utilised in isolated organs. Secondly, the effects of NAC as an antioxidant and potentiator of GTN were examined on the extent of oxidative stress in this model.

4.2 Objectives

Therefore, the experiments described in this chapter had two major objectives:

(1) To determine whether myocardial ischaemia induced by rapid pacing might affect transcoronary MDA flux in humans and correlate this to cardiac haemodynamic and metabolic changes.

(2) To determine whether NAC, alone or in combination with GTN might affect the extent of this flux.

In order to facilitate evaluation of the results of the experiments, two "control" studies were performed:

(i) Examination of the possibility that sampling through long (for example coronary sinus catheters) might introduce artefact.

This was necessary because sampling from the coronary sinus occurred through the fine lumen of a 100 cm catheter, whereas femoral artery blood sampling occurred through the 10 cm long femoral artery sheath side-arm. There is no information in the literature as to whether this difference would result in a change in the measured concentration of either lactate or MDA.

(ii) Determine the extent of MDA flux during non-ischaemic stress in the myocardial and skeletal muscle vascular beds.

The release of MDA from other vascular beds has been noted by previous studies at times of oxidative stress (Davies et al. 1990b; Coghlan et al. 1993; Royston et al. 1986) and from skeletal muscle, after extreme physical exertion (Meydani et al. 1993). However, the extent of MDA release in response to mild non-ischaemic stress is uncertain. Therefore, as part of the development of a model of myocardial oxidative stress and the role of myocardial MDA release, it was important to determine the extent of MDA metabolism and release from the non-ischaemic, stressed myocardial vascular bed. Furthermore, the extent of MDA release in a skeletal vascular bed during non-ischaemic stress would allow a comparison with cardiac muscle.

4.3 Methods

4.3.1 Protocol for determination of the extent of change of MDA and lactate concentration secondary to withdrawal of blood through a long catheter.

The procedure was carried out after routine diagnostic cardiac catheterisation and coronary angiography for the investigation of chest pain. A 7.5 or 8 F sheath was present in the right femoral artery. An 100 cm Webster thermodilution coronary sinus catheter, 100 cm 7 F right Judkins catheter (Cordis), or a 7 F Judkins catheter (Cordis) cut to a length of 50 cm was inserted into the sheath for a length of 10 to 15 cm. Blood was simultaneously withdrawn from the catheter

and the sheath side-arm. Up to triplicate samples were taken from any patient, using one or more of the different catheters. All catheters and sheath were removed after the procedure. Blood samples were stored on ice until being spun down to plasma and frozen at -20°C until assay.

4.3.2 Protocols for the assessment of plasma lactate and MDA concentrations across non-ischaemic myocardial and skeletal muscle vascular beds at rest and with exercise.

4.3.2.1 Non-ischaemic myocardial pacing-induced stress

Patients included to the study were of either sex, aged ≥ 18 years and ≤ 75 years, undergoing elective cardiac catheterisation and coronary angiography for the investigation of chest pain. Patients were included if they had no detectable stenoses at angiography in the left coronary artery, which constitutes the major source of blood draining into the coronary sinus.

All patients gave written informed consent and the protocol was approved by Human Ethics of Research Committee of The Queen Elizabeth Hospital. All long-acting nitrate therapy was stopped 24 hours prior to the procedure. Thirty minutes prior to the procedure patients were sedated with 10 mg oral diazepam and 50 mg oral diphenhydramine. Electrocardiographic monitoring on leads I and II was carried out throughout the procedure. Under local anaesthesia (1% lignocaine) 7.5F sheaths were inserted into the femoral artery and vein. Right heart catheterisation was carried out using a 7 F thermodilution Swan Ganz catheter . Cardiac output was estimated using the thermodilution technique. Left heart catheterisation, left ventriculography and coronary angiography were performed utilising the Judkins technique . Following the routine catheterisation procedure a cutdown was made to the left medial ante-cubital vein and an 8 F Pepine pacing thermodilution coronary sinus catheter (Webster, Altadena, California, USA) positioned into the

coronary sinus via this vein. Correct positioning of the catheter was confirmed by contrast injection through the catheter tip into the coronary sinus during fluoroscopic screening and determination of coronary sinus blood oxygen saturation. The catheter was then fixed in position externally. The coronary sinus catheter allowed measurement of coronary sinus blood flow using a thermodilution technique and sampling of coronary sinus blood. An 6 F bipolar temporary pacing lead was inserted via a second right femoral vein sheath to the right ventricle for rapid pacing of this chamber. A 4 F Millar micromanometer-tipped catheter was inserted via the right femoral artery sheath into the left ventricle for measurement of left ventricular pressure and determination of the first derivative, LV dP/dt.

Blood samples were simultaneously taken from the femoral artery and coronary sinus for measurement of transcoronary gradients of blood oxygen saturation, plasma lactate and malondialdehyde concentrations.

All baseline measurements were carried out while the patient was paced via the coronary sinus catheter at just above the native resting rate. Measurements included, cardiac output, left ventricular peak -dP/dt, coronary sinus blood flow, simultaneous coronary sinus and femoral artery blood sampling for blood oxygen saturation, plasma malondialdehyde and lactate concentrations.

Myocardial stress was induced by right ventricular pacing at 140 bpm for 2 to 3 minutes, with simultaneous sampling of femoral artery and coronary sinus blood for lactate, MDA and coronary sinus oxygen saturation during greater than 1 minute of stress and at 1, 2 and 5 minutes into the recovery period. Changes in LV -dP/dt associated with termination of RV pacing were recorded (Grossman, 1986). Coronary sinus blood flow was measured after 1 minute of rapid pacing, and

1.5 and 4 minutes into recovery. After a further 10 minute recovery period, the protocol was repeated.

4.3.2.2 Non-ischaemic skeletal muscle stress

The procedure was carried out after routine diagnostic cardiac catheterisation and coronary angiography for the investigation of chest pain. Two 7.5 F sheaths were present in the right femoral artery and vein respectively. Exclusion criteria included any patient with signs or symptoms suggestive of peripheral vascular disease. All patients gave written informed consent. Resting blood samples were simultaneously withdrawn from the side-arm of both vein and arterial sheaths for plasma lactate and MDA concentration determination. The patient then performed 100 straight leg raises in the supine position against resistance. Simultaneous arterial and venous samples were taken immediately following exercise and during the recovery period. Blood samples were stored in ice until being spun down to plasma and frozen at -20°C until assay. All sheaths were removed after the procedure. Proportional extraction of both lactate and MDA by the femoral vascular bed was calculated from the FV to FA gradient of plasma concentrations.

4.3.3 Protocol for comparison between rapid atrial versus ventricular pacing-induced myocardial ischaemia on cardiac haemodynamics and metabolism of lactate and MDA.

Patients included to the study were of either sex, aged ≥ 18 years and ≤ 75 years, undergoing elective cardiac catheterisation and coronary angiography for the investigation of stable exertional angina pectoris, in whom nitrate therapy had not been utilised for the previous 24 hours. At coronary angiography a significant (> 50 %) stenosis needed to be present within at least one main epicardial artery draining to the coronary sinus. Exclusion criteria included pregnant women,

patients with unstable angina pectoris, recent acute Q-wave myocardial infarction (within 3 months), haemodynamically significant impairment of left ventricular systolic function, significant valvular heart disease or significant (> 50 %) stenosis of the left main coronary artery.

4.3.3.1 Atrial pacing-induced myocardial ischaemia

Patient preparation and baseline measurements were similar to those of 4.3.2.1. Transient ischaemia was induced by incrementally increasing the pacing rate by 10 bpm each minute until the patient developed symptoms of angina or AV nodal Wenckebach occurred. Measurements and blood sampling identical to 4.3.2.1 were carried out after 1 minute of the maximum pacing. However, no sampling was performed during the recovery period.

4.3.3.2 Ventricular pacing-induced myocardial ischaemia

The patient preparation, baseline measurements, pacing protocol, measurement of cardiac haemodynamics and blood sampling for this series of experiments were identical to 4.3.2.1. In two patients the protocol was repeated after a 10 minute recovery period.

4.3.4 Protocol for determination of the effects of NAC, GTN and the combination of GTN and NAC on cardiac haemodynamics, metabolism and oxidative stress during ventricular pacing-induced myocardial ischaemia.

All patients gave written informed consent and the protocol was approved by Human Ethics of Research Committee of The Queen Elizabeth Hospital. Inclusion and exclusion criteria, patient preparation, pacing protocol, cardiac haemodynamic measurements and blood sampling at rest, during rapid pacing and into recovery was the same as for 4.3.2.1. Additional blood sampling was carried out for reduced and oxidised glutathione, and NAC concentrations in one patient.

For this series of experiments this protocol was repeated four times in each patient, unless the patient became haemodynamically unstable, causing the procedure to be immediately abandoned. One patient did not complete the protocol and results were not included in the analysis. For the first protocol, no drug was given. Due to the constraints of the relatively long plasma half-life of NAC it was not possible to randomise sequence of treatment regimens. GTN was infused intravenously at a rate of 5 μg/minute for ten minutes prior to repeating the protocol. GTN was then stopped and NAC, 5 g in 100 mL 5% dextrose was infused intravenously over ten minutes. The protocol was then repeated. GTN was then reinfused for 10 minutes (presumably in the continued presence of NAC) and the protocol was repeated for the fourth time.

4.3.5 Calculation of parameters and statistical analysis

All results were expressed as mean and standard deviation, although standard error of mean was utilised in Figure 4.8. Coronary sinus blood flow was calculated utilising the formula (Yoshida et al. 1971):

$$F_B = F_I \times 1.08 \left(\frac{\frac{\delta I}{S_I \times M_I}}{\frac{\delta B}{S_B \times M_B}} - 1 \right)$$

F_B, coronary sinus blood flow; F_I, infusion rate through catheter; δI, deflection of the indicator; S_I, indicator scale factor; M_I, indicator thermistor calibration coefficient; δB, deflection of the coronary sinus; S_B, coronary sinus scale factor; M_B, coronary sinus thermistor calibration coefficient; 1.08, constant for 5% dextrose as thermistor fluid.

A typical coronary sinus thermodilution tracing is shown in Figure 4.1. Myocardial flux was calculated as the product of the myocardial vascular gradient (coronary sinus concentration - femoral artery concentration) and coronary sinus blood flow (CSBF) at that time. To correct for variation in the positioning of the coronary sinus catheter between patients, all values of flux were

expressed as a ratio of the resting CSBF (Horowitz et al. 1986a). The slope of the relaxation phase of the left ventricular pressure curve (-dP/dt) was calculated as a mean of 3 to 5 cycles at rest and immediately after tachycardia (Grossman, 1986). Paired t test was utilised to assess the change in measured parameters with rapid pacing as compared to rest with maximal pacing-induced MDA compared with resting values in the case of MDA estimation. Comparisons between changes in measured parameters from rest to ischaemia for atrial and ventricular pacing, or between long and short catheters, were made utilising the non-paired t test. The primary comparison between the effects of NAC on relative changes in cardiac haemodynamics and metabolism and baseline measurements was performed with a paired t test. Absolute changes in lactate and MDA flux at rest and during pacing-induced ischaemia comparing no therapy with NAC were analysed utilising two way ANOVA. The relative changes between rest and pacing-induced ischaemia and the effects of GTN and GTN in combination with NAC was assessed utilising ANOVA. Results were considered statistically significant when two-tailed $p < 0.05$.

4.4 Results

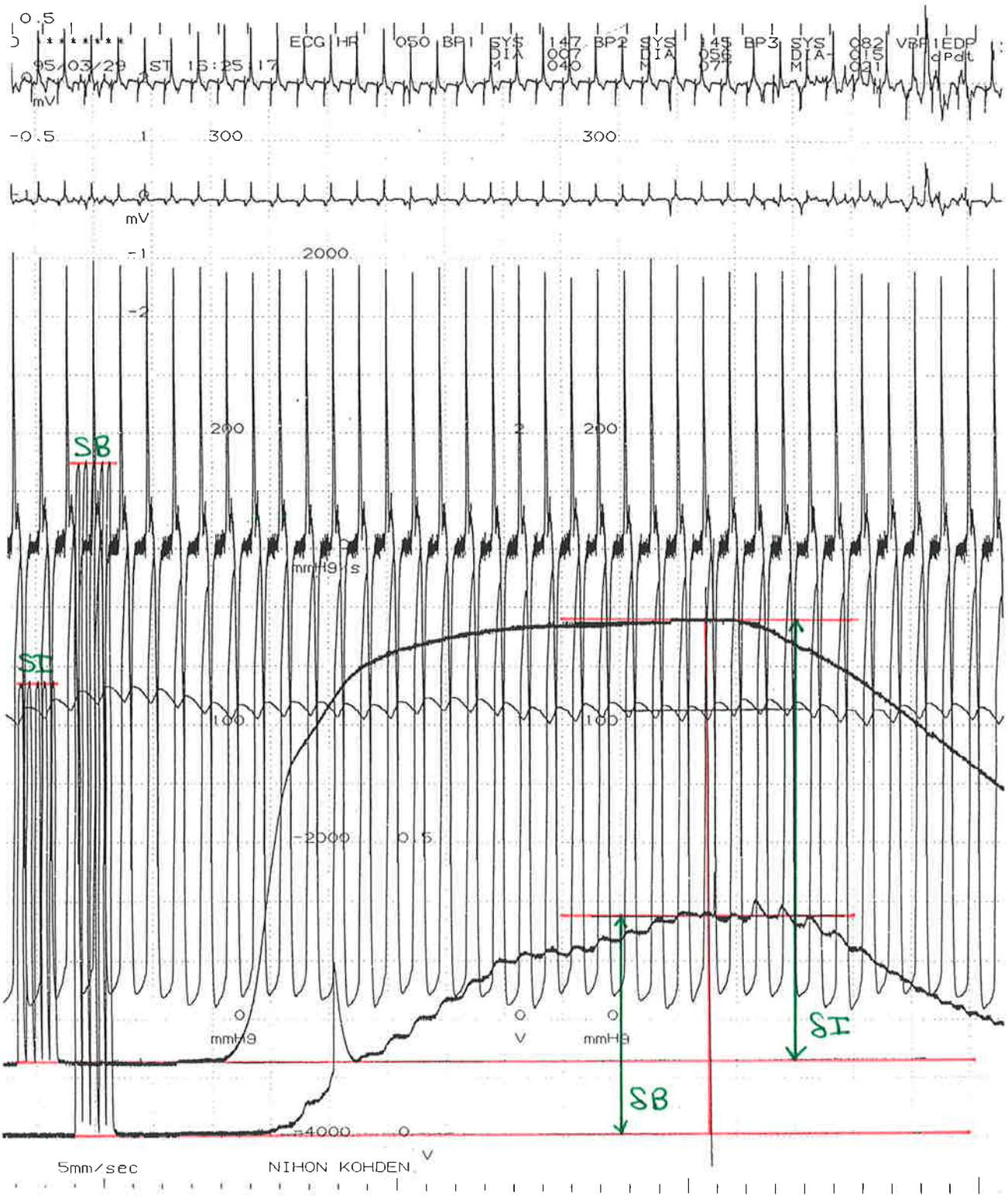
4.4.1 Determination of the extent of change of MDA and lactate concentration secondary to withdrawal of blood through a long catheter.

4.4.1.1 Effects on plasma lactate concentration

Results are summarised in Table 4.1 and Figure 4.2. There was a significant difference between plasma lactate concentrations from blood simultaneously drawn through 10 cm (0.94 ± 0.41 mmol/L) versus 100 cm (0.86 ± 0.40 mmol/L) tubing ($p = 0.002$), with a mean percentage loss of 9 ± 10% in 21 samples. There was no significant difference between the percentage loss of lactate from blood withdrawn through a coronary sinus catheter (11 ± 10%) as compared a Judkins catheter (8 ± 11%) ($p = 0.49$). The loss of lactate appeared to be at least in part dependent

FIGURE 4.1 Coronary sinus thermodilution tracing.

(Note: Increasing change of temperature of the coronary sinus blood indicates decreasing coronary sinus blood flow)



Legend: δB, change in temperature of coronary sinus blood; δI, change in temperature of indicator due to infusion of room temperature thermistor fluid; SB, coronary sinus blood scale factor; SI, indicator scale factor.

TABLE 4.1 Summary of Plasma Lactate and MDA Concentrations of Blood Drawn From the Femoral Artery Simultaneously Through the Side-Arm of a 7.5 - 8F Sheath and 50 - 100cm Coronary Sinus or Judkins Catheter.

	Plasma [lactate] (mmol/L)				Plasma [MDA] (µmol/L)			
	No	Sheath	Catheter	δlactate (%)	No	Sheath	Catheter	δMDA (%)
50cm Judkins Catheter	9	0.77±0.35	0.81±0.35	5±3	0			
100cm Judkins Catheter	11	0.98 ± 0.47	0.94±0.48	-8±11	8	0.21±0.04	0.16±0.03	-29±10
100cm Coronary Sinus Catheter	10	0.89±0.35	0.78±0.30	-11±10	6	0.19±0.05	0.17±0.03	-8±9

Legend: [lactate], concentration of lactate; [MDA], concentration of malondialdehyde; No., number; cm, centimetre; %, percent; δ, change; mmol/L, millimoles per litre; µmol/L, micromoles per litre.

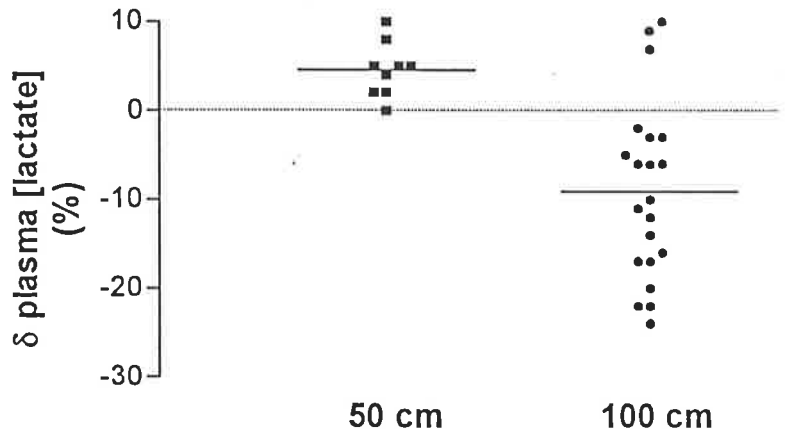


FIGURE 4.2 A Comparison of plasma lactate concentration changes of blood drawn through a 50 cm ($p = 0.001$) or 100 cm catheter ($p = 0.002$) as compared to a 10 cm Sheath Side-Arm.

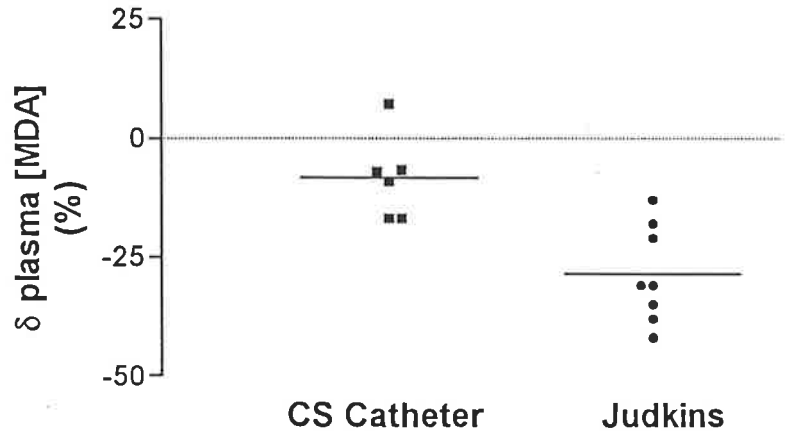


FIGURE 4.2 B Comparison of changes in plasma MDA concentration of blood withdrawn from an 100cm Coronary Sinus (CS) catheter ($p = 0.069$) or 100cm Judkins catheter ($p = 0.0002$) as compared 10cm sheath side-arm.

on tubing length, as the lactate loss was not apparent when blood was withdrawn through a 50 cm catheter. In fact, there was a small but significantly higher plasma lactate concentration measured from blood withdrawn through a 50 cm catheter (0.66 ± 0.16 mmol/L) as compared 10cm side-arm and sheath (0.69 ± 0.17 mmol/L) ($p = 0.001$), suggesting a relative loss of lactate from the sheath side-arm. There was no correlation between plasma lactate concentration in the sheath side-arm and the extent of loss of lactate through the 100 cm catheter ($r^2 = 0.002$, $p = 0.8$).

4.4.1.2 Effects on plasma MDA concentration

There was a significant relative loss of plasma MDA by the withdrawal of blood through a 100cm catheter (0.16 ± 0.03 µmol/L) as compared a 10cm sheath side-arm (0.20 ± 0.04 µmol/L) ($p = 0.0003$). However, this significant difference was only apparent when the Judkins catheter was utilised ($p = 0.0002$). The percentage relative loss of plasma MDA was 8 ± 9% from a coronary sinus catheter and 29 ± 10% from a Judkins catheter ($p = 0.002$), with an combined average loss of 20 ± 14%. (see Table 4.2 and Figure 4.2)

4.4.2 The assessment of plasma lactate and MDA concentrations across non-ischaemic myocardial and skeletal muscle vascular beds at rest and with exercise.

4.4.2.1 Myocardial metabolism and haemodynamics during non-ischaemic myocardial stress.

Two patients, one of each gender, aged 67 and 64 years were studied. There were no adverse events resulting from the procedure. Patient 2 had normal epicardial coronary arteries, and patient 1 a 70% stenosis of the postero-lateral branch of the right coronary artery, with no stenoses in the left coronary artery. Resting cardiac haemodynamics were normal and are summarised in Table

4.3 and Figure 4.3. With induction of ventricular tachycardia, patient 1 had little increase in coronary sinus blood flow and demonstrated oxygen desaturation of coronary sinus blood. There was only minor impairment of left ventricular diastolic function after ventricular tachycardia in both patients. Patient 2 increased coronary sinus blood flow during tachycardia, with a subsequent hyperaemia in the recovery period. There was no consistent change in coronary sinus blood oxygen saturation.

As regards myocardial metabolism of lactate, both patients consistently had increased uptake of lactate with induction of ventricular tachycardia suggesting a normal, non-ischaemic response to increased work. However, there was a reproducible release of myocardial MDA in these non-ischaemic patients during and/or after ventricular tachycardia.

4.4.2.2 Skeletal muscle metabolism of lactate and MDA during non-ischaemic mild exercise.

Two patients were studied. Their individual results are shown in Figure 4.4. After mild exercise and beyond 10 minutes recovery, lactate was released by the femoral vascular bed, indicating an element of anaerobic respiration within skeletal muscle. However, there was no significant release of MDA by the femoral vascular bed during rest or after mild exercise.

TABLE 4.2 Duplicate cardiac haemodynamics and myocardial metabolism in 2 patients with normal epicardial coronary arteries supplying the coronary sinus at rest and during right ventricular pacing at 140 bpm (stress).

	Rest	Stress
Cardiac index ($L/min/m^2$)	2.3 ± 0.3	-
Mean pulmonary capillary wedge pressure (mmHg)	12 ± 4	-
Left ventricular ejection fraction (%)	69 ± 8	-
Mean arterial pressure (mmHg)	106 ± 6	90 ± 4
Coronary sinus blood flow (mL/min)	134 ± 37	155 ± 49
Coronary vascular resistance (mmHg.min/mL)	0.85 ± 0.27	0.69 ± 0.29
Coronary sinus blood oxygen saturation (%)	36.4 ± 5.8	28.9 ± 3.2
LV -dP/dt (mmHg/sec)	-1660 ± 390	-1600 ± 450
Myocardial lactate flux:baseline CSBF (μmol/mL)	-0.08 ± 0.13	-0.31 ± 0.25
Myocardial MDA flux:baseline CSBF (nmol/mL)	0.03 ± 0.02	0.18 ± 0.10

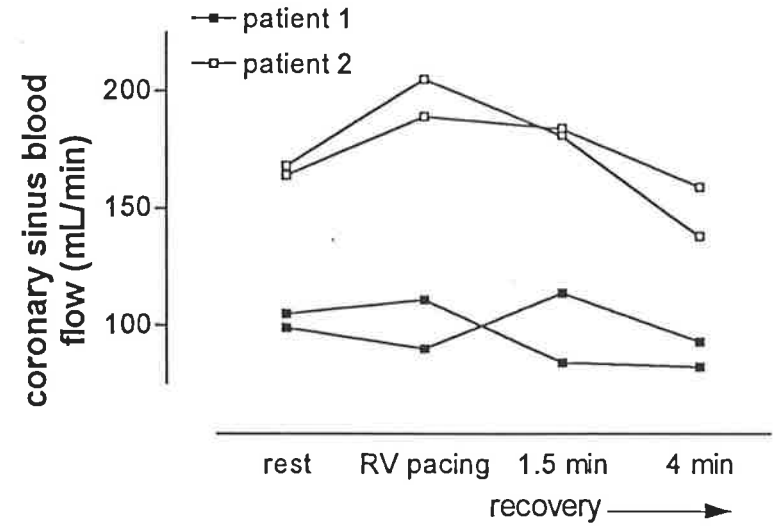


FIGURE 4.3 A Coronary sinus blood flow in 2 patients with normal left coronary arteries at rest and during RV pacing at 140 bpm and recovery. Studies carried out in duplicate.

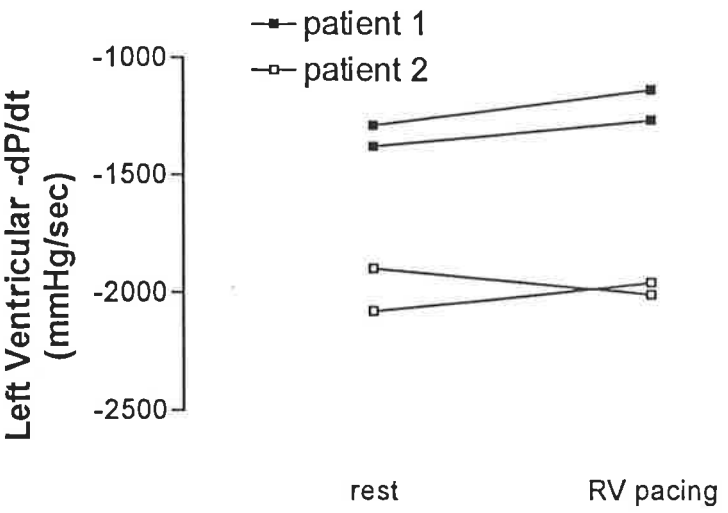


FIGURE 4.3 B Left ventricular -dP/dt in patients as in Figure 4.3 A at rest and immediately after RV pacing at 140 bpm.

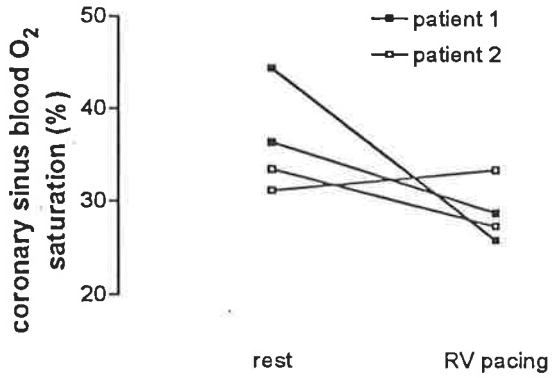


FIGURE 4.3 C Coronary sinus blood oxygen saturation in 2 patients as in Figure 4.3 A at rest and during RV pacing at 140 bpm.

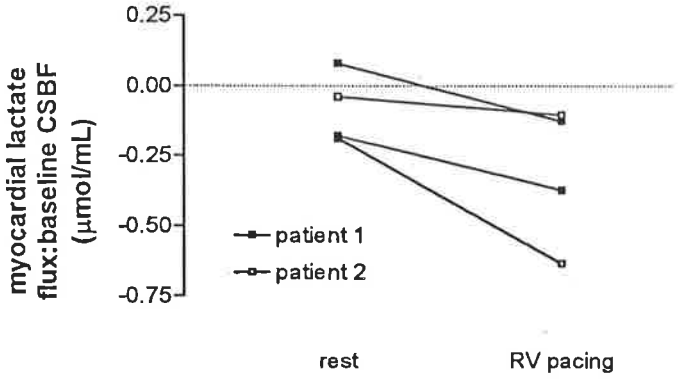


FIGURE 4.3 D Myocardial lactate flux relative to baseline coronary sinus blood flow in 2 patients as in Figure 4.3 A at rest and during RV pacing at 140 bpm.

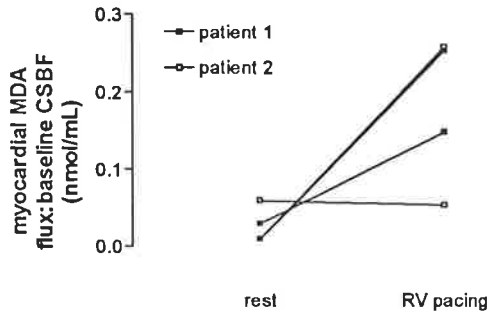


FIGURE 4.3 E Myocardial MDA flux relative to baseline coronary sinus blood flow in 2 patients as in Figure 4.3 A at rest and after RV pacing at q140 bpm.

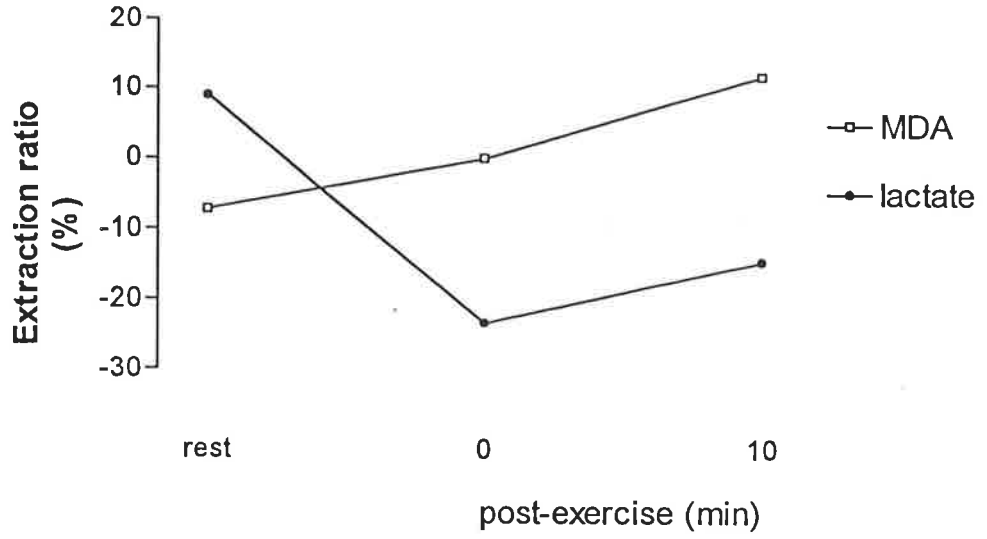


FIGURE 4.4 A Plasma Lactate and MDA Extraction by the Non-Ischaemic Femoral Vascular Bed at Rest and After Exercise. Patient 1

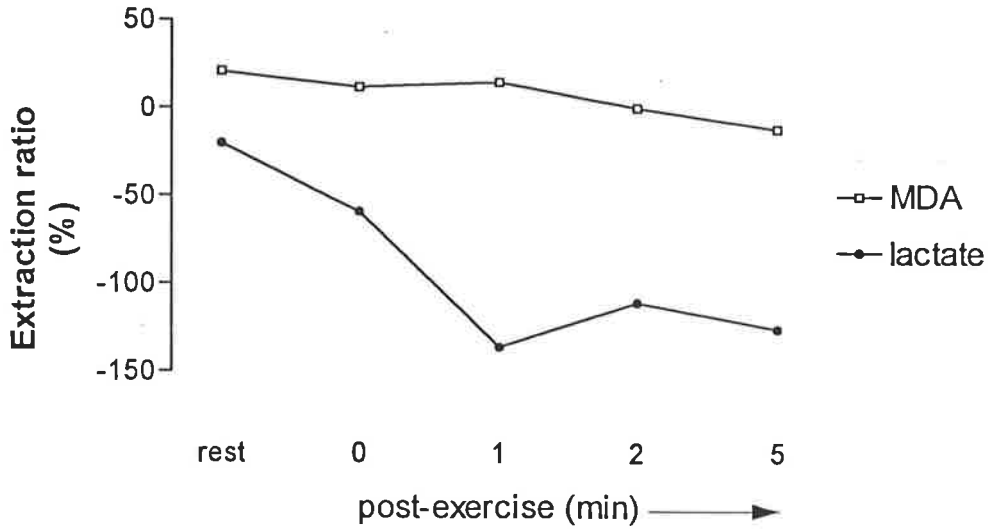


FIGURE 4.4 B Plasma Lactate and MDA Extraction by the Non-Ischaemic Femoral Vascular Bed at Rest and After Exercise. Patient 2

4.4.3 Comparison of the extent of myocardial MDA flux associated with rapid atrial versus ventricular pacing.

4.4.3.1 Rapid pacing via the coronary sinus

Five patients were included for study. There were 2 females and mean age was 63 ± 5 years. There were no adverse events associated with the procedure. Peak atrial pacing rate was 130 ± 10 bpm and was limited by symptoms of angina in 4 patients and AV nodal Wenkebach in 1. No patient developed ECG changes consistent with myocardial ischaemia during rapid pacing. Patient characteristics, cardiac haemodynamics and metabolism are summarised in Table 4.2 and Figure 4.5. One patient had a left ventricular ejection fraction less than 50% (44%) and no patient had a mean pulmonary capillary wedge pressure greater than 10 mmHg.

In general, the haemodynamic changes in this group were consistent with impaired coronary flow reserve, demonstrated by increased coronary vascular resistance during the time of increased myocardial oxygen demand and a small decrease in coronary sinus blood oxygen saturation. However, there was no significant release of lactate, suggesting the induction of only mild ischaemia, or MDA from the myocardium. Of note, there was no blood sampling during the recovery period.

4.4.3.2 Right ventricular pacing-induced tachycardia

Twelve patients were included for study. There were no adverse events associated with the procedure. Patient characteristics are shown in Table 4.2. The mean age was 62 ± 9 years and there was only one female in the group. Resting haemodynamics were normal in the majority of patients, although 2 patients had a left ventricular ejection fraction less than 50%, and 2 a mean pulmonary capillary wedge pressure greater than 15 mmHg. During ventricular pacing only 25%

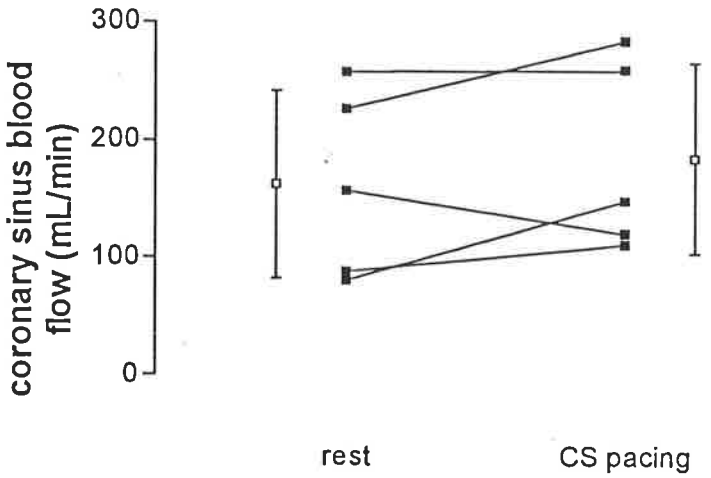


FIGURE 4.5 A Coronary sinus blood flow in patients (n = 5) with significant left coronary artery stenoses at rest and during rapid atrial pacing via the coronary sinus.

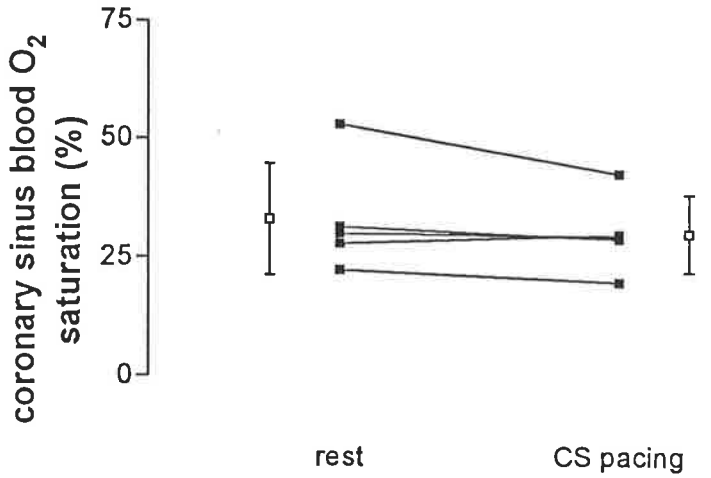


FIGURE 4.5 B Coronary sinus blood oxygen saturation in patients as in Figure 4.5 A at rest and during rapid atrial pacing via the coronary sinus. p = 0.23, NS.

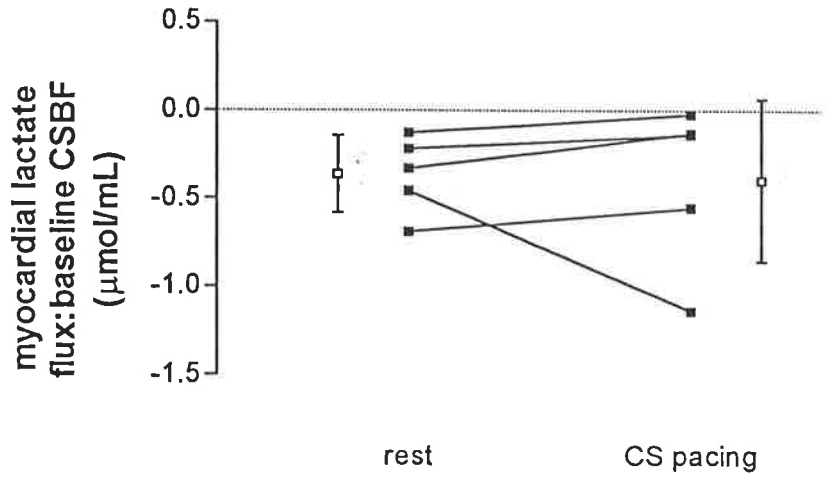


FIGURE 4.5 C Myocardial lactate flux relative to baseline coronary sinus blood flow in patients as in Figure 4.5 A at rest and during rapid atrial pacing via the coronary sinus. p = 0.85,NS.

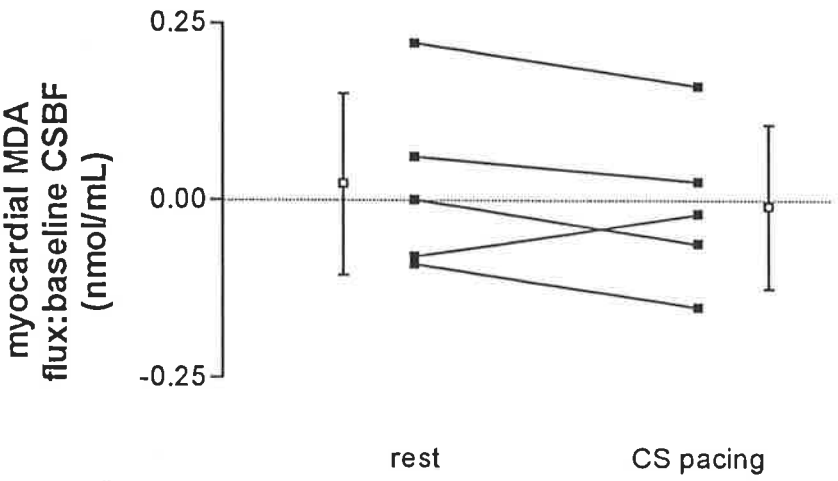


FIGURE 4.5 E Myocardial MDA flux relative to baseline coronary sinus blood flow in patients as in Figure 4.5A at rest and during rapid atrial pacing via the coronary sinus. p = 0.27, NS.

TABLE 4.3 Patient characteristics, cardiac haemodynamics and cardiac metabolism for patients included in studies of atrial versus ventricular pacing-induced myocardial ischaemia.

Parameter	Atrial pacing	Ventricular pacing	p
Number	5	12	
Age (years)	63 ± 5	62 ± 9	NS
Gender (% female)	40	8	-
Cardiac index (L/min/m^2)	2.97 ± 1.18	2.44 ± 0.24	NS
Mean pulmonary capillary wedge pressure (mmHg)	7 ± 1	10 ± 5	NS
Left ventricular ejection fraction (%)	64 ± 15	61 ± 13	NS
Increase in coronary sinus blood flow (mL/min)	23 ± 42	61 ± 42	NS
Decrease in coronary vascular resistance (mmHg.min/mL)	-0.18 ± 0.27 (increase)	0.38 ± 0.38	0.009
δ left ventricular -dP/dt (mmHg/sec)	n/a	195 ± 283 (p = 0.07)	
δ coronary sinus blood oxygen saturation (%)	-2.9 ± 4.6 (p, NS)	-1.1 ± 4.8 (p, NS)	NS
δ myocardial lactate flux as ratio of baseline CSBF (μmol/mL)	0.02 ± 0.42 (p, NS)	0.18 ± 0.24 (p = 0.037)	NS
Peak δ myocardial MDA flux as ratio of baseline CSBF (nmol/mL)	-0.03 ± 0.05 (p, NS)	0.30 ± 0.23 (p = 0.003)	0.0013

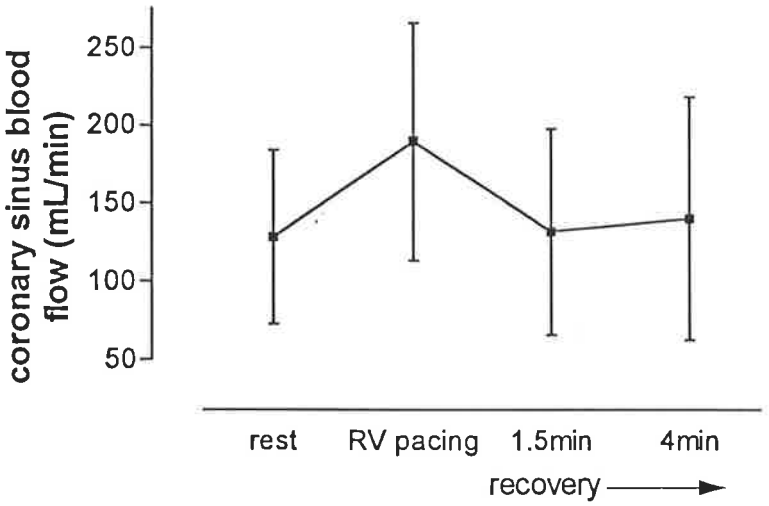


FIGURE 4.6 A Coronary sinus blood flow in patients with significant left coronary artery disease (n = 12) at rest, during RV pacing at 140 bpm and recovery.

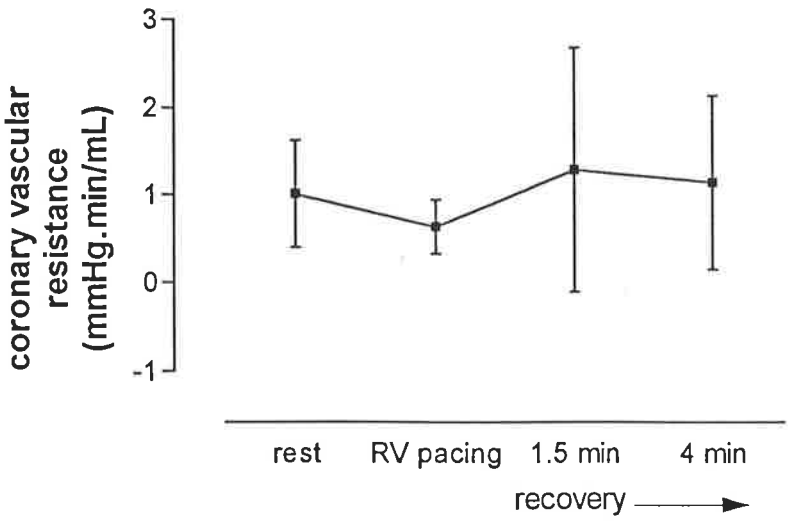


FIGURE 4.6 B Coronary vascular resistance in patients as in Figure 4.6 A (n = 12) at rest, during RV pacing at 140 bpm and recovery.

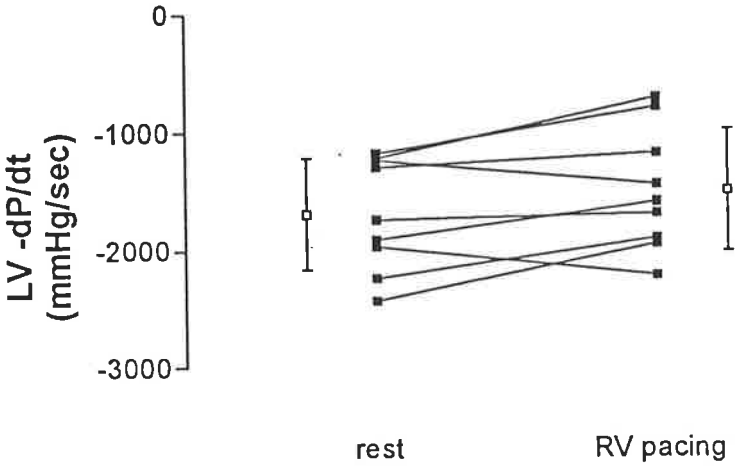


FIGURE 4.6 C Left ventricular -dP/dt in patients as in Figure 4.6 A (n = 9) at rest and immediately after RV pacing at 140 bpm. p = 0.073

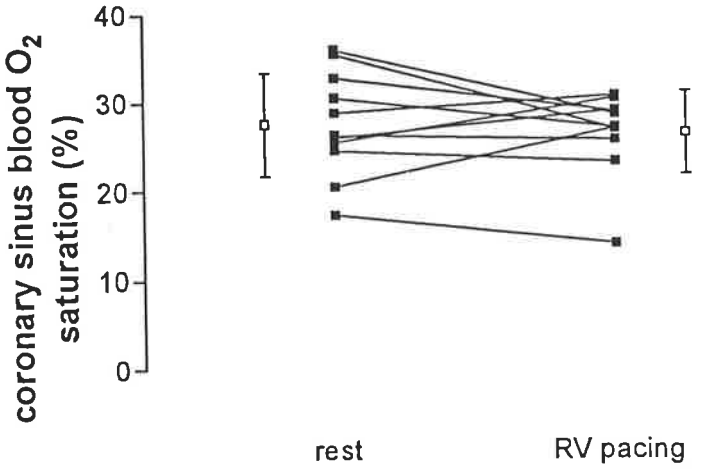


FIGURE 4.6 D Coronary sinus blood oxygen saturation in patients as in Figure 4.6 A (n = 11) at rest and during RV pacing at 140 bpm. p = 0.46, NS.

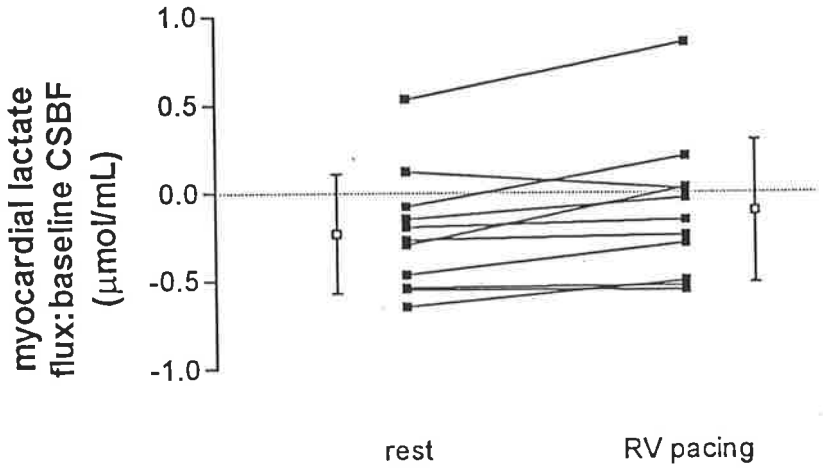


FIGURE 4.6 E Myocardial lactate flux relative to baseline coronary sinus blood flow in patients as in Figure 4.6 A (n = 11) at rest and during RV pacing at 140 bpm. p = 0.037.

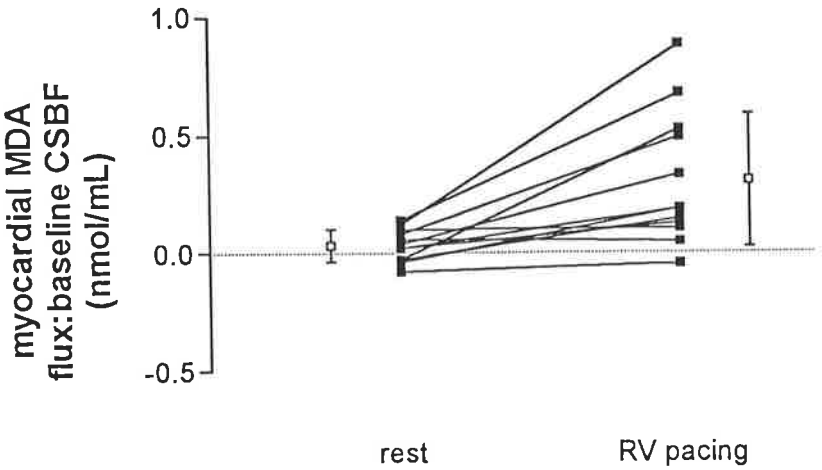


FIGURE 4.6 F Myocardial MDA flux relative to baseline coronary sinus blood flow in patients as in Figure 4.6 A (n = 12) at rest and during or after RV pacing at 140 bpm. p = 0.003.

of patients developed symptoms of angina. One patient developed transient atrial fibrillation after ventricular pacing, without haemodynamic compromise.

This group of patients had better evidence of coronary flow reserve as compared to the atrially paced group. There was a decrease in coronary vascular resistance with increased myocardial oxygen demand during tachycardia ($p = 0.009$ as compared the atrial paced group). The extent of myocardial ischaemia appeared to be mild during this protocol. There were trends towards impaired ventricular relaxation and coronary sinus blood oxygen desaturation, and a statistically significant relative release of lactate from the myocardium during tachycardia as compared with rest (p 0.037).

Myocardial release of MDA was quite marked during paced ventricular tachycardia as compared with rest ($p = 0.003$) or paced atrial tachycardia ($p = 0.008$). Peak MDA release did not necessarily occur during rapid pacing, but often appeared during the 5 minute recovery or "washout" period. There was no correlation between the change in myocardial lactate and MDA release.

4.4.4 Determine the variability of cardiac haemodynamic and metabolic response to rapid pacing-induced myocardial ischaemia

In 3 patients baseline blood samples for MDA were taken in quadruplicate. Mean values of plasma MDA concentration from the coronary sinus were 0.34 ± 0.08 µmol/L, 0.07 ± 0.01 µmol/L and 0.14 ± 0.01 µmol/L (coefficient of variation 25%, 16% and 4% respectively). For plasma MDA concentration from the femoral artery the mean values were 0.22 ± 0.04 µmol/L , 0.05 ± 0.01 µmol/L and 0.16 ± 0.01 µmol/L (coefficient of variation 17%, 13% and 4% respectively).

In two additional patients performed the baseline ventricular pacing-induced myocardial ischaemia and recovery protocol were performed in duplicate (Figure 4.7). Both were males, aged 47 and 69 years. Baseline haemodynamics included mean pulmonary capillary wedge pressure 20 and 1 mmHg, cardiac index 2.68 and 1.87 L/min/m^2 and left ventricular ejection fraction 34 and 63% respectively.

Changes in coronary sinus blood flow, coronary vascular resistance, LV -dP/dt, coronary sinus blood oxygen saturation, myocardial lactate and MDA fluxes in these two patients during duplicate RV pacing are depicted in Figure 4.7 A-E. In general, changes were closely reproducible between pacing episodes. The exception was LV -dP/dt (Figure 4.7 C), where there was a marked disparity in one patient. The cause of this disparity is uncertain.

4.4.5 Effects of GTN, NAC and their combination on cardiac haemodynamics, metabolism and extent of oxidative stress during rapid ventricular pacing-induced myocardial ischaemia.

Seven patients completed the study, with 1 female and mean age of 63 ± 9 years. There were no adverse events associated with the procedure. All patients had baseline cardiac indices above 2 L/min/m^2, 1 patient a mean pulmonary capillary wedge pressure greater than 15 mmHg (16 mmHg) and in 1 patient the left ventricular ejection fraction was less than 50% (41%). Patient results are depicted in Table 4.4 and Figure 4.8.

Resting coronary sinus blood oxygen saturation was low (25.8 ± 4.0%) as compared to patients with a normal left coronary system (36.4 ± 5.8%) , consistent with previous findings in the patients with significant left coronary artery stenoses, suggesting possible myocardial ischaemia at rest

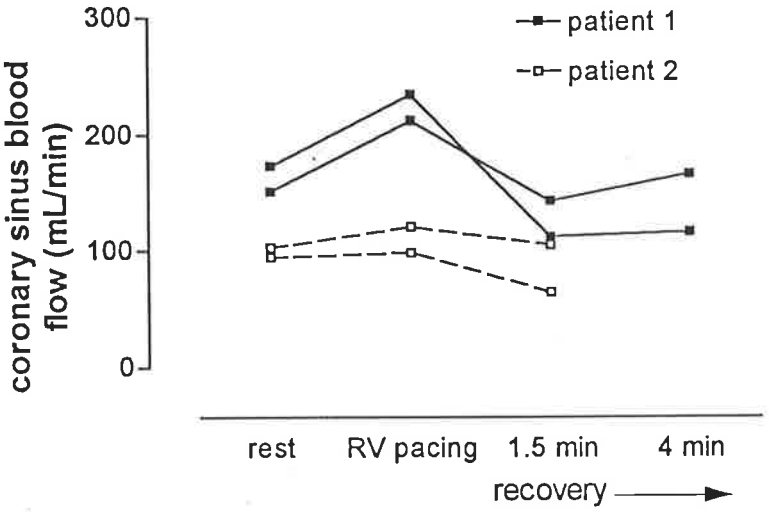


FIGURE 4.7 A Coronary sinus blood flow duplicated in 2 patients with significant left coronary artery disease at rest, during RV pacing at1 40 bpm and recovery.

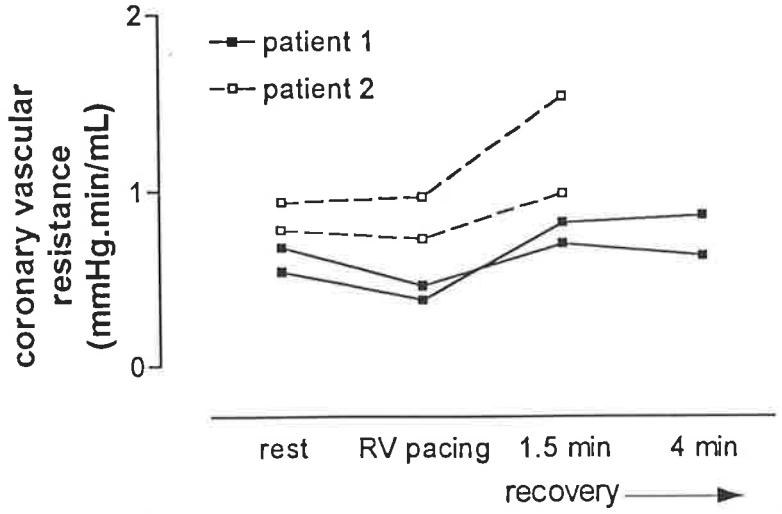


FIGURE 4.7 B Coronary vascular resistance duplicated in 2 patients as in Figure 4.7 A at rest, during RV pacing at 140 bpm and recovery.

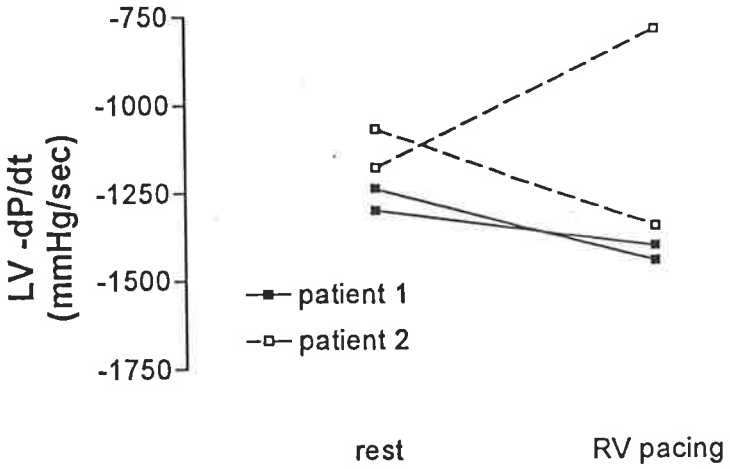


FIGURE 4.7 C Left ventricular -dP/dt duplicated in 2 patients as in Figure 4.7 A at rest and immediately after RV pacing at 140 bpm.

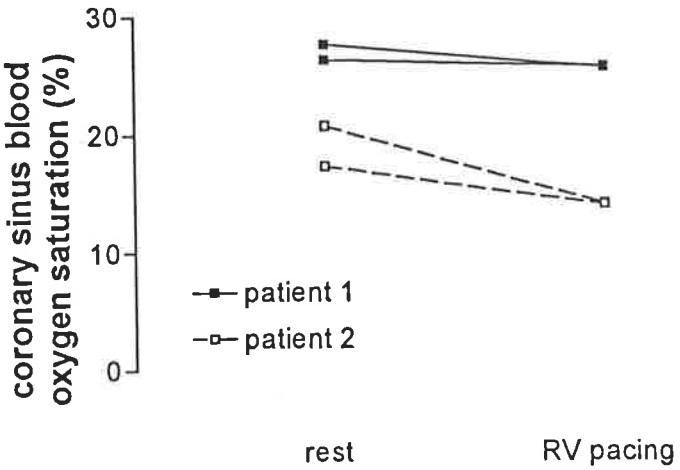


FIGURE 4.7 D Coronary sinus blood oxygen saturation duplicated in 2 patients as in Figure 4.7 A at rest and during RV pacing at 140 bpm.

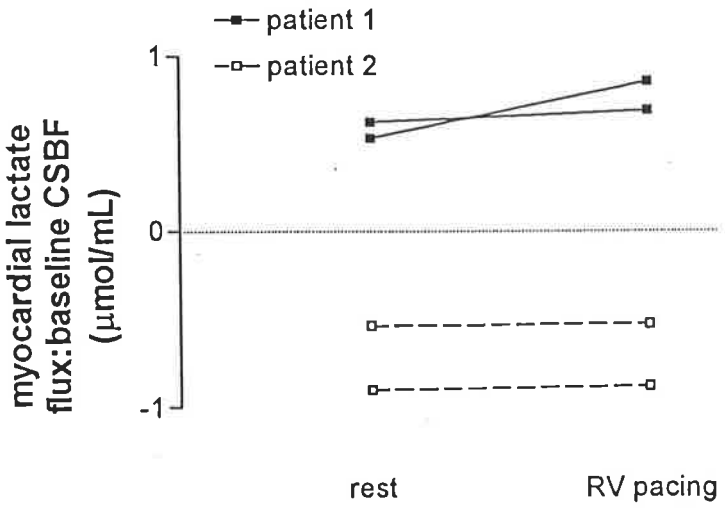


FIGURE 4.7 E Myocardial lactate flux relative to baseline coronary sinus blood flow duplicated in 2 patients as in Figure 4.7 A at rest and during RV pacing at 140 bpm.

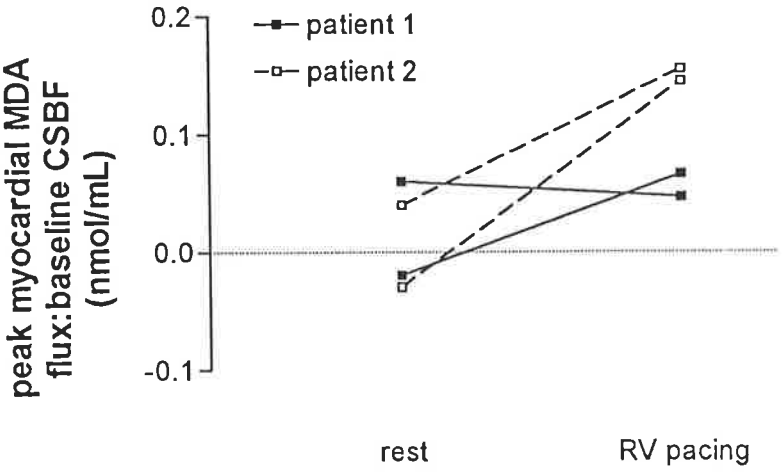


FIGURE 4.7 F Peak myocardial MDA flux relative to baseline coronary sinus blood flow duplicated in 2 patients as in Figure 4.7 A at rest and during or after RV pacing at 140 bpm.

and/or impaired coronary flow reserve. However, coronary sinus blood flow increased moderately with stress, with corresponding decrease in coronary vascular resistance adequate to prevent further coronary sinus blood oxygen desaturation.

During right ventricular pacing at 140 bpm for 2 to 3 minutes with no prior drug treatment, only one patient experienced symptoms of angina pectoris. Left ventricular -dP/dt demonstrated a non-significant trend towards a deterioration of left ventricular relaxation. Also, there was a non-significant trend for the myocardium to develop a relative lactate release. These results suggest that rapid ventricular pacing at 140 bpm induced mild ischaemia in this group of patients.

In keeping with the previous studies, there was a significant myocardial release of MDA during ventricular tachycardia-induced mild myocardial ischaemia ($p = 0.01$). In one patient glutathione redox status was estimated. Myocardial ischaemia was associated with a decrease in myocardial GSH flux and increase in myocardial GSSG flux. The plasma concentrations of NAC 10 to 30 minutes after the intravenous infusion of 5g NAC were 38 ± 4 μmol/L in this patient.

There were no statistically significant changes in cardiac haemodynamics associated with the use of NAC, GTN or the combination of both GTN and NAC. Lactate flux was significantly decreased after infusion of NAC both at rest and during tachycardia (2 way ANOVA, no drug vs NAC, $p = 0.041$), although there was no significant change in lactate flux comparing rest and tachycardia (2 way ANOVA, rest vs tachycardia, $p = 0.91$). A primary comparison of changes in lactate flux showed a significant decrease by GTN versus no prior drug treatment (paired t test, $p = 0.016$). Two way analysis of variance comparing no drug treatment and NAC showed no effect from treatment ($p = 0.61$), but a significant increase in MDA flux comparing rest and tachycardia ($p = 0.0002$). Therefore, overall trends in the changes in metabolic parameters suggested NAC and

TABLE 4.4 Patient characteristics, cardiac haemodynamics and myocardial metabolism. Effect of GTN, NAC or GTN and NAC on rapid ventricular pacing-induced myocardial ischaemia.

Parameter	Baseline	GTN	NAC	GTN+NAC	p
Number	7				
Age (years)	63 ± 9				
Gender (% female)	14				
Left ventricular ejection fraction (%)	60±11				
Mean pulmonary capillary wedge pressure (mmHg)	10±3				
Cardiac index (L/min/m^2)	2.39±0.28	2.16±0.23	2.43±0.43	2.41±0.31	NS
Increase in coronary sinus blood flow (mL/min)	71±47	95±55	96±94	76±29	NS
Decrease in coronary vascular resistance (mmHg.min/mL)	0.36±0.30	0.29±0.14	0.24±0.13	0.20±0.17	NS
Reduction in left ventricular -dP/dt (mmHg/sec)	116±240	100±142	138±232	212±152	NS
δ coronary sinus blood oxygen saturation (%)	2.7±3.9	-0.3±6.5	1.4±5.6	-0.2±5.9	NS
δ myocardial lactate flux as ratio of baseline CSBF (μmol/mL)	0.12±0.12	-0.10±0.23	-0.16±0.46	-0.11±0.29	NS
Peak δ myocardial MDA flux as ratio of baseline CSBF (nmol/mL)	0.29±0.22	0.44±0.56	0.22±0.17	0.17±0.14	NS

Legend: CSBF, coronary sinus blood flow; GTN, glyceryl trinitrate; NAC, N-acetylcysteine; NS, not significant; p, probability; δ, change.

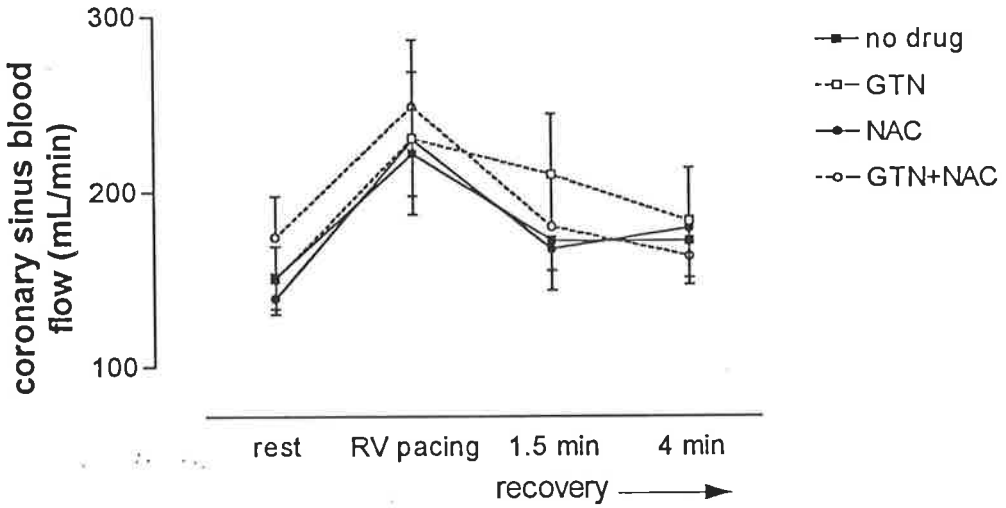


FIGURE 4.8 A Coronary sinus blood flow in patients (n = 7) with significant left coronary artery disease at rest, during RV pacing at 140 bpm and recovery, comparing no prior treatment, GTN, NAC or both GTN+NAC.

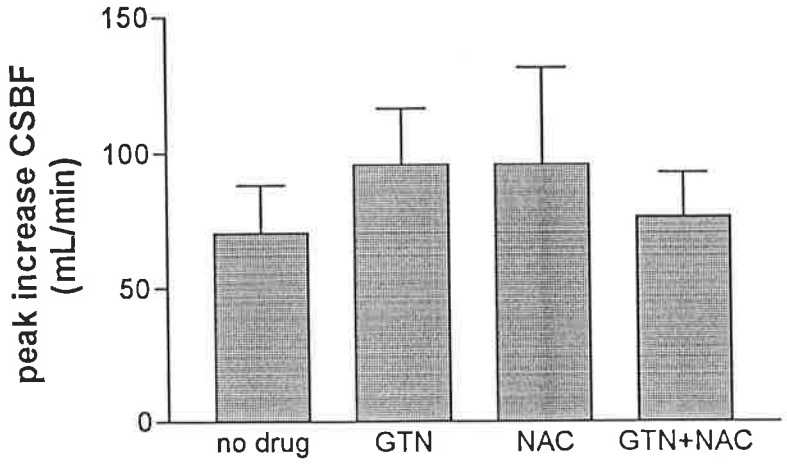


FIGURE 4.8 B Increase in coronary sinus blood flow induced by RV pacing at 140 bpm in patients (n = 7) as in Figure 4.8 A, comparing no prior treatment, GTN, NAC or both GTN+NAC. p,NS

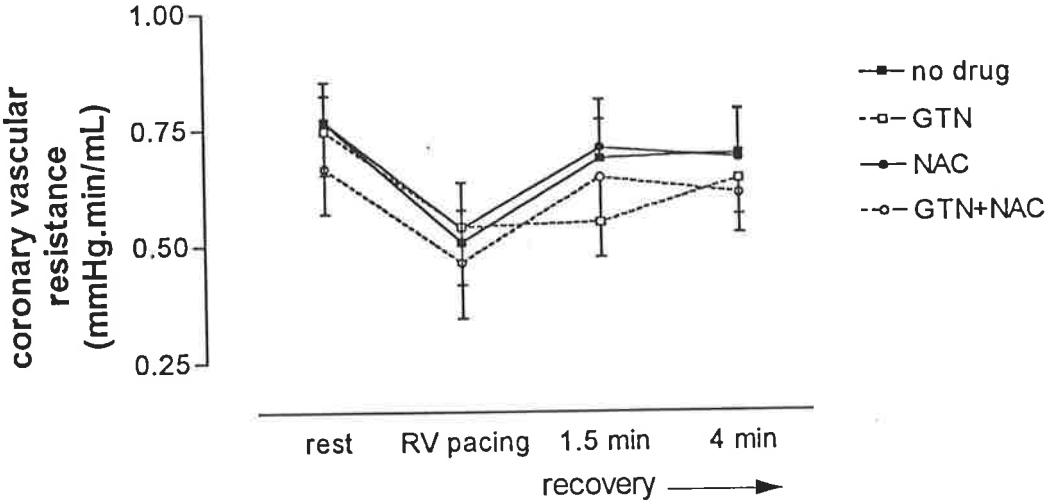


FIGURE 4.8 C Coronary vascular resistance in patients (n = 7) as in Figure 4.8A at rest, during RV pacing at 140 bpm and recovery, comparing no prior drug treatment, GTN, NAC or both GTN+NAC.

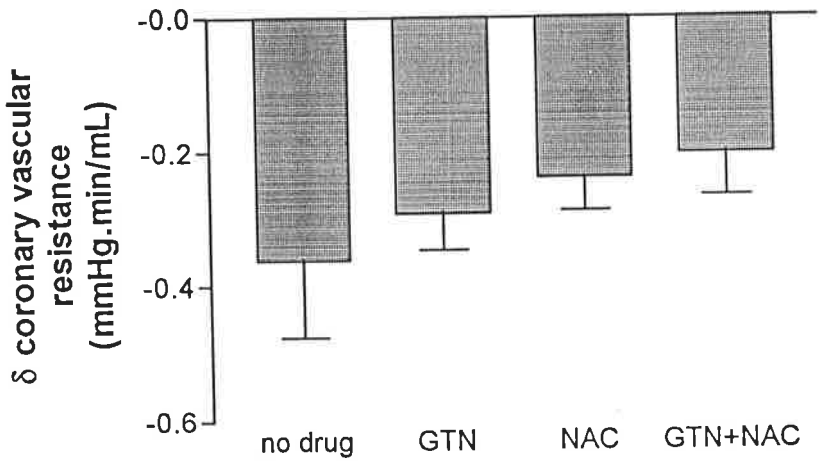


FIGURE 4.8 D Decrease in coronary vascular resistance induced by RV pacing at 140 bpm in patients (n = 7) as in Figure 4.8 A, comparing no prior drug treatment, GTN, NAC or both GTN+NAC. p,NS

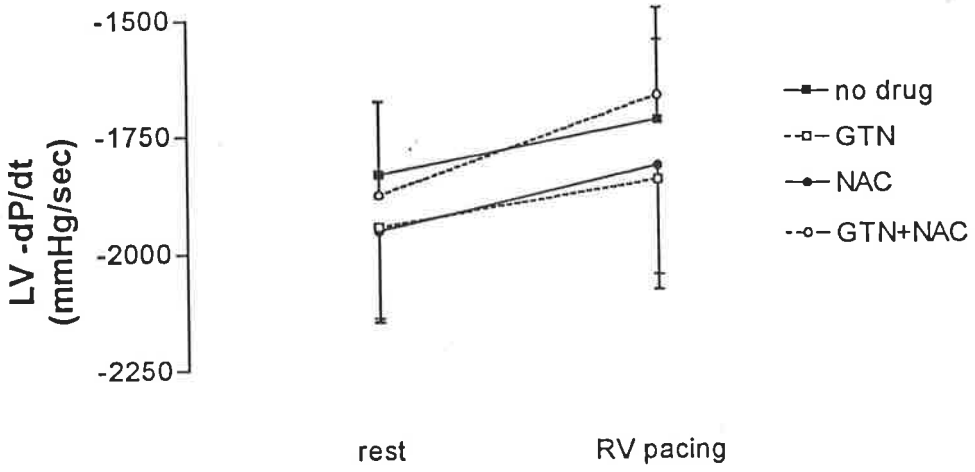


FIGURE 4.8 E Left ventricular -dP/dt in patients (n = 5) as in Figure 4.8A at rest and immediately after RV pacing at 140 bpm, comparing no prior drug treatment, GTN, NAC or both GTN+NAC.

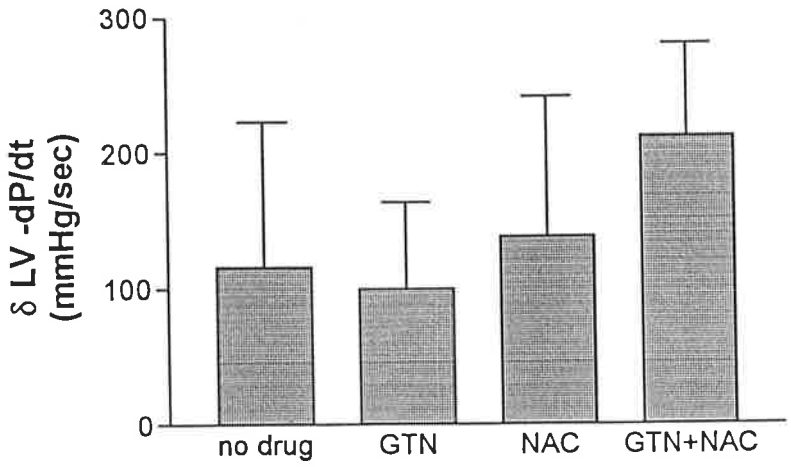


FIGURE 4.8 F Change in left ventricular -dP/dt induced by RV pacing at 140 bpm in patients (n = 5) as in Figure 4.8 A, comparing no prior drug treatment, GTN, NAC or both GTN+NAC. p, NS

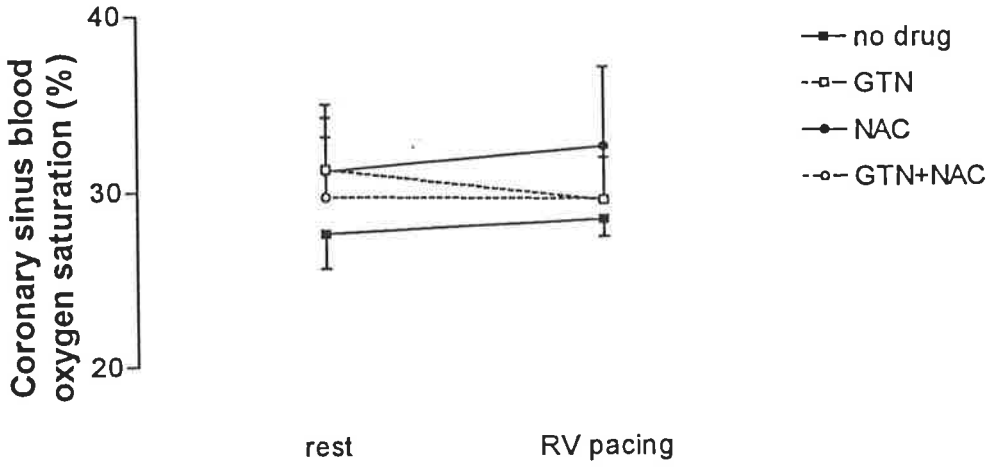


FIGURE 4.8 G Coronary sinus blood oxygen saturation in patients (n = 7) as in Figure 4.8 A at rest and during RV pacing at 140 bpm, comparing no prior drug treatment, GTN, NAC or both GTN+NAC.

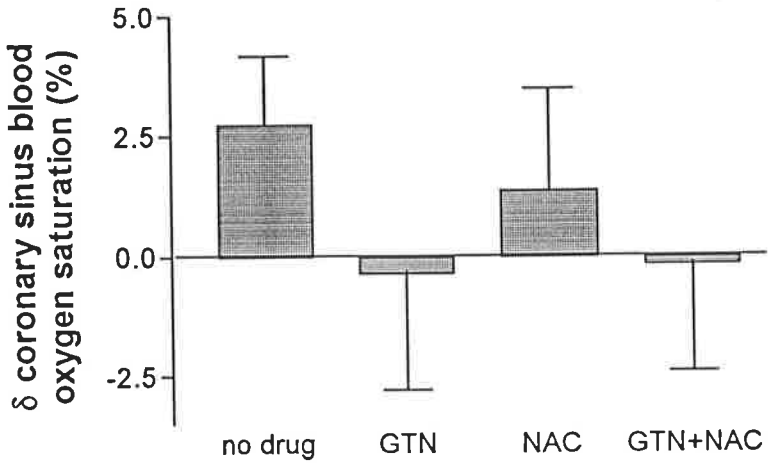


FIGURE 4.8 H Change in coronary sinus blood oxygen saturation induced by RV pacing at 140 bpm in patients (n = 7) as in Figure 4.8 A, comparing no prior drug treatment, GTN, NAC or both GTN+NAC. p, NS

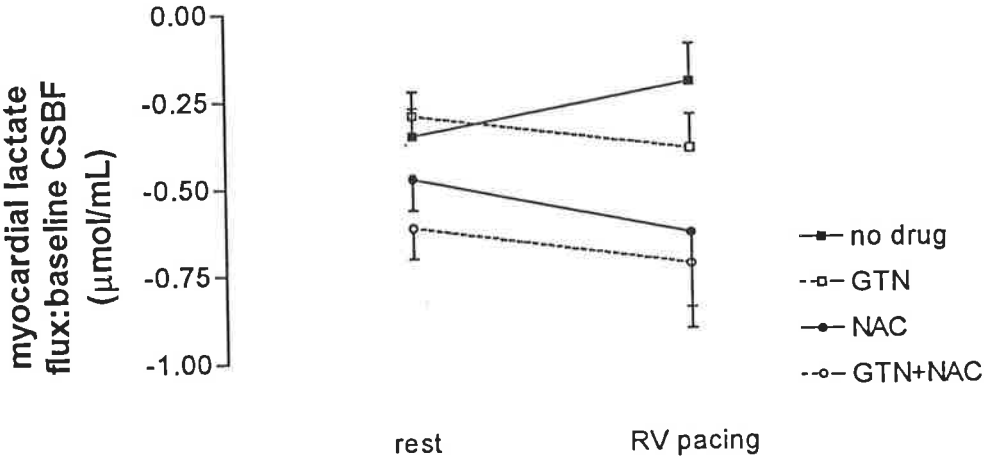


FIGURE 4.8 I Myocardial lactate flux relative to baseline coronary sinus blood flow in patients (n = 7) as in Figure 4.8 A at rest and during RV pacing at 140 bpm, comparing no prior drug treatment, GTN, NAC or both GTN+NAC. (ANOVA, p = 0.04 comparing no drug vs NAC)

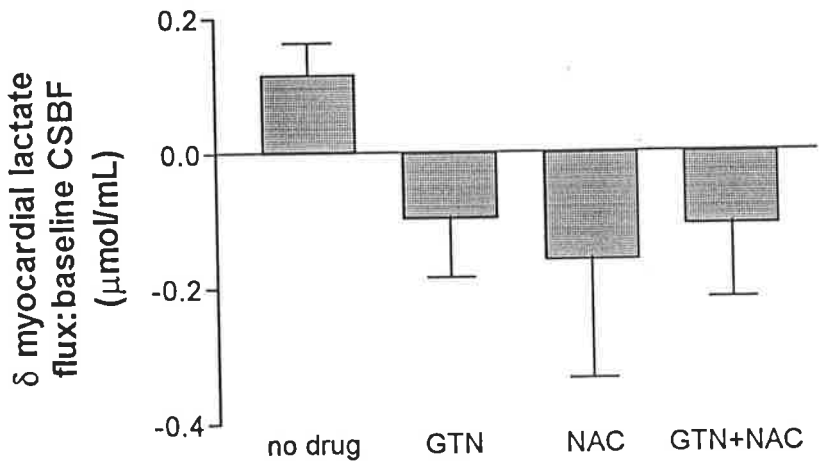


FIGURE 4.8 J Change in myocardial lactate flux relative to baseline coronary sinus blood flow induced by RV pacing at 140 bpm in patients (n = 7) as in Figure 4.8 A, comparing no prior drug treatment, GTN, NAC or both GTN+NAC. p, NS

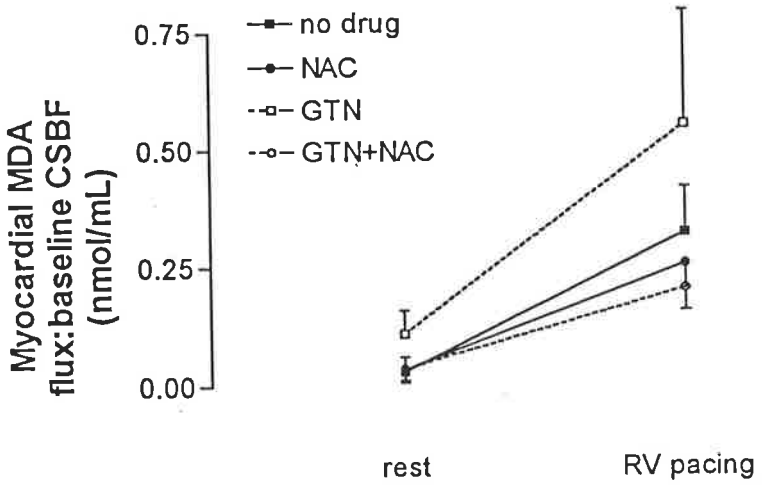


FIGURE 4.8 K Peak increase in myocardial MDA flux relative to coronary sinus blood flow in patients (n = 7) as in Figure 4.8 A at rest and during or after RV pacing at 140 bpm, comparing no prior drug treatment, GTN, NAC or both GTN+NAC. (ANOVA, p, NS comparing no drug vs NAC)

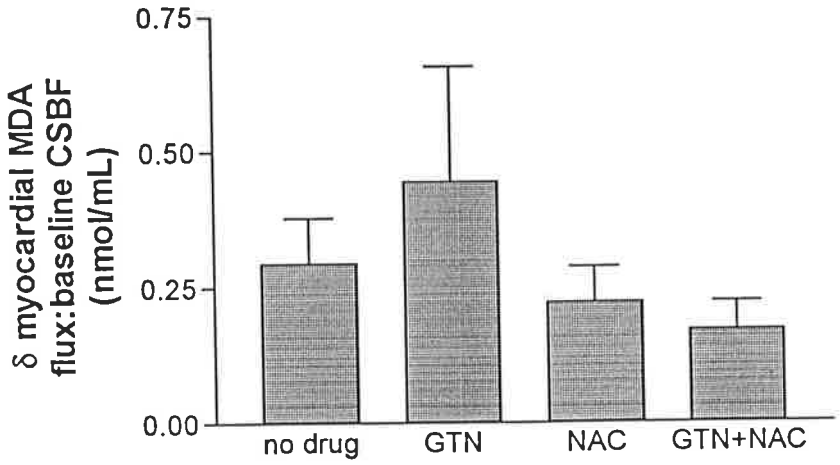


FIGURE 4.8 L Change in peak myocardial MDA flux relative to baseline coronary sinus blood flow induced by RV pacing at 140 bpm in patients (n = 7) as in Figure 4.8 A, comparing no prior drug treatment, GTN, NAC or both GTN+NAC. p, NS

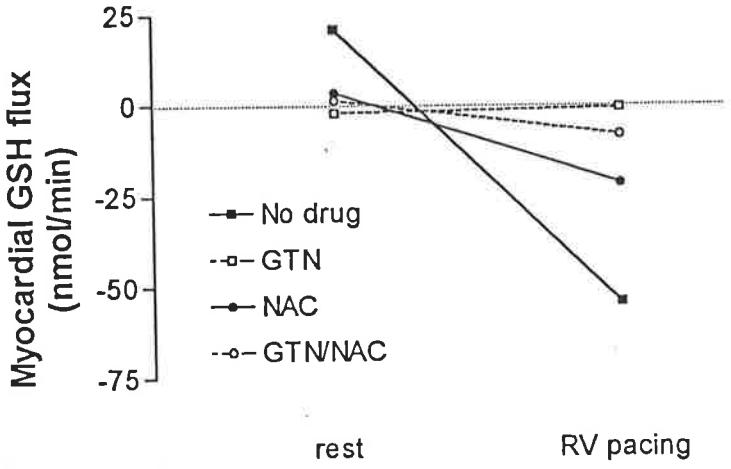


FIGURE 4.8 M Myocardial GSH flux in 1 patient as in Figure 4.8 A at rest and during RV pacing at 140 bpm, comparing no prior drug treatment, GTN, NAC or both GTN+NAC.

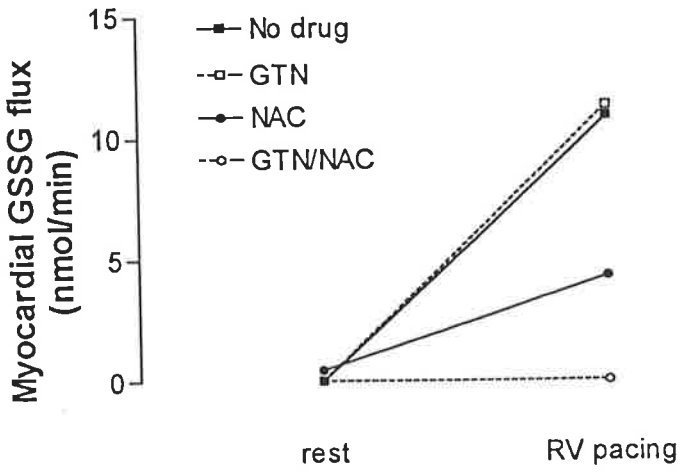


FIGURE 4.8 N Myocardial GSSG flux in 1 patient as in Figure 4.8 A at rest and during RV pacing at 140 bpm, comparing no prior drug treatment, GTN, NAC or both GTN+NAC.

possibly GTN were each associated with less anaerobic metabolism due to myocardial ischaemia, resulting in less myocardial lactate flux both at rest and/or during tachycardia. However, there was no attenuation of the increased myocardial MDA flux during tachycardia-induced myocardial ischaemia.

4.5 DISCUSSION

The results of these studies indicate that the human myocardium readily releases MDA during both non-ischaemic and ischaemic stress. Although the infusion of NAC was associated with decreased myocardial lactate flux, no significant changes in myocardial MDA flux as a parameter of oxidative stress were noted resulting from the use of NAC with or without GTN. However, the changes were small with considerable variability between individuals. This may represent type II error. Specifically, on the basis of the observed standard deviation of MDA release (0.14) between individuals, a patient sample of 22 would be associated with $\delta = 0.12$, $\beta = 0.80$, $\alpha = 0.05$. This information regarding appropriate sample size could not have been gleaned from prior studies, but clearly indicates that the current experiments were adequate only for detection of very large changes in MDA production.

Despite the lack of statistical significance for NAC as regards limitation of oxidative stress, some interesting and unique observations were made:

(1) Plasma lactate concentrations were significantly, but highly variably underestimated when blood was drawn through a long catheter.

(2) The coronary vascular bed readily released MDA in the presence or absence of left coronary artery stenoses during ventricular tachycardia, whereas the non-ischaemic femoral skeletal muscle vascular bed did not release MDA post-exercise.

(3) NAC was associated with decreased myocardial lactate flux in patients with significant left coronary artery stenoses.

4.5.1 Extraction of lactate and MDA by long catheters

Both lactate and MDA plasma concentrations decreased artefactually due to withdrawal of blood through a long catheter. This phenomenon was partly dependent upon the length of the catheter in the case of lactate, as the artefact was abolished when the catheter was only 50 cm as compared 100 cm long. It is likely that the mechanism of this extraction of both lactate and MDA was related to adherence of the measured substances to the wall of the tubing. The luminal surface area or texture, and/or chemical composition of the catheter may have influenced extraction. Although the type of catheter had no statistically significant effect when considering lactate concentrations, the Cordis Judkins catheter was associated with a significantly increased loss of MDA as compared the Coronary Sinus catheter.

The significance of these results is considerable. The only way to sample blood from the coronary sinus in humans (except during cardiac surgery) is via the 100 cm long coronary sinus catheter. A significant artefactual loss of lactate decreases the measured myocardial lactate flux, thereby falsely decreasing any significant change in one of the important markers of anaerobic respiration within ischaemic myocardium. The mean percentage extraction had a large standard deviation and was not correlated to "true" lactate concentration. Therefore a "correction factor" would be unlikely to allow for this technical artefact. The effect on MDA was less marked and not statistically significant when the coronary sinus catheter was utilised, although the same trend was present. Therefore, this extraction may also result in an underestimation of myocardial lipid peroxidation as assessed by this parameter.

4.5.2 MDA release from the myocardial vascular bed

The "control" studies demonstrated a significant disparity between vascular beds as regards metabolism of lactate and MDA. In normally perfused skeletal muscle, increased work was associated with lactate release and no change in MDA vascular gradient. However, the opposite applied to the normally perfused myocardial vascular bed. Increased work associated with ventricular tachycardia was associated with increased lactate uptake but net release of MDA from the myocardium.

In contrast, there was a lack of observed MDA release from the ischaemic myocardium during rapid atrial pacing. This could simply be related to the absence of sampling during the recovery period in these patients. During ventricular pacing, the majority of patients had a peak release of MDA during the recovery period. Therefore, any myocardial MDA release during the recovery period after atrial pacing-induced myocardial ischaemia would have been missed. It is also possible that the extent of ischaemia induced was less in the atrial pacing group. The maximum rate of pacing was slightly less (130 ± 10 bpm) as compared to the ventricular pacing group (140 bpm). However, angina pectoris was the usual factor limiting atrial pacing rate, whereas angina pectoris occurred in only 25% of ventricular pacing patients. None the less, coronary flow reserve appeared to more impaired, but resulted in less myocardial lactate release in the atrial pacing, suggesting less severe ischaemia and therefore a possible type I error. A previous comparison of atrial and atrio-ventricular rapid pacing in patients with ischaemic heart disease (Kyriakides et al. 1993) demonstrated no difference in coronary flow reserve, lactate release and coronary sinus blood oxygen saturation. These investigators concluded that the 2 types of pacing had the same effects on myocardial ischaemia. Despite the conclusions of Kyriakides *et al*, consideration of the fact that myocardial MDA release occurs during ventricular tachycardia despite the presence or absence of epicardial coronary stenoses raises questions as regards the source of MDA in human

myocardium, and the metabolic effects of pacing-induced ventricular tachycardia.

If ventricular tachycardia is metabolically similar to atrial tachycardia in the normal heart, then the source of MDA might be "physiological". Possible sources include increased enzymatic breakdown of prostaglandins (Esterbauer et al. 1991), decreased myocardial breakdown of MDA to malonate (Davydov, 1993) or decreased binding of MDA to protein (see 1.4). Camici *et al* (Camici et al. 1989) reported that myocardial stress during rapid atrial pacing in normal human subjects was associated with a rise in coronary sinus blood flow rather than increased oxygen extraction in response to the increased myocardial oxygen demand. Furthermore, glucose equivalents including lactate were the major incremental source of energy substrate over and above free fatty acids, which predominated as the energy source at rest. The increased myocardial extraction of lactate during increased myocardial work in normal hearts has been confirmed by others (Kaijser and Berglund, 1992), with the extent of lactate extraction directly correlated to arterial lactate concentration and inversely correlated to arterial free fatty acid concentration (Gertz et al. 1980). Malonic acid can be metabolised to malonyl CoA, an important regulator of free fatty acid synthesis (Esterbauer et al. 1991). Skeletal muscle concentration of malonyl CoA in rats has been shown to decrease after exercise and may be associated with increased fatty acid oxidation during exercise (Winder et al. 1989). Therefore, it might be hypothesised that decreased myocardial concentration of malonyl CoA during stress of the human heart, may be associated with decreased MDA metabolism to this product and consequently increased MDA release. However, there are no currently available studies demonstrating this relationship

In the two patients with normal left coronary arteries studied, patient 1 had a significant stenosis in a dominant right coronary artery. Myocardial stress was associated with little increase in coronary sinus blood flow and marked coronary sinus blood oxygen desaturation, suggestive of

impaired coronary flow reserve possibly related to endothelial dysfunction in the presence of anatomically normal left epicardial coronary arteries (Nabel et al. 1990). However, patient 2 behaved as would be anticipated for a normal individual with a significant increase in coronary sinus blood flow and equivocal change in coronary sinus blood oxygen saturation. Both demonstrated increased myocardial lactate uptake with stress. Therefore, even if only considering the response of patient 2 there was still a significant release of MDA from the stressed myocardium.

It is however possible that right ventricular pacing may be associated with septal ischaemia and thus oxidative stress, even with normal epicardial coronary arteries. The presence of a reversible septal perfusion defect on thallium^{201} scintigraphy, even in the absence of coronary artery disease associated with left bundle branch block is well established (Hirzel et al. 1984). Left bundle branch block is associated with a relative shortening of the left ventricular diastolic time, particularly within the interventricular septum. The subsequent asynchrony between the septum and the rest of the ventricle may result in changes in both pressure and volume throughout the cardiac cycle in both ventricles, manifest dynamically as paradoxical septal motion (Grines et al. 1989). Furthermore, Ono *et al* (Ono et al. 1992) demonstrated that septal myocardial thickening decreased and septal intramural pressure rose during diastole resulting in decreased perfusion. This was associated with relatively decreased glucose and thallium^{201} uptake within the septum and no overall release of lactate, suggesting that despite the septal hypoperfusion, the decreased work resulted in no ischaemia. Relative myocardial lactate release has previously been established as a biochemical marker of significant myocardial ischaemia (Gertz et al. 1981). In the open-chested dog experiments of Ono *et al* (Ono et al. 1992) the hearts were paced approximately 10 to 20 bpm above their native heart rate to maintain pacing dominance, but were not subjected to tachycardia equivalent to exercise. Therefore, it could be hypothesised that during exercise, the

hypoperfused septum becomes ischaemic.

The presence of a "non-ischaemic" pattern of lactate metabolism in the two patients with normal left coronary arteries was consistent with the findings of Ono *et al* (Ono et al. 1992), or in fact may reflect the more dominant normal myocardial metabolism within the ventricular free wall. It also may have been artefactually changed by extraction of lactate within the coronary sinus catheter. If the induction of ventricular tachycardia was associated with myocardial ischaemia, the release of myocardial MDA during ventricular pacing in the ischaemic and "non-ischaemic" groups, being more profound than lactate release, suggests that MDA release from the myocardium is a very sensitive marker of myocardial ischaemia and probably oxidative stress. The redox status of glutathione across the myocardial vascular bed in one patient during the induction of ischaemia was consistent with the findings of Ferrari *et al* (Ferrari et al. 1990), with decreased GSH and increased GSSG release indicative of oxidative stress. More extensive investigation of these changes is warranted in the future.

Only one other study (Oldroyd et al. 1990) has examined the extent of lipid peroxidation during and after pacing-induced myocardial ischaemia. In this investigation, estimation of the normal plasma concentration of MDA as measured by their TBA-based assay was extraordinarily high at 18.5 ± 1.4 µmol/L. It is therefore more appropriate to call the product measured TBARS. Despite these technical limitations, they demonstrated a relationship between the myocardial release of lactate and TBARS. Significant myocardial TBARS release (measured as the extraction ratio) was only seen in patients with relative lactate production. Only 1 of the 12 patients who released lactate from the myocardium had no left epicardial coronary artery disease, whereas 7 out of 14 patients who remained lactate extractors during pacing had normal coronary arteries. The significant myocardial release of MDA was only noted 5 minutes into the recovery period after

peak pacing of either the atrium or ventricle between 120 and 180 bpm. Therefore, they suggested that myocardial ischaemia (as indicated by myocardial lactate release) and recovery was associated with lipid peroxidation. No comment as to the percentage of patients with atrial and ventricular pacing was made. Furthermore, they did not comment as to why a patient with normal epicardial coronary arteries may produce myocardial MDA. In contrast, the current study utilised a more specific assay for MDA and found MDA to be released form the myocardium during ventricular tachycardia, whatever the coronary anatomy or lactate metabolic status.

From the current studies, it appears likely on balance that myocardial MDA release represented a sensitive marker of oxidative stress, present after the induction of myocardial ischaemia by rapid right ventricular pacing in the human heart.

4.5.3 Effect of GTN or NAC on the modulation of myocardial ischaemia and recovery.

There are no previous studies examining the effect of the antioxidant NAC (or in fact any other antioxidants) in humans in the setting of mild myocardial ischaemia as carried out in the current model. No statistically significant changes in cardiac haemodynamics or lipid peroxidation were noted by the use of NAC as an antioxidant or possible potentiator of the anti-ischaemic and possibly cytoprotective effects of GTN. However, NAC was associated with a significant decrease in lactate flux both at rest and during tachycardia. This suggested that NAC modulated the extent of anaerobic metabolism secondary to myocardial ischaemia, and that ischaemia was present both at rest and during tachycardia. The low coronary sinus blood oxygen saturation at rest, also supported the presence of ischaemia at this time. The mechanism by which NAC modulated the extent of myocardial ischaemia in the absence of GTN is unclear. Although not statistically confirmed, it appeared possible that NAC either alone, or in combination with GTN, was associated with less oxidative stress resulting from mild myocardial ischaemia and recovery than

baseline. As mentioned earlier, this was not associated with any discernible trend as regards cardiac haemodynamic function.

In the only patient where plasma NAC concentration was measured, there was a concentration of the drug of approximately 40 µmol/L. However, this was lower than that achieved with continuous intravenous infusion of 15g/24 hours in the patients described in Chapter 6. It is possible that the bolus dose utilised resulted in a plasma concentration insufficient to elicit maximal antioxidant effects in human myocardium. Nevertheless, increasing the size of the bolus dose would be associated with increased risk of adverse reactions related to the infusion of the drug (Holdiness, 1991; Ho and Beilin, 1983).

Assessment of the glutathione redox status and plasma NAC concentrations in a protocol of this type may aid further understanding of the effect of NAC on oxidative stress induced by myocardial ischaemia, and also determine whether the lipid peroxidation associated with ventricular tachycardia was also accompanied by changes in GSH:GSSG as seen in the one patient analysed. In chapter 6, GSH:GSSG was a more sensitive biochemical indicator of oxidative stress than lipid peroxidation, and was associated with more profound changes in response to NAC. Therefore, the putative antioxidant effect of NAC may be better reflected by changes in this parameter, especially as NAC has a direct effect on myocardial intracellular GSH concentrations that may be found in parallel extracellularly.

Chapter 5:

Modulation of the Intensity of Myocardial Ischaemia During Evolving Acute Myocardial Infarction Prior to Reperfusion: Aggravation by Streptokinase.

5.1 Introduction

The extent of myocardial salvage is determined by many factors as discussed in 1.3, but principally involve the duration and intensity of ischaemia and its modulation. Furthermore, the intensity and duration of preceding myocardial ischaemia are important determinants of the extent of reperfusion injury (see 1.5). To date, most emphasis in the management of evolving acute myocardial infarction has been placed on rapid and adequate reperfusion of the occluded infarct-related artery, rather than the modulation of the intensity of myocardial ischaemia prior to reperfusion.

Thrombolysis is well established to improve myocardial salvage, function and long-term mortality in selected patients with evolving acute myocardial infarction (see 1.2.5.2). However, thrombolytic agents are associated with a variety of adverse effects. One such effect of intravenous infusion of thrombolytic agents is the induction of hypotension (ISIS-2 (Second International Study of Infarct Survival) collaborative group, 1988; The GUSTO Investigators, 1993; Lew et al. 1985; White et al. 1989; Herlitz et al. 1993) which is usually transient, but which may require temporary cessation of the thrombolytic agent, and/or intravenous fluid administration (Lew et al. 1985; Herlitz et al. 1993). The aetiology of this phenomenon remains unclear. It has previously been felt to be generally of minor significance as regards long-term clinical outcome (Herlitz et al. 1993). Furthermore, a consistent feature of placebo-controlled thrombolytic trials for acute myocardial infarction is increased day 0-1 mortality in patients treated with thrombolytic therapy (Fibrinolytic Therapy Trialists' (FTT) Collaborative Group, 1994). Potential mechanisms for this "early hazard", which limits net benefit of thrombolysis on long-term mortality, include fatal haemorrhage, myocardial rupture and possibly reperfusion injury (Fibrinolytic Therapy Trialists' (FTT) Collaborative Group, 1994; Maggioni et al. 1992; Honan et al. 1990), although it is likely that other as yet unknown mechanisms play a role.

While monitoring patients during thrombolytic therapy for evolving acute myocardial infarction (see chapter 6) there was frequently noted a transient incremental increase in S-T segment elevation during the infusion of streptokinase suggestive of an increase in the intensity of myocardial ischaemia at this time. These observations had not previously been reported in the literature. Therefore, a more systematic study of the S-T segment trends during intravenous streptokinase infusion was undertaken as part of this thesis. Correlations with other haemodynamic and clinical parameters were sought in order to hypothesise as to the mechanism and clinical implications of any changes in the S-T segment trends in the "pre-reperfusion" period.

5.2 Hypotheses to be tested

The null hypotheses to be tested in this study were that streptokinase infusion does not affect severity of myocardial ischaemia prior to reperfusion, and that the occurrence or severity of hypotensive responses to streptokinase infusion do not affect myocardial ischaemia.

5.3 Method

5.3.1 Study protocol

The study group consisted of 20 consecutive patients, admitted to the Coronary Care Unit with evidence of evolving acute myocardial infarction fulfilling the inclusion and exclusion criteria set out in Table 5.1.

The protocol for treatment involved the intravenous infusion of glyceryl trinitrate through non-absorptive polyethylene tubing at 5 μg/ min. Verapamil was then injected at 1 mg/min intravenously, up to 5 mg as tolerated. Contraindications to the use of intravenous verapamil included AV nodal block, haemodynamically significant bradycardia or hypotension, past or

TABLE 5.1 Inclusion and Exclusion Criteria

Inclusion Criteria
1. age ≤ 75 years
2. chest pain of ≤ 6 hours duration
3. ECG evidence of transmural ischaemia: ≥ 1 mm S-T segment elevation in ≥ 2 limb leads and/or ≥ 2 mm S-T segment elevation in ≥ 2 praecordial leads
Exclusion Criteria
1. known allergy to streptokinase
2. streptokinase administration within the previous 6 months
3. surgery or cerebrovascular accident within the previous 6 weeks
4. warfarin therapy
5. active peptic ulcer disease
6. bleeding disorders
7. uncontrolled hypertension
8. diabetic proliferative retinopathy.

current clinical evidence of significant left ventricular dysfunction, previous ingestion of any β-adrenergic receptor antagonist in the last 24 hours, or allergy to the drug. At least 10 minutes after commencement of glyceryl trinitrate infusion, intravenous hydrocortisone (100 mg) was injected followed by 1.5 million units intravenous streptokinase infused in 50 mL of 5% dextrose over 30 minutes. Streptokinase-induced hypotension was managed where necessary by placing the patient in the Trendelenburg position, intravenous infusion of the plasma expander Haemaccel (Hoechst Australia) and as a last resort, slowing or temporary cessation of the streptokinase infusion. Immediately following termination of streptokinase infusion, intravenous heparin was administered at a rate of 1000 U/hr, glyceryl trinitrate infusion at 5 to 10 μg/min continued, and oral verapamil 80 mg three times daily prescribed to commence immediately unless contraindicated (Lee et al. 1988; Arstall et al. 1992)

To exclude the possibility that changes in severity of ischaemia reflected either effects of glyceryl trinitrate or verapamil or an interaction between these agents and streptokinase, a further five patients were treated with streptokinase alone, all other aspects of the treatment protocol being as for the remainder of the group.

Twelve lead continuous S-T segment monitoring was performed as an index of severity of ischaemia on all patients from admission to the Coronary Care Unit for at least 5 hours (Figure 5.1). The lead with the maximum S-T elevation was used as the reference lead and S-T elevation every 0.1 hours was measured. The first derivative of this curve (dST/dT) in millimetres per hour was obtained utilising the software package GraphPad InPlot (GraphPad Software Inc. version 4.03). Systemic systolic blood pressure was measured using an automatic sphygmomanometer (Dynamap vital signs monitor 1846 by Critikon) placed on the upper arm, at least 5 minutely from admission to 1 hour after the commencement of intravenous streptokinase infusion, and at least

15 minutely for the next 2 hours. Each patient was asked to estimate the level of chest pain at the same time intervals as systolic blood pressure monitoring, utilising a score from one to ten.

For the purpose of hypothesis testing, 4 definitions were utilised prospectively. Aggravation of myocardial ischaemia was defined as dST/dT greater than 30 millimetres per hour. Hypotension was defined as an absolute fall in systolic blood pressure of 20 mmHg or more from commencement of streptokinase infusion and an absolute minimum systolic blood pressure less than 120 mmHg. Time to reperfusion was defined as the time from the commencement of streptokinase to reduction of S-T elevation in the reference lead to 50 % of maximal value. Time zero was defined as the time of commencement of streptokinase.

5.3.2 Statistical analysis

In view of previous investigations indicating that streptokinase-induced hypotension occurs most frequently during infusion of the drug (Lew et al. 1985; Herlitz et al. 1993) and that the mean time of reperfusion is at least 60 minutes after commencement of streptokinase infusion (Krucoff et al. 1986; Hackett et al. 1987), the first 40 minutes were taken as representing in general, a pre-reperfusion period. The significance of changes in S-T elevation and systolic blood pressure was evaluated via Dunnett's test. Occurrence of systolic blood pressure and/or degree of ischaemia was categorised in absolute terms and assessed via Wilcoxon rank sum test. Furthermore such variations were grouped in 20 minute segments from time zero for purposes of statistical analysis. Distribution of times of aggravated ischaemia and of hypotension was evaluated utilising chi-squared test involving these 20 minute segments. Correlations were sought between times of occurrence of aggravated ischaemia and of hypotension using chi-squared test, and between extent of aggravation of ischaemia and degree of hypotension via linear regression. Pain levels at commencement of streptokinase, time of maximum dST/dT during streptokinase infusion, end of

streptokinase and time to 50% of maximum dST/dT were analysed via Dunnett's test. All analyses involved two-tailed methods and a p value of less than 0.05 was used to indicate statistical significance. Normally distributed data are reported, unless otherwise stated as mean plus or minus the standard deviation.

5.4 Results

5.4.1 Patient characteristics

Twenty patients were studied initially, including 13 males. Mean age was 55 ± 12 years. Patients presented 2.8 ± 2.2 hours after the onset of chest pain. The infarct site was anterior in 11 (55%) of patients and infero-posterior in the remainder. No patient had isolated right ventricular or posterior infarction. Median maximum S-T elevation was 5.5 millimetres (range 2.1 - 17.1). Initial systolic blood pressure was 128 ± 16 mmHg and heart rate 70 ± 12 beats per minute.

Fifteen patients (75%) received intravenous verapamil at a median dose of 5 milligrams (range 2 - 5). Streptokinase infusion was slowed or temporarily interrupted in 8 patients (40%) primarily due to hypotension. The overall median infusion time was still 30 minutes (range 28 - 75). A median of 100 millilitres (range < 50 - 500) of the plasma expander Haemaccel was required for the management of hypotension in 9 patients (45%). Other variations from the normal treatment regimen were intravenous dopamine infusion prior to and during streptokinase in one patient, and reduced prescribed doses of streptokinase (0.5 to 0.75 million units) in 4 patients.

No patient developed clinically overt hypersensitivity to streptokinase. There were no deaths over the first 24 hours of treatment, no haemodynamically significant bleeding or arrhythmias. In all

cases, there was eventual reduction of S-T elevation consistent with reperfusion, with median time to reduction of S-T elevation to 50% of maximal value of 62 minutes (range 9 - 174 minutes). (Table 5.2)

5.4.2 Effects on systolic blood pressure and extent of electrocardiographic S-T segment deviation

The maximum positive dST/dT was 217 ± 152 millimetres/hour occurring at 13 ± 13 minutes after commencement of streptokinase. Increases in positive dST/dT, representing aggravation of myocardial ischaemia, were more common in the first 40 minutes as compared to 41 to 80 minutes ($p < 0.0001$) and the maximum dST/dT was significantly greater in the first 40 minutes as compared to 41 to 80 minutes ($p < 0.01$). Maximum dST/dT within the first 1 hour occurred in 19 (95%) patients before 30 minutes. Maximal dST/dT within the first 40 minutes occurred at less than 20 minutes in 18 patients, versus 21 to 40 minutes in 2 patients ($p< 0.001$) (Table 5.2). Therefore, aggravation of ischaemia, a phenomenon of the pre-reperfusion period, occurred most commonly within the early phase of the streptokinase infusion. Mean S-T elevation within the first 40 minutes of treatment increased from 4.4 ± 3.0 millimetres at zero minutes to a maximal value of 5.3 ± 3.7 millimetres at 12 minutes ($p< 0.05$) (Figure 5.2).

Hypotension developed in 16 patients (80%) and was always transient, resolving either spontaneously or with treatment in all patients. The mean minimum systolic blood pressure over the first 1 hour was 92 ± 22 mmHg occurring 16 ± 5 minutes after the commencement of streptokinase ($p < 0.01$) (Table 5.2, Figure 5.2). The minimum blood pressure occurred before 20 minutes in 16 patients (80%) and after 20 minutes in 4 patients (20%) ($p < 0.01$). Only 1 patient developed hypotension greater than 20 minutes after commencement of streptokinase. The mean

TABLE 5.2: Changes in Myocardial Ischaemia and Systolic Blood Pressure (During the First 1 Hour of Therapy).

Patient Number	Maximum ST Elevation		Aggravation of Ischaemia		Systolic Blood Pressure Changes			Time to 50% ST Maximum (minutes)
	Lead	Extent (mm)	dST/dT Maximum (mm/hour)	Time of dST/dT Maximum (minutes)	Initial BP (mmHg)	Minimum BP (mmHg)	Time of Minimum BP (minutes)	
1	V6	2.1	57	7	129	116	11	78
2	V2	3.7	81	27	152	84	20	54
3	V3	5.5	162	16	136	112	15	42
4	V2	3.1	122	14	112	76	16	42
5	V2	7.3	401	52	138	87	13	69
6	V2	7.8	331	10	127	97	16	33
7	III	17.1	562	9	152	103	14	54
8	V2	4.5	174	-2	122	87	17	9
9	V3	6.3	175	20	124	72	25	156
10	III	7.5	217	8	125	121	21	77
11	V3	8.2	263	28	122	114	25	93
12	III	4.5	328	8	99	49	15	24
13	III	5.5	202	16	102	50	20	42
14	III	3.7	37	8	145	83	6	114
15	V2	11.2	568	8	120	101	11	36
16	III	11.8	111	3	117	75	13	162
17	III	4.3	177	4	141	102	10	81
18	III	3.3	109	8	148	106	10	144
19	V2	9.3	87	20	139	128	25	174
20	V1	2.2	172	-11	119	72	11	42
Mean ± SD	-	**Media n=5.5**	**217±152**	**13±13**	**128±15**	**92±22**	**16±5**	**Median= 62**

relative fall in blood pressure between baseline and minimum blood pressure was 29 ± 15 %.

Thus average maximum dST/dT coincided with the onset of the hypotensive episode (Figure 5.2). There were no significant correlations between minimum systolic blood pressure and maximum dST/dT, or between relative fall in systolic blood pressure and maximum dST/dT at any stage of treatment. Thus, the association between hypotension and aggravation of myocardial ischaemia was limited to concurrence.

Patients' estimation of pain severity showed a significant reduction from 6 ± 3 (out of a score of 10) at the commencement of streptokinase to 4 ± 3 ($p < 0.05$) at end of the streptokinase infusion and 3 ± 3 ($p < 0.01$) at the time of halving of the maximum S-T segment elevation (Figure 5.3). There was no statistically significant change in pain severity at the time of maximum dST/dT during streptokinase infusion (6 ± 3).

Among the additional five patients treated without glyceryl trinitrate and verapamil, both an initial hypotensive phase and a transient aggravation of ischaemia were also noted (Figure 5.4). The extent of both these changes was comparable to that occurring in the presence of glyceryl trinitrate and verapamil.

5.5 Discussion

This findings of this study appear to implicate streptokinase *per se*, rather than the reperfusion process, as a cause of transient aggravation of myocardial injury early after initiation of treatment for acute myocardial infarction. Coincident with the frequent, and previously well documented, blood pressure fall that occurred with infusion of streptokinase, S-T elevation increased,

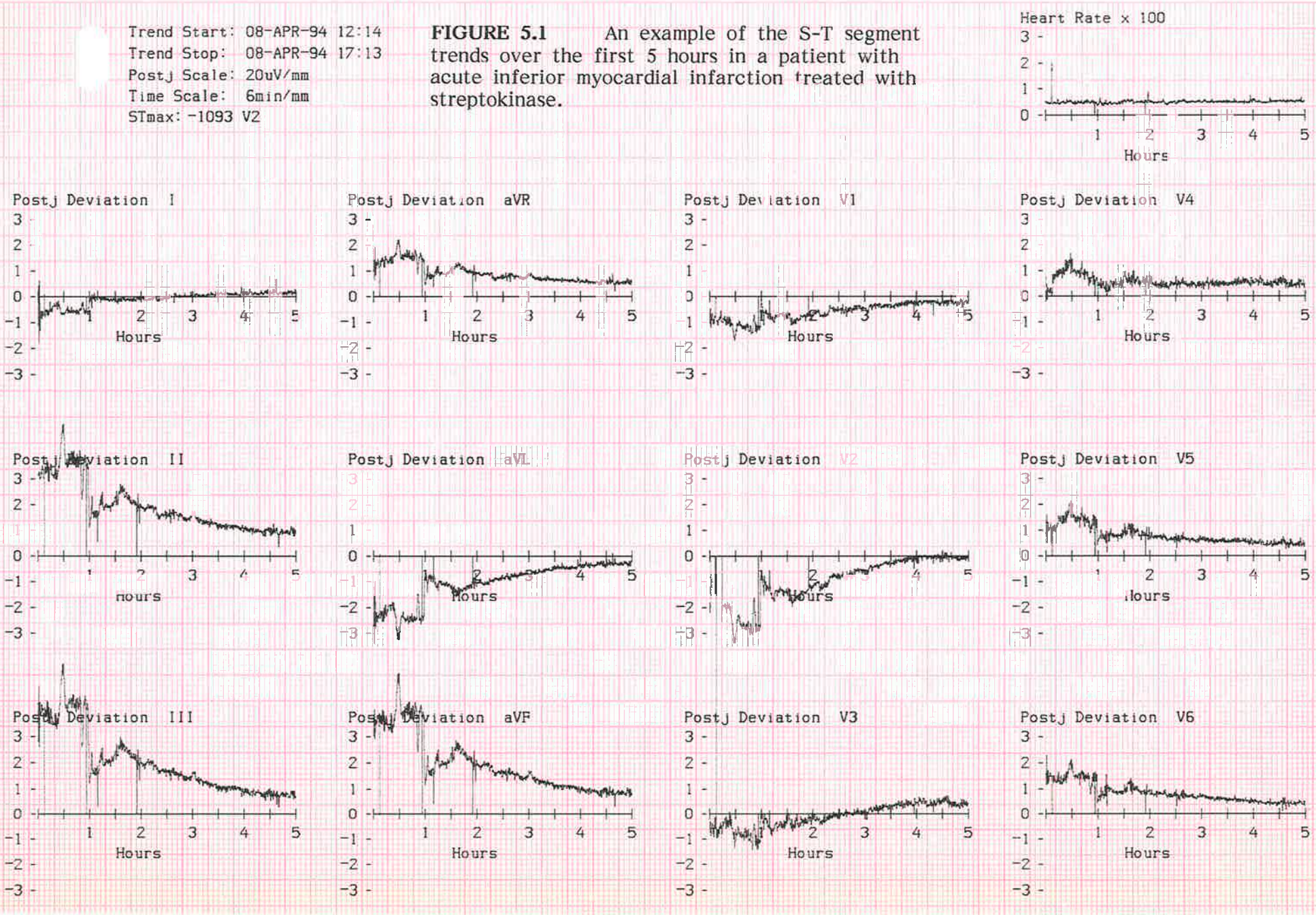
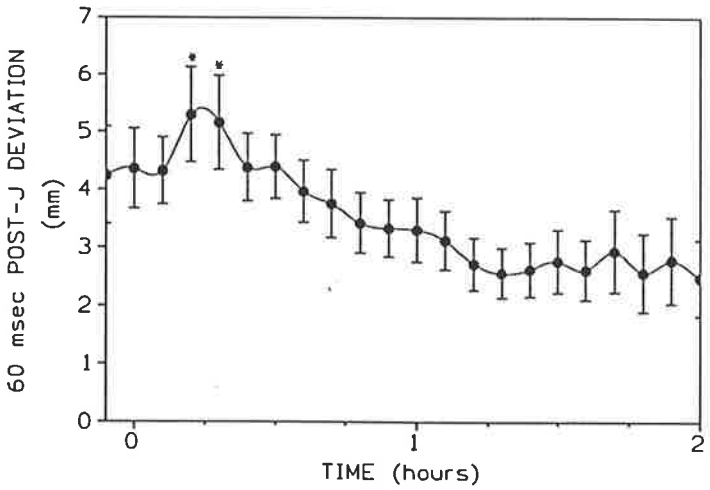
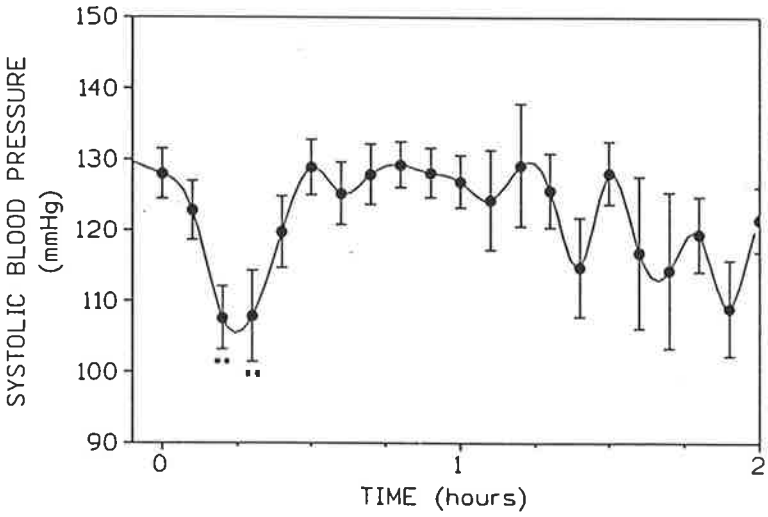


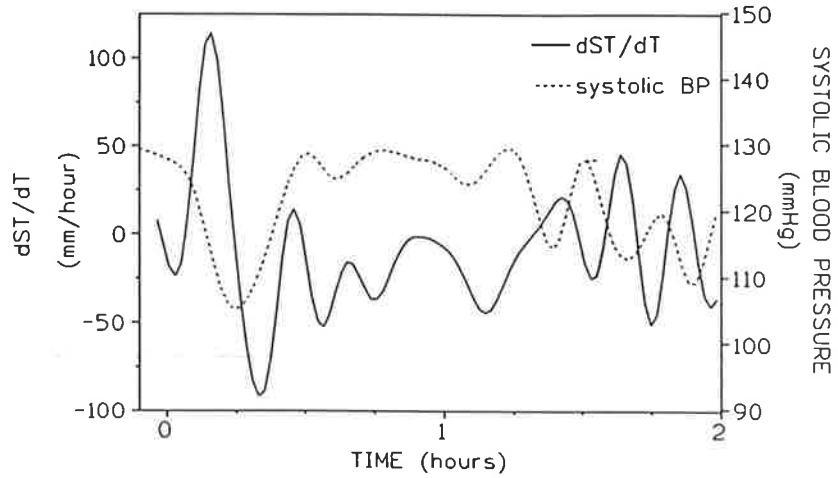
FIGURE 5.1 An example of the S-T segment trends over the first 5 hours in a patient with acute inferior myocardial infarction treated with streptokinase.



A S-T segment deviation in the reference lead. * $p < 0.05$



B Systolic blood pressure ** $p < 0.01$



C dST/dT and systolic blood pressure

FIGURE 5.2 Mean data (± standard error) of measured and derived parameters.

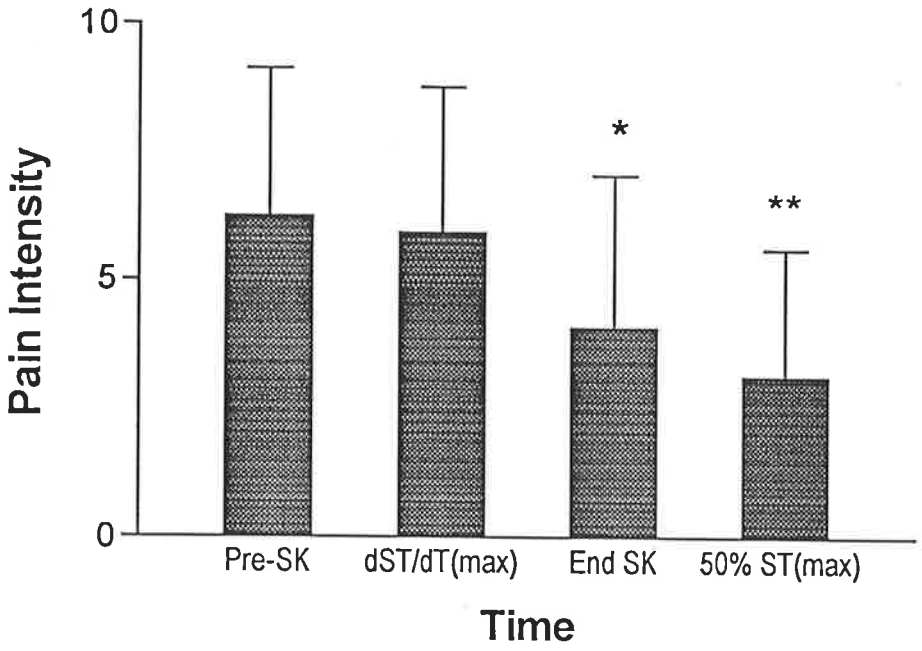


FIGURE 5.3 Pain intensity during streptokinase infusion, at maximum dST/dT and at reperfusion.
(*p<0.05, **p<0.01 as compared pre-streptokinase)

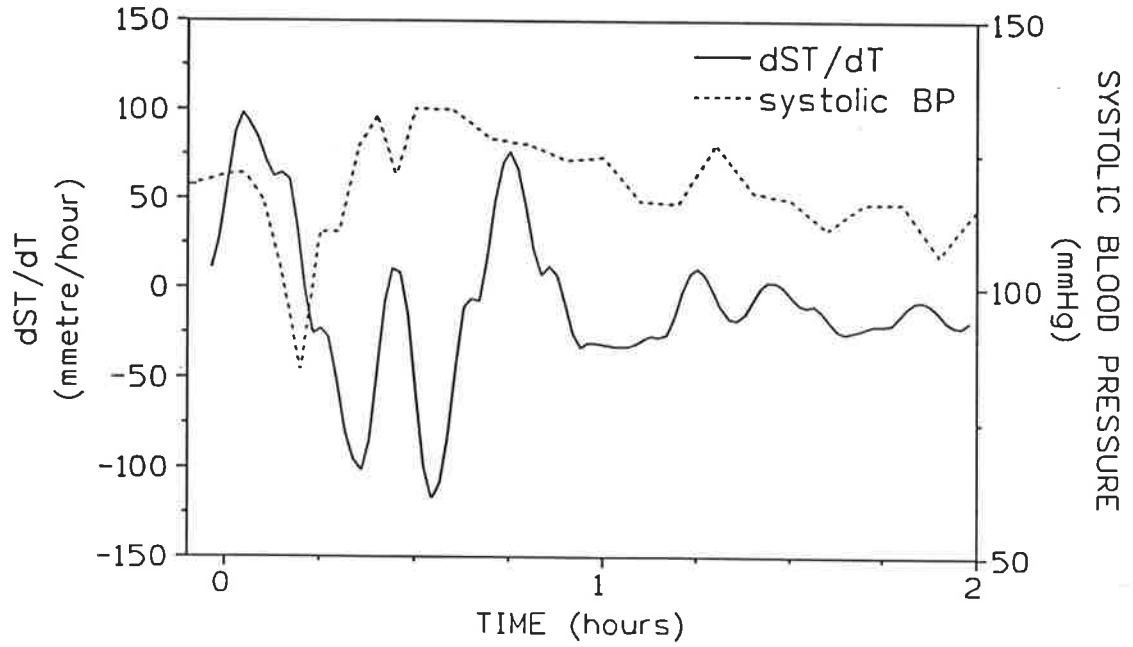


FIGURE 5.4 dST/dT and systolic blood pressure for 5 patients treated with streptokinase alone without intravenous glyceryl trinitrate or verapamil.

suggesting marked aggravation of myocardial injury at that time. This was a phenomenon that appeared usually within 20 minutes of the commencement of streptokinase infusion and antedated ECG evidence of reperfusion. In view of the concurrence of this phenomenon with the early phase of streptokinase infusion, it is virtually certain that this represents an effect of streptokinase, rather than a manifestation of the evolution of myocardial infarction or the effect of concomitant nitrate or verapamil therapy. The demonstration of aggravation of injury in patients receiving streptokinase without glyceryl trinitrate and verapamil also tends to exclude a significant interaction between these agents and streptokinase in the pathogenesis of aggravated injury. In this study all patients appeared to reperfuse subsequently.

Several previous investigators have reported the occurrence in some patients of transient increases in S-T segment elevation following thrombolysis (Hackett et al. 1987; Kwon et al. 1991; Krucoff et al. 1993a; Shah et al. 1993; Kondo et al. 1993; Shechter et al. 1992; Dissman et al. 1993). However, in general, such changes have developed only in a minority of patients, late after infusion of thrombolytic agents, usually either immediately antecedent to or following reperfusion (Hackett et al. 1987; Kwon et al. 1991; Krucoff et al. 1993a; Shah et al. 1993; Kondo et al. 1993; Shechter et al. 1992). The only previous report of relatively early (less than 1 hour) incremental S-T elevation did not attempt to elucidate either the precise time course of this change nor its relationship to fluctuations in blood pressure (Dissman et al. 1993). In the present study, on the other hand, it was demonstrated that very early aggravation of S-T segment elevation was virtually universal in its occurrence, irrespective of infarct site or of eventual infarct size.

The pathophysiology of myocardial injury-induced S-T segment elevation on ECG is discussed in 1.2.4.3. It is reasonable to presume from this discussion that the extent of S-T elevation reflects the severity of myocardial ischaemia at the site being monitored. Furthermore, the method of

continuous monitoring for the extent of abnormalities of S-T segments on 12 lead ECG, to assess fluctuations of S-T segment elevation during acute myocardial infarction has been discussed in 1.3.1.4. and has been shown to be a useful non-invasive assessment of both changes in the intensity of myocardial ischaemia (Madias et al. 1975) and infarct-related artery patency in individual patients (Krucoff et al. 1986; Hackett et al. 1987; Kwon et al. 1991; Krucoff et al. 1993a). Rapid resolution of S-T segment elevation has been shown to correlate with restoration of patency of the infarct related artery in patients receiving thrombolytic therapy (Krucoff et al. 1993a; Hackett et al. 1987). If S-T elevation is directly proportional to the intensity of injury (Kubota et al. 1993; Madias et al. 1975), then the rate of change in S-T elevation with time (dST/dT) is a logical measure of rate of change in the intensity of injury. Although this parameter has not been utilised in previous clinical investigations, it was essential for optimal evaluation of fluctuating ischaemia, as it provides a more precise and sensitive signal of individual episodes of S-T fluctuation than grouped S-T segment data.

In the light of the close correlation in time, but not extent, between streptokinase-induced hypotension and aggravation of myocardial injury, it becomes necessary to consider the mechanisms that induce hypotension. A critical issue is whether aggravation of myocardial injury is directly induced by hypotension, or by some other haemodynamic effect of streptokinase which occurs at about the same time as hypotension. To date, the mechanism of streptokinase-induced hypotension has not been clearly elucidated. It is also uncertain to what extent hypotension is due to peripheral vasodilatation (the usual presumption) or to negative inotropic changes. Another important consideration is how aggravation of myocardial injury might impact on the clinical outcome of patients undergoing thrombolytic therapy.

The working definition of hypotension utilised in this study was arbitrary. The definition of a 20

mmHg fall in blood pressure with the minimum systolic blood pressure less than 120 mmHg was prospectively chosen. In this study, not all of the patients defined as hypotensive were symptomatic or required any treatment. Indeed, there is no consistent definition of streptokinase-induced hypotension in the literature. Some studies give only a qualitative definition such as "significant hypotension" (The GUSTO Investigators, 1993). However, we also analysed data with respect to absolute minimum blood pressure, percentage blood pressure fall and overall averaged data, rather than relying solely on a threshold.

Hypotension during the intravenous infusion of thrombolytic agents is common and occurs with all thrombolytic agents. In the ISIS-2 study hypotension occurred in 10% of patients treated with streptokinase as compared to 2 % of those who received placebo (ISIS-2 (Second International Study of Infarct Survival) collaborative group, 1988). Herlitz *et al* (Herlitz et al. 1993) noted that 90% of 306 patients treated with a 60 minute infusion of 1.5 million units streptokinase had some fall in blood pressure; 31 % requiring treatment for this. The magnitude of this effect is directly related to the infusion rate (Lew et al. 1985). In comparison, all our patients had some blood pressure fall, even if not significant by our definition (Table 5.2), probably consistent with the rapid rate of infusion (Lew et al. 1985). In the GUSTO study "sustained hypotension" was noted in 12.9 % of patients treated with streptokinase and similarly 10.1% of patients treated with tissue plasminogen activator (The GUSTO Investigators, 1993). Also, a controlled comparison of streptokinase and tissue plasminogen activator revealed relative incidences of hypotension (defined as a fall in blood pressure of less than 80 mmHg during the first 3 hours of infusion) of 25% and 18% for streptokinase and tissue plasminogen activator respectively (White et al. 1989). There is little information as to whether these hypotensive episodes may impose long-term adverse effects on outcome. However Herlitz *et al* found no correlation between streptokinase-induced hypotension and probability of survival to one year after infarction (Herlitz et al. 1993).

Gemmill *et al* (Gemmill et al. 1993) have provided data suggesting that hypersensitivity, manifest biochemically as pre-treatment streptokinase resistance titre or anti-streptokinase IgG concentration, does not contribute to the development of hypotension. Also, they showed no relationship between streptokinase-induced hypotension and plasma fibrinogen concentration, or other indices of thrombin activation and fibrin breakdown, which may decrease plasma viscosity or activate the prostacyclin-prostaglandin system.

Activation of the Bezold-Jarisch reflex is only likely to be involved in the development of "late" or reperfusion-induced hypotension (Koren et al. 1986). This "late" hypotension may involve several pathophysiological mechanisms. The current study showed aggravation of injury to occur early at 13 ± 13 minutes and hypotension at 16 ± 5 minutes, whereas reperfusion occurred later (median 62 minutes) consistent with previous experience with streptokinase. In all but one patient, the aggravation of injury and associated minimum systolic blood pressure occurred before evidence of reperfusion on ECG.

A study in rabbits suggested that platelet activating factor (PAF) release may mediate streptokinase-induced hypotension (Montrucchio et al. 1993a). Streptokinase stimulates the intravascular synthesis of PAF in humans being treated for evolving acute myocardial infarction (Montrucchio et al. 1993b), but correlation with early streptokinase-induced hypotension in humans has not been proven. This is of theoretical interest, especially as PAF has been shown to reduce coronary blood flow in animal models (Benveniste et al. 1983; Tamura and Shibamoto, 1991) and might represent a mechanism for transient aggravation of injury.

However, a further potential mechanism for aggravation of injury is the production of "coronary steal", via dilatation of vessels supplying non-ischaemic myocardium (Warltier et al. 1980).

Release of vasodilator materials by streptokinase may represent a basis for development of steal, particularly in the presence of systemic hypotension (Patterson and Kirk, 1983).

Therefore, possible mechanisms for transient aggravation of myocardial injury during transient hypotension include:

(1) a reduction in collateral flow to the ischaemic zone at a time when anterograde flow is negligible,

(2) a decrease in perfusion pressure resulting in a further decrease in anterograde flow down the infarct-related artery if the vessel was not completely occluded.

and

(3) Induction of coronary "steal", as outlined above.

The failure to demonstrate a precise correlation between extent of hypotension and that of aggravation of myocardial injury suggests that more than one of the above mechanisms may operate.

A limitation of this study was the lack of control groups. Further confirmation of the pivotal role of thrombolytic agents in the aggravation of myocardial ischaemia could be sought by a comparison with:

(1) patients treated with thrombolytic agents other than streptokinase, such as rTPA, and

(2) patients treated with mechanical reperfusion by immediate PTCA.

A comparable effect on dST/dT in patients treated with other thrombolytic agents would extend this phenomenon to all agents that induce systemic thrombolysis. Also, lack of a significant change in dST/dT during immediate PTCA would clearly implicate rather than infer streptokinase as the causative agent.

The central finding of this observational study, that streptokinase aggravates myocardial ischaemia, raises several questions of potential direct clinical significance as regards the management of evolving acute myocardial infarction.

(1) Is this effect due to systemic thrombolysis *per se*, and thereby avoided with true thrombus-specific thrombolytic agents ?

(2) If this phenomenon is purely secondary to arteriolar vasodilatation, might it also occur with other agents with such actions if administered early during the evolution of acute infarction? (The HINT Research Group, 1986)

(3) If a patient receiving streptokinase develops aggravation of myocardial injury, but fails to subsequently reperfuse, does this contribute to the increased "early mortality" associated with the use of thrombolytic therapy ?

It is feasible that these findings could become the basis of a case towards new priorities regarding pharmacological and/or mechanical protection of the ischaemic area in the pre-reperfusion period, which to date have received very little direct attention. Should it be found that this problem occurs with other routinely available thrombolytic regimens, this issue may increase the impetus towards primary PTCA. However, primary PTCA in unlikely to represent a means for eliminating the need for thrombolytics in some patients (given logistic difficulties inherent in widespread availability of PTCA). Furthermore, suggestion of increased myocardial salvage with rapid thrombolytic regimens, recently demonstrated in the GUSTO-1 study (The GUSTO Investigators, 1993) indicates that slower infusion of thrombolytic agents to minimise hypotension is unlikely to be beneficial overall. Hence the problem of aggravation of ischaemia by thrombolytic agents needs to be addressed specifically.

There are three possible solutions to the problem:

(1) minimisation of oxygen demand by the ischaemic myocardium,

(2) improvement of coronary collateral flow, and

(3) mechanical coronary retroperfusion.

Co-infusion of nitrates with thrombolytic agents may increase collateral flow (Forman et al. 1983), decrease the extent of coronary "steal" (Patterson and Kirk, 1983), and decrease cardiac work via reduction in preload and afterload (Boesgaard et al. 1994b). NAC co-infusion may potentiate all these effects. In this regard it is possible that the impressive results of Judgutt *et al* (Jugdutt and Warnica, 1988) in the pre-thrombolytic era reflect nitrate effects on myocardial ischaemia in the setting of an occluded vessel. Similarly, it has been suggested that the benefit of GTN in patients treated with thrombolytic agents is seen in those who fail to reperfuse (Hildebrandt et al. 1992). These issues merit further detailed investigations.

It is not certain the extent myocardial salvage would be improved by the minimisation of the aggravation of ischaemia prior to reperfusion. Certainly, the more prolonged and intense the ischaemia, the larger the necrotic area. However the time period was generally short, although no patient failed to reperfuse on ECG criteria or had prolonged and sustained hypotension in this study, making further speculation on the role of reperfusion and systolic blood pressure on resolution of this aggravated ischaemia difficult. Certainly, S-T segment analysis of a larger number of patients, some of whom suffer sustained hypotension, fail to reperfuse, or die in the early day 0 - 1 period would facilitate resolution of these issues.

Chapter 6:

N-acetylcysteine in Combination With Glyceryl Trinitrate and Streptokinase for the Treatment of Evolving Acute Myocardial Infarction: Reduction in Oxidative Stress, Haemodynamic and Clinical Effects

6.1 Background

The most clinically relevant example of myocardial ischaemia/reperfusion is acute myocardial infarction followed by "successful" thrombolysis. There is evidence that this process is associated with oxidative stress (see 1.5.2.1), but no data are available concerning the clinical relevance of modulating oxidative stress in humans. A few clinical trials have addressed this issue in humans. The clinical and haemodynamic effects of the antioxidants SOD (Flaherty et al. 1994) and Fluosol (Wall et al. 1994) showed no significant benefit in myocardial salvage. Again no biochemical assessment of oxidative stress was made so it was unclear whether the lack of beneficial effect represented failure to modify oxidative stress or whether amelioration of oxidative stress proved to be without benefit.

The only sulphydryl-containing agent to be assessed in this clinical setting has been captopril (Kingma et al. 1994). However this agent is both an ACE inhibitor and antioxidant, hence the mechanism of putative benefit would be uncertain. Furthermore, the use of an oral agent and the early dosage limitation due to the drug's hypotensive effects suggested it was potentially not ideal as an antioxidant in the early reperfusion period. Certainly, no assessment of the effect on oxidative stress was made. As discussed in 1.7.3.3, there has been no previous systematic assessment of NAC during treatment with reperfusion for the management of evolving acute myocardial infarction. It is not known whether utilisation of NAC as an antioxidant will decrease oxidative stress and/or improve myocardial salvage or function in this setting. Furthermore, the clinical implications of potentiation of the systemic haemodynamic, coronary microvascular and antiplatelet effects of GTN in the setting of acute myocardial infarction are unexplored.

6.2 Aims

The aims of this study were to:

(1) Assess the safety of intravenous NAC in combination with intravenous GTN and streptokinase in the management of evolving acute myocardial infarction. In particular, the incidence of haemorrhage, symptomatic hypotension and headache were to be noted.

(2) Assess the efficacy of NAC. In particular, the primary objective was to assess the effect of NAC on biochemical markers of oxidative stress.

(3) Other secondary objectives were to perform a preliminary assessment of the effect of NAC on speed and rate of reperfusion, myocardial infarct size and haemodynamic function in the early post-infarction period.

6.3 Method

6.3.1 Protocol for pharmacological intervention

This study was approved by the Ethics Committee on Human Research of The Queen Elizabeth Hospital and informed consent was obtained from all patients before entry into the trial. Inclusion and exclusion criteria are listed in Table 6.1.

In this open-labelled study, patients were randomised in a ratio of 3:1 for NAC:no NAC treatment to permit an assessment of the efficacy of NAC. All potentially eligible patients received GTN intravenously infused through non-absorptive polyethylene tubing at 5 μg/min prior to trial entry. Intravenous NAC infusion was commenced at 20 mg/min for the first hour, then 10 mg/min for the subsequent 23 hours, to give a total dose of 15 grams over 24 hours. Fifteen minutes after commencement of NAC infusion, an intravenous bolus of 100 mg hydrocortisone was administered, immediately followed by 1.5 million units intravenous streptokinase over 30 minutes.

TABLE 6.1 Inclusion and Exclusion Criteria

Inclusion Criteria
1. age ≤ 75 years
2. chest pain of ≤ 4 hours duration
3. ECG evidence of transmural ischaemia: ≥ 1 mm S-T segment elevation in ≥ 2 limb leads and/or ≥ 2 mm S-T segment elevation in ≥ 2 praecordial leads
Exclusion Criteria
1. past Q-wave myocardial infarction
2. previous severe (New York Heart Association class III to IV) cardiac failure
3. admission systolic blood pressure<90 mmHg
4. acute myocardial infarction within the preceding 7 days
5. haemodynamically significant valvular heart disease
6. ingestion of allopurinol, penicillamine, gold salts, ethacrynic acid or captopril within the previous 7 days
7. known allergy to any of the protocol medications
8. contraindications to the use of streptokinase: a) streptokinase administration within the previous 6 months b) surgery or cerebrovascular accident within the previous 6 weeks c) warfarin therapy d) active peptic ulcer disease e) bleeding disorders f) uncontrolled hypertension g) diabetic proliferative retinopathy.

Additional therapy thereafter was intravenous GTN infusion at 5 to 10 μg/min for 48 ± 12 hours, followed by oral isosorbide dinitrate 10 mg three times daily for at least 7 days, heparin infusion immediately following streptokinase, and verapamil after 8 hours (80 mg 8 hourly) unless contraindicated (Lee et al. 1988; Arstall et al. 1992). Aspirin and β-adrenoceptor antagonists were not administered during the first 12 hours of the study.

Criteria for withdrawal of trial medications included the onset of cardiogenic shock, complications of myocardial infarction requiring emergency cardiac surgery, persistent ischaemic chest pain requiring emergency coronary revascularisation or severe adverse reactions to GTN, NAC or streptokinase.

6.3.2 Protocol for the assessment of haemodynamic effects

Left ventricular systolic function was assessed at day 1 and 7 by gated heart pool scanning, utilising 900 mbq intravenous technetium 99m and a General Electric 300 mobile gamma camera on day 1 and Triad triple headed gamma camera (Trionics, Cleveland) on day 7. Right and left cardiac catheterisation with estimation of cardiac index via the Fick method and coronary angiography was performed between days 2 and 5.

6.3.3 Protocol for the non-invasive assessment of reperfusion

Twelve lead continuous S-T segment monitoring was performed from admission to the Coronary Care Unit for 12 to 24 hours, utilising a MAC 15 ST Guard (Marquette Electronics, Wisconsin, USA) with measurement of S-T deviation from baseline 60 msec after the J point, every 30 seconds, and 12 lead ECG stored every 15 minutes. Data were stored on computer disc and printed as hard copy for each patient. The lead with the maximum S-T elevation was used as the

reference lead. The time to reperfusion of the infarct-related artery was defined as that from trial entry to that at which the S-T segment elevation had declined to 50% of the maximum level in the reference lead. Infarct related coronary artery patency was graded according to the TIMI-1 criteria at coronary angiography 2 to 5 days after trial entry.

6.3.4 Protocol for the assessment of biochemical effects

Peripheral venous blood was serially sampled to measure several biochemical parameters. Plasma creatine kinase concentration was measured at 0, 1, 2, 4, 8, 12, 16, 20, 24 and 48 hours after trial onset. Plasma MDA concentration was measured at 0, 0.5, 1, 2, 4, 8, 12, 24 and 48 hours after trial onset. Plasma reduced and oxidised glutathione and N-acetylcysteine concentrations were measured at 0, 4 and 24 hours.

6.3.5 Statistical analysis

Sample sizing was prospectively determined as regards the biochemical endpoints of plasma MDA concentrations. Utilising a 3:1 randomisation protocol, 20 patients in the treatment group were required to detect a 50% difference in peak MDA plasma concentration with a power of 87%. Normally distributed data were analysed by the Student's t test and skewed data via the Mann-Whitney U test. Variance within and between groups was assessed using two-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. Correlations were sought between different measured values utilising linear regression. All normally distributed data are expressed as mean ± standard deviation unless otherwise stated. Median with 95% confidence intervals are used to describe skewed data. All analyses were two tailed and a $p < 0.05$ was defined as a statistically significant result.

6.4 Results

6.4.1 Patient characteristics

Of 29 consecutive eligible patients, 28 patients were enrolled to the study. One patient was not included in further analysis because his final diagnosis was acute viral pericarditis with S-T segment elevation in the precordial ECG leads. The two groups were well matched for age, sex, previous myocardial infarction, site of infarction and the length of time from onset of symptoms to trial entry as outlined in Table 6.2.

6.4.2 Adverse events

Adverse reactions and cardiac events are summarised in Table 6.3. There were no deaths during hospital admission or allergic reactions to trial medication. Among patients receiving NAC, 3 had minor episodes of haemorrhage (two Mallory - Weiss tears and one spontaneous haemarthrosis), none of which were associated with haemodynamic compromise or required transfusion. Four patients developed headache. No patient developed sustained hypotension, and one patient with inferior infarction sustained a transient episode of extreme sinus bradycardia. No patient treated with streptokinase and GTN alone had any adverse effects.

No-one within the NAC group developed re-infarction within 7 days of trial entry, but one patient developed symptomatic and persistent left ventricular failure on day 6 after trial entry. One patient in the control group was withdrawn from the trial at 18 hours due to re-infarction and continued unstable angina pectoris requiring urgent revascularisation with coronary artery bypass grafting.

6.4.3 Plasma concentration of N-acetylcysteine

In the NAC treated patients, plasma NAC concentrations were 172 ± 79 μmol/L at 4 hours and

TABLE 6.2 Patient Characteristics

	Treatment Group	
	NAC/GTN/SK	**GTN/SK**
Number	20	7
Age (years)	59 ± 11	58 ± 9
Sex (% male)	80	86
Past AMI (n)	1	0
Time from symptom onset (hours)	2.2 ± 1.0	2.4 ± 1.3
Site of AMI (% anterior)	35	14

Legend: AMI, acute myocardial infarction; NAC, N-acetylcysteine; GTN, glyceryl trinitrate; SK, streptokinase.

151 ± 78 μmol/L at 24 hours, suggesting attainment of steady state plasma NAC concentrations within 4 hours. In one patient receiving NAC, plasma NAC concentrations were assayed more frequently to make a preliminary assessment of variation in this parameter over the whole duration of the study. These results are seen in Figure 6.1. In this patient, plasma NAC concentrations >100μmol/L were present within 1 hour. Furthermore, 24 hours after cessation of NAC infusion, plasma NAC concentration was 52 μmol/L.

6.4.4 Haemodynamic effects

Comparisons of haemodynamic status between the two treatment groups are summarised in Table 6.4. Consistent with the small sample size of the control group and anticipated extensive inter-individual variability, there were no significant differences between groups for the majority of parameters measured. However, patients receiving NAC had significantly greater cardiac indices (median 3.3, 95% CI 3.0 - 3.8 versus 2.5, 95% CI 2.3 - 2.9; p = 0.009), with trends towards lower mean pulmonary capillary wedge pressure (p = 0.09) and greater left ventricular ejection fraction. Left ventricular systolic function was generally well preserved.

6.4.5 Rate of reperfusion

Median time to reperfusion on ECG criteria tended to be shorter (59, 95% CI 48 - 98 minutes versus 95, 5% CI 59 - 106 minutes, p = 0.17) in patients receiving NAC (see Figure 6.2), with somewhat more rapid time to peak plasma creatine kinase concentrations. Infarct-related artery patency at day 2 to 5 was 90% in NAC treated patients and 100% in the control group. These results are displayed in Table 6.5.

TABLE 6.3 Adverse Events During Hospital Admission

	NAC/GTN/ SK (n=20)	GTN/SK (n=7)
Death	0	0
Haemorrhage	3	0
Allergic reaction	0	0
Sustained hypotension	0	0
Significant headache	4	0
Re-infarction within 7 days	0	1
Haemodynamically significant arrhythmias	1	0
Post-infarction cardiac failure	1	0
Post-infarction angina or positive exercise ECG	6	1

Legend: ECG, electrocardiogram; GTN, glyceryl trinitrate; n, number of patients; NAC, N-acetylcysteine; SK, streptokinase.

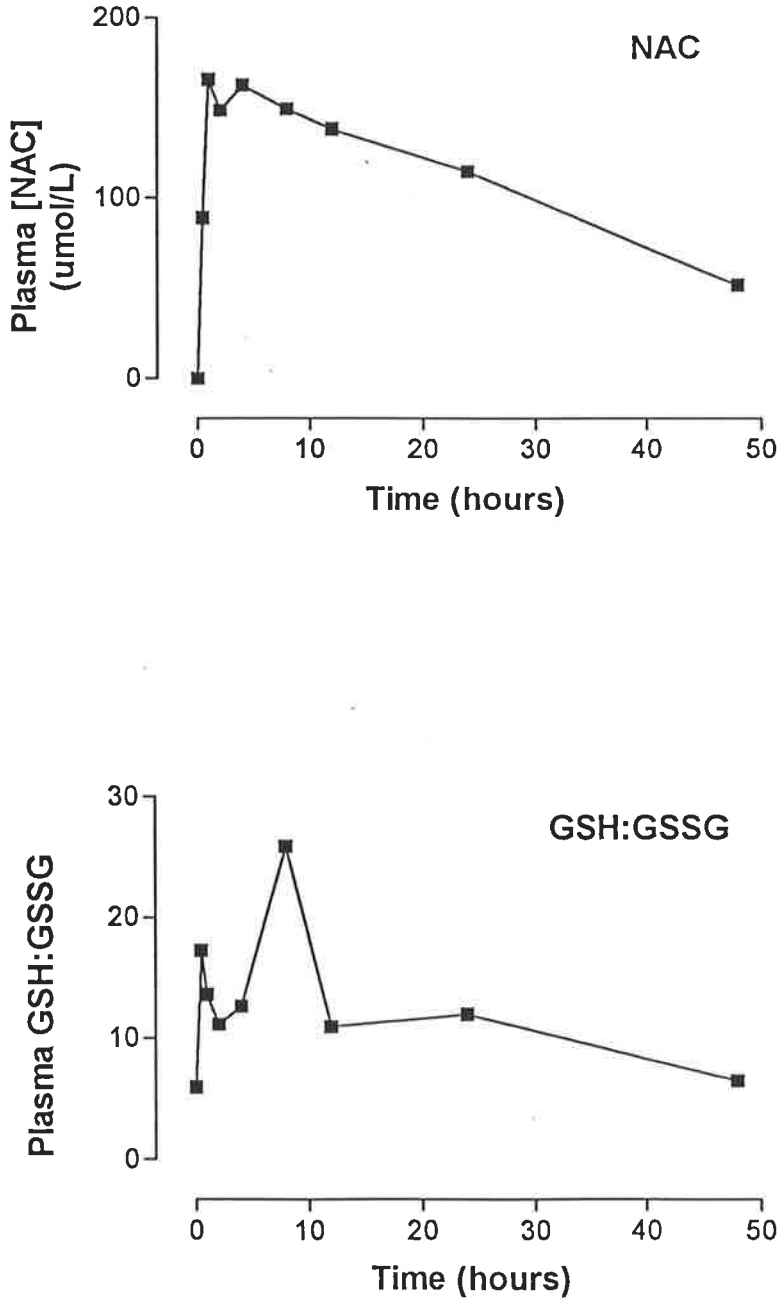


FIGURE 6.1 Plasma NAC concentration and glutathione ratio (GSH:GSSG) over time in an individual patient

TABLE 6.4 Haemodynamic Effects

		Treatment Group	
		NAC/GTN/SK (n=20)	**GTN/SK (n=7)**
LVEF (%)	DAY 1	57 ± 12	53 ± 9
	DAY 7	54 ± 12	51 ± 11
PCWP (mm Hg)		10 ± 4	13 ± 6‡
Cardiac Index (L/min/m^2)		3.3	2.5*
95% CI (L/min/m^2)		3.0-3.8	2.3-2.9
Number of Coronary Arteries with ≥ 70% Stenosis (n)	0	3	0
	1	12	4
	2	4	2
	3	1	1

Legend: LVEF, left ventricular ejection fraction; n, number of patients; NAC, N-acetylcysteine; GTN, glyceryl trinitrate; PCWP, mean pulmonary capillary wedge pressure; SK, streptokinase; 95% CI, 95 percent confidence interval. * p = 0.009 ‡p=0.09

6.4.6 Effects on oxidative stress

The extent of lipid peroxidation in this setting was estimated by measurement of peripheral plasma MDA. Plasma MDA concentrations (Figure 6.4) peaked at a median of 1 hour (95% CI 0.9 - 8.0 hours) in the NAC treated patients and 2 hours (95% CI 0.7 - 3.9) in the control patients (p, not significant). Peak plasma MDA concentrations within 24 hours were 0.22 ± 0.07 μmol/L and 0.30 ± 0.14 μmol/L in NAC treated and control patients respectively ($p = 0.097$). Plasma concentrations of MDA were significantly lower over the first 8 hours of the study in NAC treated patients ($p < 0.001$, ANOVA).

Effects of NAC on plasma concentrations of GSH and on the ratio of GSH:GSSG are summarised in Table 6.6 and Figure 6.5. GSH concentrations were significantly higher in NAC treated patients than in control patients (mean 87 ± 119 % increase versus 19 ± 47 % decrease at 24 hours, $p < 0.002$, ANOVA). Furthermore, there was a significantly lower concentration of GSSG in the NAC treated groups ($p = 0.012$, ANOVA), with significant changes with time in both groups ($p = 0.001$, ANOVA). Therefore, the GSH:GSSG ratios were significantly higher for NAC treated patients ($p < 0.001$, ANOVA), which increased with time as compared to control patients ($p = 0.01$, ANOVA). In the single patient in whom frequent measurements of GSH:GSSG were performed (Figure 6.1) there was an increase in this ratio within 30 minutes of commencement of NAC infusion.

Plasma concentrations of NAC were correlated with plasma GSH concentrations at 4 and 24 hours ($r^2 = 0.298$, $p = 0.006$, Figure 6.6), and percentage change of GSH at 4 hours ($r^2 = 0.235$, $p = 0.042$). No other statistically significant correlations between biochemical and/or clinical parameters were found.

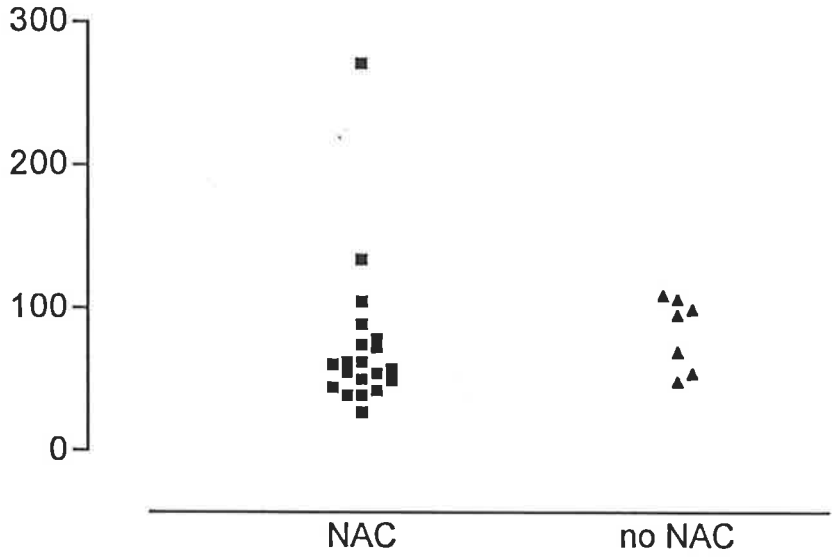


FIGURE 6.2 Time to Reperfusion (minutes)

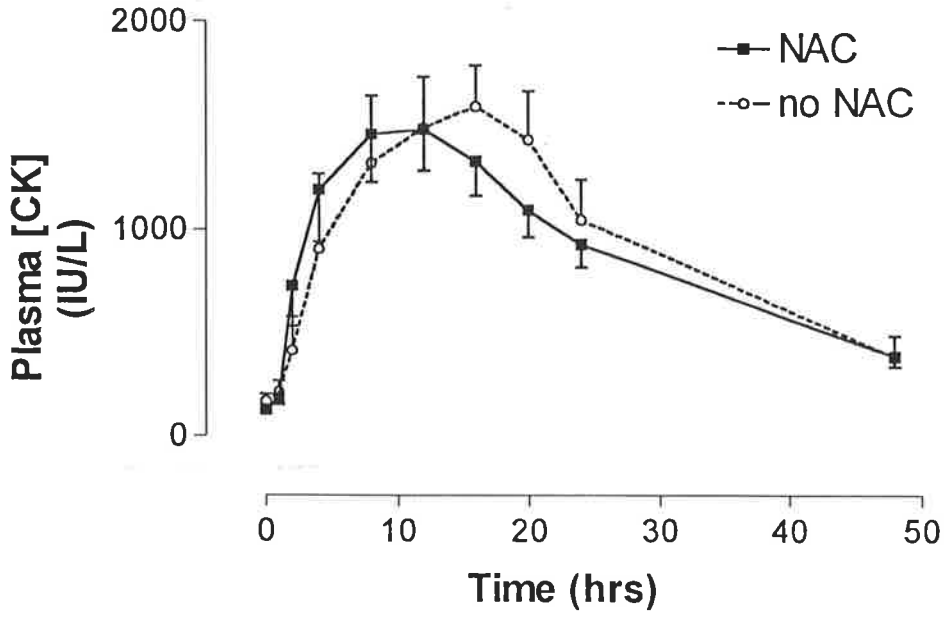


FIGURE 6.3 Plasma Creatine Kinase Concentration Over Time

TABLE 6.5 Parameters of Reperfusion

		Treatment Group	
		NAC/GTN/SK (n=20)	**GTN/SK (n=7)**
Reperfusion time (minutes) 95% CI		59 48 -98	95 59 -106
Plasma CK	Peak CK (IU/L)	1730 ± 1050	1870 ± 680
	AUC (U.hour/L)	53700 ± 29300	57500 ± 21000
	Time to peak (hours)	11 ± 6	13 ± 5
TIMI Grade of IRA (n)	0	2	0
	1	0	0
	2	2	0
	3	16	7

Legend: AUC, area under curve of CK versus time curve; CK, plasma creatine kinase concentration; IU/L, international units per litre; n, number; NAC, N-acetylcysteine; GTN, glyceryl trinitrate; IRA, infarct-related artery; Reperfusion Time, time to 50% maximum S-T elevation; SK, streptokinase; 95% CI, 95 percent confidence interval.

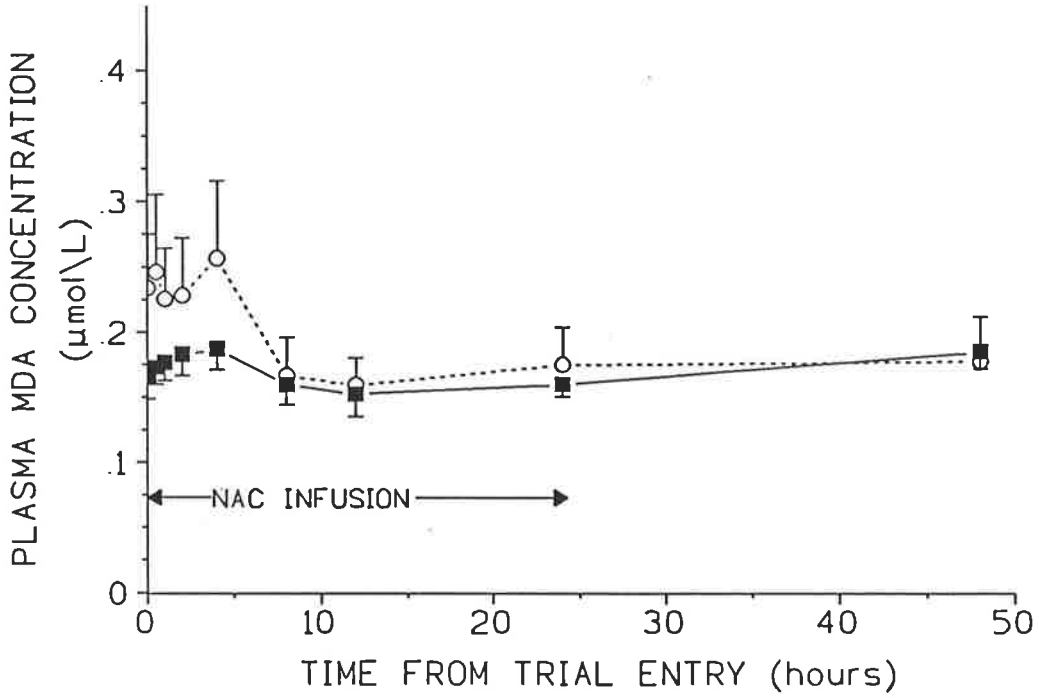


FIGURE 6.4 Changes in plasma malondialdehyde (MDA) concentration over 48 hours in patients receiving (■) or not receiving (○) N-acetylcysteine (NAC). (Mean with standard error of mean displayed.)

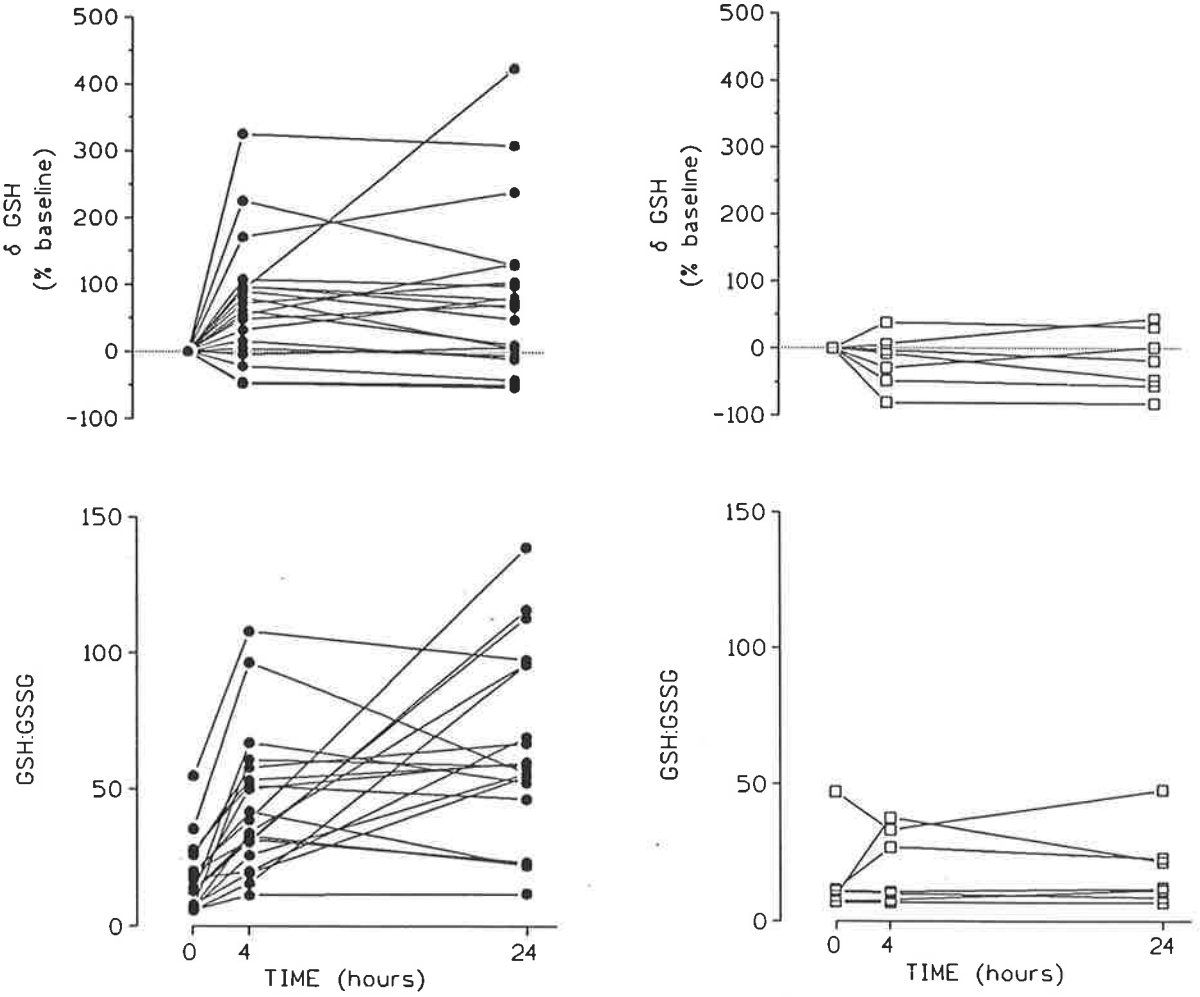


FIGURE 6.5 Changes in plasma reduced glutathione (GSH) concentration and the ratio of reduced to oxidised glutathione (GSH:GSSG) over 24 hours in patients receiving (●) or not receiving (□) N-acetylcysteine (NAC).

TABLE 6.5 Effects of NAC on plasma GSH concentrations, plasma GSSG concentrations and GSH:GSSG at 0, 4 and 24 hours.

	Time	Treatment Group		p
	(hours)	NAC/GTN/SK (n=20)	GTN/SK (n=7)	
GSH (μmol/L)	0	2.2 ± 2.4	2.5 ± 2.4	ns
	4	2.8 ± 1.3	1.4 ± 0.3	< 0.05
	24	2.8 ± 1.2	1.4 ± 0.4	< 0.05
Change in GSH (%)	0	0	0	-
	4	+73 ± 90	-18 ± 39	< 0.05
	24	+82 ± 122	-18 ± 47	ns
GSSG (μmol/L)	0	0.14 ± 0.11	0.23 ± 0.23	ns
	4	0.08 ± 0.05	0.12 ± 0.08	ns
	24	0.05 ± 0.03	0.11 ± 0.07	< 0.01
GSH:GSSG	0	18 ± 12	15 ± 14	ns
	4	43 ± 26	19 ± 13	< 0.05
	24	66 ± 34	19 ± 14	< 0.01

Legend: GSH, reduced glutathione; GSSG, oxidised glutathione; GSH:GSSG, ratio of reduced to oxidised glutathione; n, number of patients; NAC, N-acetylcysteine; GTN, glyceryl trinitrate; SK, streptokinase.

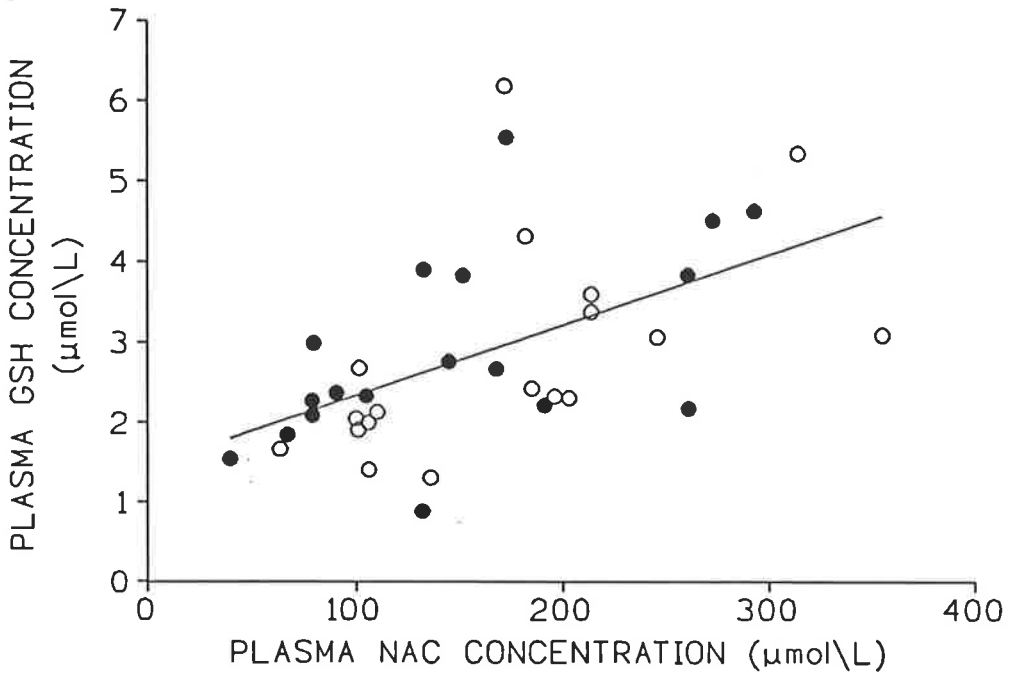


FIGURE 6.6 Correlation between plasma reduced glutathione (GSH) concentrations and plasma N-acetylcysteine (NAC) concentrations at 4 (○) and 24 (●) hours.

($r^2 = 0.298$, $p = 0.006$)

6.5 Discussion

This study assessing the safety and biochemical effects of NAC in the management of evolving acute myocardial infarction has demonstrated no significant adverse events associated with this treatment regime in this small group of patients. There was also a significant benefit as regards reduction of oxidative stress, trends towards more rapid reperfusion and improved cardiac haemodynamics post-AMI. It is possible that NAC was acting via multiple pathways to achieve these apparently disparate effects. Firstly, NAC may act as an antioxidant, secondly it may be potentiating the peripheral and coronary vasodilator, antiplatelet and potentially cytoprotective effects of GTN, and thirdly preventing GTN tolerance.

The results of this study suggest that NAC can be co-administered safely in combination with GTN and streptokinase in the treatment of uncomplicated AMI. Prospectively, several potential hazards were anticipated, related to the potentiation of both the vasodilator and anti-platelet effects of GTN by NAC, namely sustained hypotension (Horowitz et al. 1988) headache (Iversen, 1992) and haemorrhage (Loscalzo, 1985). Although 3 patients had minor haemorrhages, the most common problem was that of headache. This was controllable with simple analgesia and no patient needed to be withdrawn from the study as a consequence of this problem.

The size of the study was not adequate for reliable analysis of the clinical and haemodynamic effects of NAC, but was designed to permit assessment of its biochemical effects. Therefore, the lack of statistically significant differences between treatment groups may reflect type II error. Despite this, two trends were noted. Firstly, reperfusion tended to occur more rapidly in the NAC treated patients. Although the mechanism for this was not assessed during this study, it is possible that this either reflects a potentiation of effects of GTN or even an anti-aggregatory effect of NAC *per se* (del Principe et al. 1990) Mean plasma concentration of NAC at 4 hours (172 ± 79 μmol/L)

was similar to those for which NAC had been shown to inhibit platelet aggregation *in vitro* (del Principe et al. 1990). Secondly, haemodynamics tended to be better preserved in the NAC treated patients. Although only cardiac index showed a statistically significant difference between treatment groups, all measured parameters suggested a beneficial effect of NAC treatment.

Both MDA and glutathione redox status indicated less oxidative stress in NAC treated patients. As regards MDA, the effect of NAC appeared to be limited to approximately 25% reduction of plasma MDA concentrations during the first 8 hours of treatment; thereafter there was no difference between treatment groups. This lack of difference in MDA concentrations over the 8 to 24 hour period is not explained on the basis of current investigations. On the other hand, the effects of NAC on GSH, GSSG and GSH:GSSG were apparent both at 4 and 24 hours, and were more marked than changes in MDA concentrations. These biochemical effects are similar to the preliminary report of NAC utilisation in patients undergoing coronary revascularisation (Ferrari et al. 1991a). The lower GSSG concentration at 24 hours in the NAC treated patients is consistent with the role of NAC as a scavenger of radical species *per se*. On the other hand, the correlation between GSH and NAC concentrations in plasma supports previous animal and pharmacokinetic studies whereby NAC is in part metabolised to GSH (Ceconi et al. 1988; Ferrari et al. 1991a; De Caro et al. 1989). This process appears to occur rapidly (Figure 6.1), but the terminal half-life of NAC may be somewhat longer than documented by previous investigations (Olsson et al. 1988)

In previous studies measuring oxidative stress associated with AMI, reported plasma concentrations of MDA vary widely (see 1.5.2.1), probably reflecting different and sometimes very nonspecific assay methodology, and thereby making direct comparisons difficult. However, in the study of Giardiana *et al* (Giardina et al. 1993) mean peak plasma MDA concentrations in their patients who reperfused were considerably higher than those in either of our treatment

groups. This suggests a greater degree of reperfusion injury, perhaps due to later reperfusion. Alternatively GTN (not routinely administered in the abovementioned study) may have also exerted anti-ischaemic, antioxidant and/or cytoprotective effects (Cooke and Tsao, 1993; Lefer et al. 1993) (see 1.5.1.6).

There were several limitations to this study. The small size of the study, and of the control group in particular, limited determination of NAC effects on the measured clinical parameters, such as the time to reperfusion and post-infarct haemodynamic status. Therefore the results in general show only trends and no firm conclusions can be made. As regards the biochemical parameters, the changes in the glutathione redox pathway were profound, allowing confident discernment of a limitation in redox stress by NAC. The plasma MDA concentration differences were less marked between groups, although NAC prevented the initial rise in MDA concentration ($p < 0.001$, ANOVA). However, the differences in peak MDA concentrations were not statistically significant.

Although NAC/GTN/streptokinase appeared to be relatively safe in this study, a much larger investigation would be required to detect a small incremental risk of adverse effect such as haemorrhage. Furthermore, it must be noted that aspirin therapy was not routinely initiated until after the first 48 hours of treatment in these patients. Hence it remains possible that concomitant aspirin therapy may increase haemorrhagic risk in the presence of NAC/GTN/streptokinase.

The precise mechanism of biochemical and haemodynamic effects of the NAC/GTN/streptokinase treatment regimen cannot be deduced from this study. Indeed, it is uncertain that NAC must be administered prior to reperfusion to exert a beneficial effect, although the current regimen and previous animal models of myocardial ischaemia/reperfusion (Ceconi et al. 1988) are concordant with the hypothesised major mechanism of effect. The timing of therapy, and the examination of

components of NAC/GTN or NAC/streptokinase interaction versus direct NAC effect would require future larger studies, comparing NAC/GTN, NAC and GTN with streptokinase monotherapy both before and after reperfusion with streptokinase. Furthermore, the safety and effect of other thrombolytic agents with NAC/GTN cannot be determined (Nicolini et al. 1994).

The potential clinical benefits of this treatment regimen are uncertain, as it remains unclear to what extent reperfusion injury is a determinant of outcome post-AMI, either overall or in particular subgroups. However, it appears that this strategy which significantly reduces oxidative stress can be further utilised for the evaluation of the putative benefit of the limitation of reperfusion injury in this clinical setting.

Chapter 7:

General Discussion

The primary objective of this thesis was to expand on the understanding of the role of NAC as an antioxidant during oxidative stress induced by myocardial ischaemia and reperfusion and to assess the potential interactive role of GTN with this process. In particular, the potential of NAC noted by many previous reports in animal models as regards amelioration of oxidative stress, limitation of myocardial necrosis and stunning was considered in both animal and human models.

In Chapter 2 results of modifications to the non-specific TBA test for measurement of MDA in plasma were presented. The changes enabled this relatively simple test to more specifically measure plasma free MDA than previously achieved. Furthermore, a development of the measurement of plasma GSH, GSSG and NAC, with improved sensitivity was presented. These assays became the basis for the measurement of biochemical markers of oxidative stress for the studies reported in subsequent chapters.

The studies in Chapter 3 utilised a Langendorff-perfused rat heart model. Only severe stress in the form of a combination of hypoglycaemia and myocardial ischaemia was associated with a significant increase in the concentration of MDA in the coronary effluent during early reperfusion compared to myocardial ischaemia alone. However, even though there was a significant release of myocardial MDA, it was not sustained for more than 2 minutes into the reperfusion period and represented an inadequate biochemical marker of oxidative stress in this model. In contrast, 30 minutes of total global ischaemia followed by 30 minutes of reperfusion resulted in marked impairment of left ventricular function. The addition of 200 μmol/L NAC into the perfusate, either before the onset of ischaemia or at reperfusion was not associated with a significant recovery of left ventricular function as compared to Krebs solution alone.

In Chapter 4, coronary sinus catheterisation in humans was utilised, measuring cardiac

haemodynamics and trans-coronary gradients of MDA and lactate. Rapid ventricular pacing with or without significant left coronary artery disease was associated with a significant increase in myocardial MDA flux. In a study of 7 patients with significant left coronary artery disease, an intravenous bolus of 5 g NAC was associated with a significant decrease in myocardial lactate flux both before and after rapid ventricular pacing, but no change in myocardial MDA flux. The addition of intravenous GTN at 5 μg/min was not associated with any significant effect.

Chapter 5 reported the modulation of myocardial ischaemia prior to reperfusion of an occluded coronary artery in humans. This was measured by the use of 12 lead ECG S-T segment monitoring and measurement of the slope of the S-T segment versus time curve in patients with evolving acute myocardial infarction. Infusion of intravenous streptokinase was generally associated with transient hypotension and a corresponding aggravation of myocardial ischaemia early after the onset of the streptokinase infusion.

An open-labelled randomised trial involving 27 patients assessing the safety and biochemical effects of intravenous NAC infusion (15 g/24 hours) in combination with GTN and streptokinase was reported in Chapter 6. Treatment with NAC was associated with a significant decrease in plasma MDA concentration in the first 4 hours after onset of treatment and significant improvement in plasma GSH:GSSG. There were trends towards more rapid reperfusion and better cardiac haemodynamics in NAC treated patients.

The two patient studies in chapters 4 and 6 were unique, having not previously been reported in similar form in the literature. Therefore the results of these studies, even if not always conclusive from a statistical point of view, require careful examination to assess trends, safety and potential areas for further study and clinical application.

An important observation was the difference between the human *in vivo* and rat *in vitro* heart as regards extent of myocardial lipid peroxidation in response to oxidative stress. The human heart readily released MDA in response to oxidative stress as compared to the small, non-sustained response of the Langendorff-perfused rat heart, which required combined ischaemia and metabolic stress to induce significant lipid peroxidation. The reason for the discrepancy is unclear from these studies. However, it can be concluded that the rat heart is not necessarily an appropriate model for exanination of and/or extrapolation to the circumstances within the human heart.

Furthermore, the redox status of glutathione in plasma was an even more sensitive biochemical marker of oxidative stress, and the effect of NAC, than plasma MDA concentration during evolving acute myocardial infarction in humans. Therefore, these two biochemical markers in combination appear to be powerful tools for the assessment of oxidative stress in human studies. Examples of the use of two biochemical markers to improve confidence in the interpretation of results could be found in the study of the effect of NAC during myocardial infarction. On the other hand, the demonstration of release of myocardial MDA during ventricular tachycardia, whether associated with significant left coronary artery disease or not, raised the question as to the specificity of myocardial MDA release as a marker of oxidative stress. Confirmation of this by the measurement of plasma glutathione redox status in all patients, rather than one, would have permitted a more confident conclusion.

From the results contained in this thesis, it can be confidently concluded that NAC limited the extent of oxidative stress in patients with evolving acute myocardial infarction treated with a combination of streptokinase and GTN. In the case of mild myocardial ischaemia, there was a trend towards a beneficial effect on the limitation of oxidative stress but more patients would have to be studied to confirm this. No such trend could be discussed from the Langendorff-perfused rat

heart, although the biochemical marker utilised was poor.

This thesis made some preliminary assessments of the cardiac haemodynamic effect of the limitation of oxidative stress with NAC. Again the rat heart differed to the human heart. The rat heart function during reperfusion after myocardial ischaemia showed no benefit from the perfusion of NAC, but during evolving acute myocardial infarction treated with GTN and streptokinase, the addition of NAC showed trends towards beneficial effect in all measured parameters of myocardial salvage and residual function. However, this study was not primarily designed to make any conclusions as regards these functional parameters, and was thus prone to type II error for any of the haemodynamic end-points. No conclusions could be made of the effects of NAC on cardiac haemodynamics during mild myocardial ischaemia as any changes appeared to be small and would require a larger number of patients in the study to give clear results.

No clear conclusions as to the interactive role of GTN with the antioxidant effect of NAC could be made from the results of these studies. Certainly, there was no marked potentiation of NAC effect on the amelioration of oxidative stress during mild myocardial ischaemia and recovery in humans. However, during evolving acute myocardial infarction, the trend towards more rapid reperfusion in the NAC treated group possibly represented potentiation of the antiplatelet effect of GTN acting in synergy with streptokinase. Although no definitive conclusions could be made without a group of patients receiving no GTN, the plasma MDA concentration was lower in both treatment groups, than that reported previously in patients treated with streptokinase alone (Giardina et al. 1993). The assay used in this report was a direct HPLC method with a normal plasma MDA concentration of 0.05 μmol/L, compared to a higher concentration of 0.16 μmol/L noted with the methodology utilised in this thesis. This suggests that GTN itself may have an effect on oxidative stress, possibly by the limitation of the extent of ischaemia prior to reperfusion, rate

of reperfusion and adequacy of endothelial function in the reperfused vasculature. Whether NAC acted to potentiate GTN effect only, rather than acting as an antioxidant *per se* was unclear from the study. However, it is more probable that mechanisms of beneficial effects of NAC are multiple, and that maximum benefit would be achieved when utilised in combination with GTN.

The important observation that streptokinase infusion results in aggravation of myocardial ischaemia prior to reperfusion of the infarct-related artery in acute myocardial infarction, demonstrated the need for better protection of the myocardium at risk in the pre-reperfusion period as well as after. As yet, this issue has received little attention in the "thrombolytic era" of myocardial infarction management with the emphasis being generally place on speed of reperfusion. How this issue should be addressed requires further investigation but may be another incremental step in improved myocardial salvage and avoidance of "Day 1 hazard" noted with the use of thrombolytic agents.

A number of residual issues of considerable potential importance might be addressed in view of the results of the currently reported studies. Firstly, it would be helpful to compare the modified TBA test developed during this thesis with a direct MDA assay of adequate sensitivity, such as that described by Giardina *et al* (Lazzarino et al. 1991). This would allow validation of the specificity of the assay. If free plasma MDA concentration in any sample was similar by both methods, this would confirm the worth of continuing the simpler TBA fluorometric method for the rapid processing of large numbers of samples in the future.

Secondly, determination of the changes in GSH:GSSG in Langendorff-perfused coronary effluent during myocardial ischaemia and reperfusion would allow a comparison with lipid peroxidation. One previous report suggests that oxidative stress in the rat heart is not associated with significant

changes in myocardial GSH:GSSG (Verbunt et al. 1995), but the converse has been shown in the rabbit (Ceconi et al. 1988). If coronary effluent GSH:GSSG were a better biochemical marker of oxidative stress than MDA in the rat heart, it would permit further utilisation of this model for assessment of the antioxidant potential of agents of interest.

From the coronary sinus experiments, there are several points that require clarification. Firstly, conformation that ventricular tachycardia is associated with oxidative stress by assessment of plasma glutathione redox status is needed. Secondly, an increase in the number of patients studied to approximately 20 would allow adequate power for meaningful statistical assessment of the small changes seen in myocardial MDA release so far. Thirdly, to further assess the effect of left bundle branch block and pacing-induced ventricular tachycardia on lipid peroxidation and oxidative stress, a comparison could be performed to determine the extent of myocardial MDA, GSH and GSSG flux during ischaemia and recovery, associated with sinus rhythm and rapid atrial and ventricular pacing in patients with and without significant left coronary artery stenoses.

With the compelling results of the antioxidant effect of NAC in combination with GTN and streptokinase for the management of evolving acute myocardial infarction, further clinical trials are now necessary to firstly examine the haemodynamic effect of this combination, and secondly to try to separate the individual and interactive roles of the three agents used. If using rate of reperfusion of streptokinase with GTN and all three agents in combination as the primary end-point, it is likely that 30 patients in each group would give adequate power to assess this end-point. Furthermore, comparison of streptokinase alone or in combination with GTN and/or NAC would allow a comparison of the separate effects of these agents.

Lastly, consideration of a clinical trial to assess the antioxidant role of NAC during cardioplegia

for cardiac surgery would be appropriate. Ferrari *et al* (Ferrari et al. 1991a) reported some preliminary results of such a study, but this has never been published in a complete form. As cardiac surgery represents controlled myocardial ischaemia and reperfusion, NAC administration can begin before the onset of ischaemia permitting maximum antioxidant effect. It is therefore theoretically possible that the beneficial effect of NAC on the amelioration of oxidative stress in this setting will be even more marked than in evolving acute myocardial infarction.

With the favourable safety profile of NAC in humans, continued studies to assess the potential role of NAC as an antioxidant in the management of acute myocardial ischaemia and/or infarction with reperfusion are feasible and justifiable. Until larger studies assessing haemodynamic end-points are performed, the definitive clinical extent and relevance of oxidative stress in humans cannot be appreciated fully.

Bibliography

Abrams J (1991) Interactions between organic nitrates and thiol groups. Am J Med 91[suppl 3C]: 106S-112S

Adachi H, Motomatsu K, and Yara I (1979) Effect of allopurinol (zyloric) on patients undergoing open heart surgery. Jpn Circ J 43: 395-401

Adams DH, and Shaw S (1994) Leucocyte-endothelial interactions and regulation of leucocyte migration. Lancet 343: 831-836

AIMS Trial Study Group (1988) Effect of intravenous APSAC on mortality after acute myocardial infarction: preliminary report of a placebo-controlled clinical trial. AIMS Trial Study Group. Lancet 1: 545-549

AIRE Study Investigators (1993) Effect of ramipril on mortality and morbidity of survivors of acute myocardial infarction with clinical evidence of heart failure. The Acute Infarction Ramipril Efficacy (AIRE) Study Investigators. Lancet 342: 821-828

Albert C, and Lullmann Rauch R (1983) Ultrastructural alterations in peripheral nerve trunks of rats subchronically treated with chlorphentermine or perhexiline. Arzneimittelforschung 33: 125-127

Albrich JM, McCarthy CA, and Hurst JK (1981) Biological reactivity of hypochlorous acid: implications for microbicidal mechanisms of leukocyte myeloperoxidase. Proc Natl Acad Sci U S A 78: 210-214

Alderman EL, Bourassa MG, Cohen LS, Davis KB, Kaiser GG, Killip T, Mock MB, Pettinger M, and Robertson TL (1990) Ten-year follow-up of survival and myocardial infarction in the randomized Coronary Artery Surgery Study. Circulation 82: 1629-1646

Allison LA, and Shoup RE (1983) Dual electrode liquid chromatography detector for thiols and disulfides. Anal Chem 55: 8-12

Alpert JS (1985) Coronary vasomotion, coronary thrombosis, myocardial infarction and the camel's back. J Am Coll Cardiol 5: 617-8

Ambrose JA, Tannenbaum MA, Alexopoulos D, Hjemdahl Monsen CE, Leavy J, Weiss M, Borrico S, Gorlin R, and Fuster V (1988) Angiographic progression of coronary artery disease and the development of myocardial infarction. J Am Coll Cardiol 12: 56-62

Ambrose JA, Winters SL, Arora RR, Eng A, Riccio A, Gorlin R, and Fuster V (1986) Angiographic evolution of coronary artery morphology in unstable angina. J Am Coll Cardiol 7: 472-478

Ambrosio G, Weisman HF, Mannisi JA, and Becker LC (1989) Progressive impairment of regional myocardial perfusion after initial restoration of postischemic blood flow. Circulation 80: 1846-1861

Ambrosio G, Zweier JL, Duilio C, Kuppusamy P, Santoro G, Elia PP, Tritto I, Cirillo P, Condorelli M, Chiariello M, and et al (1993) Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. J Biol Chem 268: 18532-18541

Anderson JL, Marshall HW, Bray BE, Lutz JR, Frederick PR, Yanowitz FG, Datz FL, Klausner SC, and Hagan AD (1983) A randomized trial of intracoronary streptokinase in the treatment of acute myocardial infarction. N Engl J Med 308: 1312-1318

Ando H, Nakano E, Ueno Y, and Tokunaga K (1989) New technique for analysis of cardiac energetics using a modified Fenn equation. J Thorac Cardiovasc Surg 97: 565-574

Ardehali A, and Ports TA (1990) Myocardial oxygen supply and demand. Chest 98: 699-705

Arstall MA, Beltrame JF, Mohan P, Wuttke RD, Esterman AJ, and Horowitz JD (1992) Incidence of adverse events during treatment with verapamil for suspected acute myocardial infarction. Am J Cardiol 70: 1611-1612

Aruoma OI, Halliwell B, Hoey BM, and Butler J (1989) The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Rad Biol Med 6: 593-597

Atar D, Mortensen SA, Flachs H, and Herzog WR (1993) Coenzyme Q10 protects ischemic myocardium in an open-chest swine model. Clin Investig 71: S103-11

Axelsson KL, and Ahlner J (1987) Nitrate tolerance from a biochemical point of view. Drugs 33 Suppl 4: 63-68

Aznar J, Santos MT, Valles J, and Sala J (1983) Serum malondialdehyde-like material in acute myocardial infarction. J Clin Pathol 36: 712-715

Bagchi D, Das DK, Engelman RM, Prasad MR, and Subramanian R (1990) Polymorphonuclear leucocytes as potential source of free radicals in the ischaemic-reperfused myocardium. Eur Heart J 11: 800-813

Baim DS (1992) Interventional catheterisation techniques: percutaneous transluminal balloon angioplasty, valvuloplasty, and related procedures. In: Braunwald E (ed) Heart disease. W. B. Saunders Company, Philadelphia, pp 1365-1381

Bainton D, Miller NE, Bolton CH, Yarnell JW, Sweetnam PM, Baker IA, Lewis B, and Elwood PC (1992) Plasma triglyceride and high density lipoprotein cholesterol as predictors of ischaemic heart disease in British men. The Caerphilly and Speedwell Collaborative Heart Disease Studies. Br Heart J 68: 60-66

Balkau B, Eschwege E, Fontbonne A, Claude JR, and Warnet JM (1992) Cardiovascular and alcohol-related deaths in abnormal glucose tolerant and diabetic subjects. Diabetologia 35: 39-44

Ballagi Pordany G, Richter J, Koltai M, Aranyi Z, Pogatsa G, and Schaper W (1991) Is malondialdehyde a marker of the effect of oxygen free radicals in rat heart tissue? Basic Res Cardiol 86: 266-272

Balligand JL, Ungureanu D, Kelly RA, Kobzik L, Pimental D, Michel T, and Smith TW (1993) Abnormal contractile function due to induction of nitric oxide synthesis in rat cardiac myocytes follows exposure to activated macrophage-conditioned medium. J Clin Invest 91: 2314-2319

Baroldi G (1975) Different types of myocardial necrosis in coronary heart disease: a pathophysiologic review of their functional significance. Am Heart J 89: 742-752

Barrington PL, Meier CF, Jr., and Weglicki WB (1988) Abnormal electrical activity induced by free radical generating systems in isolated cardiocytes. J Mol Cell Cardiol 20: 1163-1178

Barta E, Pechan I, Cornak V, Luknarova O, Rendekova V, and Verchovodko P (1991) Protective effect of alpha-tocopherol and L-ascorbic acid against the ischemic-reperfusion injury in patients during open-heart surgery. Bratisl Lek Listy 92: 174-183

Beckman JS, Beckman TW, Chen J, Marshall PA, and Freeman BA (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A 87: 1620-1624

Behrens WA, and Madere R (1991) Malonaldehyde determination in tissues and biological fluids by ion-pairing high-performance liquid chromatography. Lipids 26: 232-236

Belch JJF, Bridges AB, Scott N, and Chopra M (1991) Oxygen free radicals and congestive cardiac failure. Br Heart J 65: 245-248

Bell D, Jackson M, Nicoll JJ, Millar A, Dawes J, and Muir AL (1990) Inflammatory response, neutrophil activation, and free radical production after acute myocardial infarction: effect of thrombolytic treatment. Br Heart J 63: 82-87

Benditt EP, and Benditt JM (1973) Evidence for a monoclonal origin of human atherosclerotic plaques. Proc Natl Acad Sci U S A 70: 1753-1756

Bennett BM, Leitman DC, Schroder H, Kawamoto JH, Nakatsu K, and Murad F (1989) Relationship between biotransformation of glyceryl trinitrate and cyclic GMP accumulation in various cultured cell lines. J Pharmacol Exp Ther 250: 316-323

Benveniste J, Boullet C, Brink C, and Labat C (1983) The actions of Paf-acether (platelet-activating factor) on guinea-pig isolated heart preparations. Br J Pharmacol 80: 81-83

Bergmann SR, Herrero P, Markham J, Weinheimer CJ, and Walsh MN (1989) Noninvasive quantitation of myocardial blood flow in human subjects with oxygen-15-labeled water and positron emission tomography. J Am Coll Cardiol 14: 639-652

Bernier M, and Hearse DJ (1988) Reperfusion-induced arrhythmias: mechanisms of protection by glucose and mannitol. Am J Physiol 254: H862-H870

Betts WH, Zhang Y, Rokicinski M, Horowitz JD (1993) N-acetylcysteine and captopril inhibit but S-nitroso-N-acetylcysteine stimulates human neutrophil free radical production. Circulation 88 [Suppl I]: I-90(Abstract)

Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, and Gimbrone MA, Jr. (1985) Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. J Clin Invest 76: 2003-2011

Bevilacqua MP, and Nelson RM (1993) Selectins. J Clin Invest 91: 379-387

BHAT Investigators (1982) A randomized trial of propranolol in patients with acute myocardial infarction. I. Mortality results. JAMA 247: 1707-1714

Bical O, Gerhardt MF, Paumier D, Gaillard D, Comas J, Landais P, Fischer M, Trivin F, and Vanetti A (1991) Comparison of different types of cardioplegia and reperfusion on myocardial metabolism and free radical activity. Circulation 84: III375-III379

Bird RP, and Draper HH (1984) Comparative studies on different methods of malonaldehyde determination. Methods Enzymol 105: 299-305

Blanke H, von Hardenberg D, Cohen M, Kaiser H, Karsch KR, Holt J, Smith H, Jr., and Rentrop P (1984) Patterns of creatine kinase release during acute myocardial infarction after nonsurgical reperfusion: comparison with conventional treatment and correlation with infarct size. J Am Coll Cardiol 3: 675-680

Blann A, Midgley H, Burrows G, Maxwell S, Utting S, Davies M, Waite M, and McCollum C (1993) Free radicals, antioxidants, and endothelial cell damage after percutaneous transluminal coronary angioplasty. Coron Artery Dis 4: 905-910

Blaustein A, Deneke SM, Stolz RI, Baxter D, Healey N, and Fanburg BL (1989) Myocardial glutathione depletion impairs recovery after short periods of ischemia. Circulation 80: 1449-1457

Boesgaard S, Petersen JS, Aldershvile J, Poulsen HE, and Flachs H (1991b) Nitrate tolerance: effect of thiol supplementation during prolonged nitroglycerin infusion in an in vivo rat model. J Pharmacol Exp Ther 258: 851-856

Boesgaard S, Aldershvile J, and Poulsen HE (1992) Preventive administration of intravenous N-acetylcysteine and development of tolerance to isosorbide dinitrate in patients with angina pectoris. Circulation 85: 143-149

Boesgaard S, Poulsen HE, Aldershvile J, Loft S, Anderson ME, and Meister A (1993) Acute effects of nitroglycerin depend on both plasma and intracellular sulphydryl compound levels in vivo. Effect of agents with different sulphydryl-modulating properties. Circulation 87: 547-553

Boesgaard S, Aldershvile J, Pedersen F, Pietersen A, Madsen JK, and Grande P (1991a) Continuous oral N-acetylcysteine treatment and development of nitrate tolerance in patients with stable angina pectoris. J Cardiovasc Pharmacol 17: 889-893

Boesgaard S, Aldershvile J, Poulsen HE, Loft S, Anderson ME, and Meister A (1994a) Nitrate tolerance in vivo is not associated with depletion of arterial or venous thiol levels. Circ Res 74: 115-120

Boesgaard S, Iversen HK, Wroblewski H, Poulsen HE, Frandsen H, Kastrup J, and Aldershvile J (1994b) Altered peripheral vasodilator profile of nitroglygerin during long-term infusion of N-acetylcysteine. J Am Coll Cardiol 23: 163-169

Bolli R, Patel BS, Zhu WX, O'Neill PG, Hartley CJ, Charlat ML, and Roberts R (1987a) The iron chelator desferrioxamine attenuates postischemic ventricular dysfunction. Am J Physiol 253: H1372-80

Bolli R, Zhu WX, Hartley CJ, Michael LH, Repine JE, Hess ML, Kukreja RC, and Roberts R (1987b) Attenuation of dysfunction in the postischemic 'stunned' myocardium by dimethylthiourea. Circulation 76: 458-468

Bolli R, Patel BS, Jeroudi MO, Lai EK, and McCay PB (1988a) Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap alpha-phenyl N-tert-butyl nitrone. J Clin Invest 82: 476-485

Bolli R, Zhu WX, Thornby JI, O'Neill PG, and Roberts R (1988b) Time course and determinants of recovery of function after reversible ischemia in conscious dogs. Am J Physiol 254: H102-14

Bolli R, Patel BS, Hartley CJ, Thornby JI, Jeroudi MO, and Roberts R (1989) Nonuniform transmural recovery of contractile function in stunned myocardium. Am J Physiol 257: H375-85

Bolli R (1990) Mechanism of myocardial "stunning". Circulation 82: 723-738

Bolli R (1992) Myocardial 'stunning' in man. Circulation 86: 1671-1691

Bolli R (1993) Role of neutrophils in myocardial stunning after brief ischaemia: the end of a six year old controversy (1987-1993) [comment]. Cardiovasc Res 27: 728-730

Borgstrom L, Kagedal B, and Paulsen O (1986) Pharmacokinetics of N-acetylcysteine in man. Eur J Clin Pharmacol 31: 217-222

Bowry VW, Stanley KK, and Stocker R (1992) High density lipoprotein is the major carrier of lipid hydroperoxides in human blood plasma from fasting donors. Proc Natl Acad Sci U S A 89: 10316-10320

Brand FN, Kiely DK, Kannel WB, and Myers RH (1992) Family patterns of coronary heart disease mortality: the Framingham Longevity Study. J Clin Epidemiol 45: 169-174

Brasch H, Schoenberg MH, and Younes M (1989) No evidence for an increased lipid peroxidation during reoxygenation in Langendorff hearts and isolated atria of rats. J Mol Cell Cardiol 21: 697-707

Braunwald E (1982) Mechanism of action of calcium-channel-blocking agents. N Engl J Med 307: 1618-1627

Brazier J, Cooper N, and Buckberg G (1974) The adequacy of subendocardial oxygen delivery: the interaction of determinants of flow, arterial oxygen content and myocardial oxygen need. Circulation 49: 968-977

Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, and Snyder SH (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 351: 714-718

Breisblatt WM, Stein KL, Wolfe CJ, Follansbee WP, Capozzi J, Armitage JM, and Hardesty RL (1990) Acute myocardial dysfunction and recovery: a common occurrence after coronary bypass surgery. J Am Coll Cardiol 15: 1261-1269

Brunelli C, Spallarossa P, Ghigliotti G, Iannetti M, and Caponnetto S (1991) Thrombolysis in refractory unstable angina. Am J Cardiol 68: 110B-118B

Brutsaert DL, Sys SU, and Gillebert TC (1993) Diastolic failure: pathophysiology and therapeutic implications. J Am Coll Cardiol 22: 318-325

Brutsaert DL, and Sys SU (1989) Relaxation and diastole of the heart. Physiol Rev 69: 1228-1315

Buchalter MB, Suntharalingam G, Jennings I, Hart C, Luddington RJ, Chakraverty R, Jacobson SK, Weissberg PL, and Baglin TP (1992) Streptokinase resistance: when might streptokinase administration be ineffective? Br Heart J 68: 449-453

Butler AR, Flitney FW, and Williams DLH (1995) NO, nitrosonium ions, nitroxide ions, nitrosothiols and iron-nitrosyls in biology: a chemist's perspective. T i P S 16: 18-22

Butler WJ, Ostrander LD, Jr., Carman WJ, and Lamphiear DE (1985) Mortality from coronary heart disease in the Tecumseh study. Long-term effect of diabetes mellitus, glucose tolerance and other risk factors. Am J Epidemiol 121: 541-547

Buttery JE, Chamberlain BR, Milner CR, Pannall PR (1985) Colormetric measurement of plasma lactate. Am J Clin Path 84: 363-365

Califf RM, Topol EJ, Stack RS, Ellis SG, George BS, Kereiakes DJ, Samaha JK, Worley SJ, Anderson JL, Harrelson Woodlief L, and et al (1991) Evaluation of combination thrombolytic therapy and timing of cardiac catheterization in acute myocardial infarction. Results of thrombolysis and angioplasty in myocardial infarction--phase 5 randomized trial. TAMI Study Group. Circulation 83: 1543-1556

Califf RM, Fortin DF, Tenaglia AN, and Sane DC (1992) Clinical risks of thrombolytic therapy. Am J Cardiol 69: 12A-20A

Cameron A, Davis KB, Green GE, Myers WO, and Pettinger M (1988) Clinical implications of internal mammary artery bypass grafts: the Coronary Artery Surgery Study experience. Circulation 77: 815-819

Camici P, Marraccini P, Marzilli M, Lorenzoni R, Buzzigoli G, Puntoni R, Boni C, Bellina CR, Klassen GA, L'Abbate A, and et al (1989) Coronary hemodynamics and myocardial metabolism during and after pacing stress in normal humans. Am J Physiol 257: E309-E317

Camici P, Marraccini P, Lorenzoni R, Ferrannini E, Buzzigoli G, Marzilli M, and L'Abbate A (1991) Metabolic markers of stress-induced myocardial ischemia. Circulation 83: III8-II13

Capucci A, Bassein L, Bracchetti D, Carini G, Maresta A, and Magnani B (1983) Propranolol v. verapamil in the treatment of unstable angina. A double-blind cross-over study. Eur Heart J 4: 148-154

Cargnoni A, Comini L, Boraso A, De Giuli F, Scotti C, and Ferrari R (1992) The effects of L-arginine mono(2-mercaptoethanesulfonate) on the ischemic and reperfused heart. Cardioscience 3: 179-187

Carroll JD, Hess OM, Hirzel HO, and Krayenbuehl HP (1983) Exercise-induced ischemia: the influence of altered relaxation on early diastolic pressures. Circulation 67: 521-528

Caulfield J, and Klionsky B (1959) Myocardial ischemia and early infarction: an electron microscopic study. Am J Pathol 35: 489-523

Cavarocchi NC, England MD, O'Brien JF, Solis E, Russo P, Schaff HV, Orszulak TA, Pluth JR, and Kaye MP (1986a) Superoxide generation during cardiopulmonary bypass: is there a role for vitamin E? J Surg Res 40: 519-527

Cavarocchi NC, England MD, Schaff HV, Russo P, Orszulak TA, Schnell WA, Jr., O'Brien JF, and Pluth JR (1986b) Oxygen free radical generation during cardiopulmonary bypass: correlation with complement activation. Circulation 74: III130-3

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

Charlat MI, O'Neill PG, Egan JM, Abernethy DR, Michael LH, Myers ML, Roberts R, and Bolli R (1987) Evidence for a pathogenetic role of xanthine oxidase in the "stunned" myocardium. Am J Physiol 252: H566-77

Chatham JC, Seymour AML, Harmsen E, and Radda GK (1988) Depletion of myocardial glutathione: its effects on heart function and metabolism during ischaemia and reperfusion. Cardiovasc Res 22: 833-839

Cheeseman KH, and Slater TF (1993) An introduction to free radical biochemistry. Br Med Bull 49: 481-493

Chello M, Mastroroberto P, Romano R, Bevacqua E, Pantaleo D, Ascione R, Marchese AR, and Spampinato N (1994) Protection by coenzyme Q10 from myocardial reperfusion injury during coronary artery bypass grafting. Ann Thorac Surg 58: 1427-1432

Chenoweth DE, Cooper SW, Hugli TE, Stewart RW, Blackstone EH, and Kirklin JW (1981) Complement activation during cardiopulmonary bypass: evidence for generation of C3a and C5a anaphylatoxins. N Engl J Med 304: 497-503

Chesebro JH, Knatterud G, Roberts R, Borer J, Cohen LS, Dalen J, Dodge HT, Francis CK, Hillis D, Ludbrook P, and et al (1987) Thrombolysis in Myocardial Infarction (TIMI) Trial, Phase I: A comparison between intravenous tissue plasminogen activator and intravenous streptokinase. Clinical findings through hospital discharge. Circulation 76: 142-154

Chirkov YY, Gee DJ, Naujalis JI, Sage RE, and Horowitz JD (1993a) Reversal of ADP-induced platelet aggregation by S-nitrosothiols, nitroglycerine and nitroglycerine/N-acetylcysteine. Pharm Com 3: 97-105

Chirkov YY, Naujalis JI, Sage RE, and Horowitz JD (1993b) Antiplatelet effects of nitroglycerin in healthy subjects and in patients with stable angina pectoris. J Cardiovasc Pharmacol 21: 384-389

Clancy RM, Leszczynska Piziak J, and Abramson SB (1992) Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. J Clin Invest 90: 1116-1121

Clausen J, and Jensen G (1992) Blood pressure and mortality: an epidemiological survey with 10 years follow-up. J Hum Hypertens 6: 53-59

Coghlan JG, Flitter WD, Clutton SM, Ilsley CD, Rees A, and Slater TF (1993) Lipid peroxidation and changes in vitamin E levels during coronary artery bypass grafting. J Thorac Cardiovasc Surg 106: 268-274

Coghlan JG, Flitter WD, Clutton SM, Panda R, Daly R, Wright G, Ilsley CD, and Slater TF (1994) Allopurinol pretreatment improves postoperative recovery and reduces lipid peroxidation in patients undergoing coronary artery bypass grafting. J Thorac Cardiovasc Surg 107: 248-256

Coghlan JG, Flitter WD, Holley AE, Norell M, Mitchell AG, Ilsley CD, and Slater TF (1991) Detection of free radicals and cholesterol hydroperoxides in blood taken from the coronary sinus of man during percutaneous transluminal coronary angioplasty. Free Radic Res Commun 14: 409-417

Cohen M, and Rentrop KP (1986) Limitation of myocardial ischemia by collateral circulation during sudden controlled coronary artery occlusion in human subjects: a prospective study. Circulation 74: 469-476

Cohn PF, Maddox D, Holman BL, Markis JE, Adams DF, See JR, and Idoine J (1977) Effect of sublingually administered nitroglycerin on regional myocardial blood flow in patients with coronary artery disease. Am J Cardiol 39: 672-678

Cohn JN, and Rector TS (1988) Prognosis of congestive heart failure and predictors of mortality. Am J Cardiol 62: 25A-30A

Cole PL, Beamer AD, McGowan N, Cantillon CO, Benfell K, Kelly RA, Hartley LH, Smith TW, and Antman EM (1990) Efficacy and safety of perhexiline maleate in refractory angina. A double-blind placebo-controlled clinical trial of a novel antianginal agent. Circulation 81: 1260-1270

Collins P (1993) Coronary flow reserve [editorial]. Br Heart J 69: 279-281

Cooke JP, and Tsao PS (1993) Cytoprotective effects of nitric oxide [editorial; comment]. Circulation 88: 2451-2454

Cordis GA, Maulik N, Bagchi D, Engelman RM, and Das DK (1993) Estimation of the extent of lipid peroxidation in the ischemic and reperfused heart by monitoring lipid metabolic products with the aid of high-performance liquid chromatography. J Chromatogr 632: 97-103

Corr PB, Gross RW, and Sobel BE (1982) Arrhythmogenic amphiphilic lipids and the myocardial cell membrane. J Mol Cell Cardiol 14: 619-626

Corretti MC, Koretsune Y, Kusuoka H, Chacko VP, Zweier JL, and Marban E (1991) Glycolytic inhibition and calcium overload as consequences of exogenously generated free radicals in rabbit hearts. J Clin Invest 88: 1014-1025

Cotgreave IA, Eklund A, Larsson K, and Moldeus PW (1987) No penetration of orally administered N-acetylcysteine into bronchoalveolar lavage fluid. Eur J Respir Dis 70: 73-77

Cotgreave IA, Moldeus P, and Orrenius S (1988) Host biochemical defense mechanisms against prooxidants. Annu Rev Pharmacol Toxicol 28: 189-212

Covell JW, Ross J, Jr., Taylor R, Sonnenblick EH, and Braunwald E (1967) Effects of increasing frequency of contraction on the force velocity relation of left ventricle. Cardiovasc Res 1: 2-8

Craven PA, and DeRubertis FR (1978) Restoration of the responsiveness of purified guanylate cyclase to nitrosoguanidine, nitric oxide, and related activators by heme and hemeproteins. Evidence for involvement of the paramagnetic nitrosyl-heme complex in enzyme activation. J Biol Chem 253: 8433-8443

Crichton RR, and Charloteaux Wauters M (1987) Iron transport and storage. Eur J Biochem 164: 485-506

Culotta E, and Koshland DEJ (1992) NO news is good news. Science 258: 1862-1865

Curfman GD, Heinsimer JA, Lozner EC, and Fung HL (1983) Intravenous nitroglycerin in the treatment of spontaneous angina pectoris: a prospective, randomized trial. Circulation 67: 276-282

Das DK, and Maulik N (1994) Antioxidant effectiveness in ischemia-reperfusion tissue injury. Methods Enzymol 233: 601-610

Davies MJ, Woolf N, Rowles PM, and Pepper J (1988) Morphology of the endothelium over atherosclerotic plaques in human coronary arteries. Br Heart J 60: 459-464

Davies SW, Underwood SM, Wickens DG, Feneck RO, Dormandy TL, and Walesby RK (1990b) Systemic pattern of free radical generation during coronary bypass surgery. Br Heart J 64: 236-240

Davies SW, Ranjadayalan K, Wickens DG, Dormandy TL, and Timmis AD (1990a) Lipid peroxidation associated with successful thrombolysis. Lancet 335: 741-743

Davies SW, Ranjadayalan K, Wickens DG, Dormandy TL, Umachandran V, and Timmis AD (1993) Free radical activity and left ventricular function after thrombolysis for acute myocardial infarction. Br Heart J 69: 114-120

Davies MJ, and Thomas A (1984) Thrombosis and acute coronary-artery lesions in sudden cardiac ischemic death. N Engl J Med 310: 1137-1140

Davydov VV (1993) [Pathways of endogenous malonate formation in the rat liver]. Ukr Biokhim Zh 65: 85-88

de Zwaan C, Willems GM, Vermeer F, Res J, Verheugt FW, van der Laarse A, Simoons ML, Lubsen J, and Hermens WT (1988) Enzyme tests in the evaluation of thrombolysis in acute myocardial infarction. Br Heart J 59: 175-183

de Jong JW, van der Meer P, Nieukoop AS, Huizer T, Stroeve RJ, and Bos E (1990) Xanthine oxidoreductase activity in perfused hearts of various species, including humans. Circ Res 67: 770-773

de Servi S, Mazzone A, Ricevuti G, Fioravanti A, Bramucci E, Angoli L, Stefano G, and Specchia G (1990) Granulocyte activation after coronary angioplasty in humans. Circulation 82: 140-146

De Bruyne B, Bronzwaer JG, Heyndrickx GR, and Paulus WJ (1993) Comparative effects of ischemia and hypoxemia on left ventricular systolic and diastolic function in humans. Circulation 88: 461-471

De Scheerder IK, van de Kraay AM, Lamers JM, Koster JF, de Jong JW, and Serruys PW (1991) Myocardial malondialdehyde and uric acid release after short-lasting coronary occlusions during coronary angioplasty: potential mechanisms for free radical generation. Am J Cardiol 68: 392-395

De Caro L, Ghizzi A, Costa R, Longo A, Ventresca GP, and Lodola E (1989) Pharmacokinetics and bioavailability of oral acetylcysteine in healthy volunteers. Arzneimittelforschung 39: 382-386

DeBoer LW, Ingwall JS, Kloner RA, and Braunwald E (1980) Prolonged derangements of canine myocardial purine metabolism after a brief coronary artery occlusion not associated with anatomic evidence of necrosis. Proc Natl Acad Sci U S A 77: 5471-5475

del Principe D, Menichelli A, de Matteis W, di Giulio S, Giordani M, Savini I, and Finazzi-Agro A (1990) The effect of cyst(e)ine on human platelets is mediated by hydrogen peroxide. Clin Chem Enzym Com 3: 9-17

Des Rosiers C, Rivest MJ, Boily MJ, Jette M, Carrobe Cohen A, and Kumar A (1993) Gas chromatographic-mass spectrometric assay of tissue malondialdehyde, 4-hydroxynonenal, and other aldehydes after their reduction to stable alcohols. Anal Biochem 208: 161-170

Deutsch N, Klitzner TS, Lamp ST, and Weiss JN (1991) Activation of cardiac ATP-sensitive K^+ current during hypoxia: correlation with tissue ATP levels. Am J Physiol 261: H671-H676

Devries SR, Jaffe AS, Geltman EM, Sobel BE, and Abendschein DR (1989) Enzymatic estimation of the extent of irreversible myocardial injury early after reperfusion. Am Heart J 117: 31-36

Devries SR, Sobel BE, and Abendschein DR (1986) Early detection of myocardial reperfusion by assay of plasma MM-creatine kinase isoforms in dogs. Circulation 74: 567-572

DeWood MA, Notske RN, Berg R, Jr., Ganji JH, Simpson CS, Hinnen ML, Selinger SL, and Fisher LD (1989) Medical and surgical management of early Q wave myocardial infarction. I. Effects of surgical reperfusion on survival, recurrent myocardial infarction, sudden death and functional class at 10 or more years of follow-up. J Am Coll Cardiol 14: 65-77

DeWood MA, Stifter WF, Simpson CS, Spores J, Eugster GS, Judge TP, and Hinnen ML (1986) Coronary arteriographic findings soon after non-Q-wave myocardial infarction. N Engl J Med 315: 417-423

Di Pasquale P, Paterna S, Cannizzaro S, and Bucca V (1994) Does captopril treatment before thrombolysis in acute myocardial infarction attenuate reperfusion damage? Short-term and long-term effects. Int J Cardiol 43: 43-50

Di Pasquale P, Paterna S, Valenza M, Valdes L, Albano V, Trombino G, Vitrano G, Cannizzaro S, Licata G, and Albiero R (1993) Effects of captopril on myocardial protection during cardioplegia. Int J Cardiol 42: 225-230

Di Pasquale P, Barone G, Paterna S, Cannizzaro S, and Giubilato A (1990) Efficacy of captopril before thrombolysis in acute myocardial infarction: preliminary findings. Drugs Exp Clin Res 16: 581-589

Di Pasquale P, Paterna S, Cannizzaro S, Albano V, Valdes L, Licata G, and Barone G (1992) Captopril and glutathione before thrombolysis in acute myocardial infarction: a pilot study. Drugs Exp Clin Res 18: 401-406

Diamond MS, Staunton DE, de Fougerolles AR, Stacker SA, Garcia Aguilar J, Hibbs ML, and Springer TA (1990) ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). J Cell Biol 111: 3129-3139

Dillard CJ, and Tappel AL (1984) Fluorescent damage products of lipid peroxidation. Methods Enzymol 105: 337-341

Dissman R, Linderer T, Goerke M, von Ameln H, Rennhak U, and Schroder R (1993) Sudden increase of the ST segment elevation at time of reperfusion predicts extensive infarcts in patients with intravenous thrombolysis. Am Heart J 126: 832-839

Dobson AJ, Alexander HM, Heller RF, and Lloyd DM (1991) How soon after quitting smoking does risk of heart attack decline? J Clin Epidemiol 44: 1247-1253

Dodge HT, Sandler H, Ballew DW, and Lord JD (1960) The use of biplane angiocardiography for the measurement of left ventricular volume in man. Am Heart J 60: 762-776

Dousset JC, Trouilh M, and Foglietti MJ (1983) Plasma malondialdehyde levels during myocardial infarction. Clin Chim Acta 129: 319-322

Draper HH, and Hadley M (1990) Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol 186: 421-431

Drossos G, Lazou A, Panagopoulos P, and Westaby S (1995) Deferoxamine cardioplegia reduces superoxide radical production in human myocardium. Ann Thorac Surg 59: 169-172

Drost E, Lannan S, Bridgeman MM, Brown D, Selby C, Donaldson K, and MacNee W (1991) Lack of effect of N-acetylcysteine on the release of oxygen radicals from neutrophils and alveolar macrophages. Eur Respir J 4: 723-729

Dubois Rande JL, Artigou JY, Darmon JY, Habbal R, Manuel C, Tayarani I, Castaigne A, and Grosgogeat Y (1994) Oxidative stress in patients with unstable angina. Eur Heart J 15: 179-183

Dubrow R, Burnett CA, Gute DM, and Brockert JE (1988) Ischemic heart disease and acute myocardial infarction mortality among police officers. J Occup Med 30: 650-654

Duling BR (1972) Microvascular responses to alterations in oxygen tension. Circ Res 31: 481-489

Dupuis J, Lalonde G, Lemieux R, and Rouleau JL (1990) Tolerance to intravenous nitroglycerin in patients with congestive heart failure: role of increased intravascular volume, neurohumoral activation and lack of prevention with N-acetylcysteine. J Am Coll Cardiol 16: 923-931

Duthie GG, Morrice PC, Ventresca PG, and McLay JS (1992) Effects of storage, iron and time of day on indices of lipid peroxidation in plasma from healthy volunteers. Clin Chim Acta 206: 207-213

Dykewicz MS, McGrath KG, Davison R, Kaplan KJ, and Patterson R (1986) Identification of patients at risk for anaphylaxis due to streptokinase. Arch Intern Med 146: 305-307

Efthymiadis A, Lefkos N, Liatsis I, Bountonas G, Styliadis IG, Aroditis K, and Tsapas G (1994) The diagnostic value of the determination of troponin T in the diagnosis of unstable angina. Acta Cardiol 49: 419-424

el Tamimi H, Mansour M, Wargovich TJ, Hill JA, Kerensky RA, Conti CR, and Pepine CJ (1994) Constrictor and dilator responses to intracoronary acetylcholine in adjacent segments of the same coronary artery in patients with coronary artery disease. Endothelial function revisited. Circulation 89: 45-51

Elayda MA, Mathur VS, Hall RJ, Massumi GA, Garcia E, and de Castro CM (1985) Collateral circulation in coronary artery disease. Am J Cardiol 55: 58-60

Ellis SG, Henschke CI, Sandor T, Wynne J, Braunwald E, and Kloner RA (1983) Time course of functional and biochemical recovery of myocardium salvaged by reperfusion. J Am Coll Cardiol 1: 1047-1055

Ellis SG, Wynne J, Braunwald E, Henschke CI, Sandor T, and Kloner RA (1984) Response of reperfusion-salvaged, stunned myocardium to inotropic stimulation. Am Heart J 107: 13-19

Ellis SG, Topol EJ, George BS, Kereiakes DJ, Debowey D, Sigmon KN, Pickel A, Lee KL, and Califf RM (1989) Recurrent ischemia without warning. Analysis of risk factors for in-hospital ischemic events following successful thrombolysis with intravenous tissue plasminogen activator. Circulation 80: 1159-1165

Ely DL, and Mostardi RA (1986) The effect of recent life events stress, life assets, and temperament pattern on cardiovascular risk factors for Akron City police officers. J Human Stress 12: 77-91

Eng C, Patterson RE, Horowitz SF, Halgash DA, Pichard AD, Midwall J, Herman MV, and Gorlin R (1982) Coronary collateral function during exercise. Circulation 66: 309-316

England MD, Cavarocchi NC, O'Brien JF, Solis E, Pluth JR, Orszulak TA, Kaye MP, and Schaff HV (1986) Influence of antioxidants (mannitol and allopurinol) on oxygen free radical generation during and after cardiopulmonary bypass. Circulation 74: III134-7

Engler RL, Dahlgren MD, Peterson MA, Dobbs A, and Schmid Schonbein GW (1986b) Accumulation of polymorphonuclear leukocytes during 3-h experimental myocardial ischemia. Am J Physiol 251: H93-100

Engler RL, Dahlgren MD, Morris DD, Peterson MA, and Schmid Schonbein GW (1986a) Role of leukocytes in response to acute myocardial ischemia and reflow in dogs. Am J Physiol 251: H314-23

Enos WF, Beyer JC, and Holmes RH (1955) Pathogenesis of coronary disease in american soldiers killed in Korea. J A M A 158: 912-914

Entman ML, Youker K, Shappell SB, Siegel C, Rothlein R, Dreyer WJ, Schmalstieg FC, and Smith CW (1990) Neutrophil adherence to isolated adult canine myocytes. Evidence for a CD18-dependent mechanism. J Clin Invest 85: 1497-1506

Erbel R, Pop T, Meinertz T, Olshausen KV, Treese N, Henrichs KJ, Schuster CJ, Rupprecht HJ, Schlurmann W, and Meyer J (1988) Combination of calcium channel blocker and thrombolytic therapy in acute myocardial infarction. Am Heart J 115: 529-538

Ernster L, and Forsmark Andree P (1993) Ubiquinol: an endogenous antioxidant in aerobic organisms. Clin Investig 71: S60-S65

Esterbauer H, Schaur RJ, and Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 11: 81-128

Esterbauer H, Wag G, and Puhl H (1993) Lipid peroxidation and its role in atherosclerosis. Br Med Bull 49: 566-576

Esterbauer H, and Zollner H (1989) Methods for determination of aldehydic lipid peroxidation products. Free Radic Biol Med 7: 197-203

Euler DE (1994) Reperfusion induced arrhythmias are not caused by generation of free radicals [comment]. Cardiovasc Res 28: 423-432

Ezra D, Boyd LM, Feuerstein G, and Goldstein RE (1983) Coronary constriction by leukotriene C4, D4, and E4 in the intact pig heart. Am J Cardiol 51: 1451-1454

Fabiani JN, Farah B, Vuilleminot A, Lecompte T, Emerit I, Chardigny C, and Carpentier A (1993) Chromosomal aberrations and neutrophil activation induced by reperfusion in the ischaemic human heart. Eur Heart J 14 Suppl G: 12-17

Falk E (1992) Why do plaques rupture? Circulation 86: III30-42

Farb A, Kolodgie FD, Jenkins M, and Virmani R (1993b) Myocardial infarct extension during reperfusion after coronary artery occlusion: pathological evidence. J Am Coll Cardiol 21: 1245-1253

Farber JL, Chien KR, and Mittnacht S, Jr. (1981) Myocardial ischemia: the pathogenesis of irreversible cell injury in ischemia. Am J Pathol 102: 271-281

Feelisch M, and Noack EA (1987) Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. Eur J Pharmacol 139: 19-30

Fernandez Ortiz A, Badimon JJ, Falk E, Fuster V, Meyer B, Mailhac A, Weng D, Shah PK, and Badimon L (1994) Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture. J Am Coll Cardiol 23: 1562-1569

Ferrari R, Ceconi C, Curello S, Cargnoni A, Pasini E, and Visioli O (1991b) The occurrence of oxidative stress during reperfusion in experimental animals and men. Cardiovasc Drugs Ther 5 Suppl 2: 277-287

Ferrari R, Ceconi C, Curello S, Cargnoni A, Alfieri O, Pardini A, Marzollo P, and Visioli O (1991a) Oxygen free radicals and myocardial damage: protective role of thiol-containing agents. Am J Med 91 [suppl 3C]: 95S-105S

Ferrari R, Alfieri O, Curello S, Ceconi C, Cargnoni A, Marzollo P, Pardini A, Caradonna E, and Visioli O (1990) Occurrence of oxidative stress during reperfusion of the human heart. Circulation 81: 201-211

Ferrari R, Ceconi C, Curello S, Cargnoni A, Agnoletti G, Boffa GM, and Visioli O (1986) Intracellular effects of myocardial ischaemia and reperfusion: role of calcium and oxygen. Eur Heart J 7 Suppl A: 3-12

Ferrari R, Ceconi C, Curello S, Cargnoni A, and De Giuli F (1992) Occurrence of oxidative stress during myocardial reperfusion. Mol Cell Biochem 111: 61-69

Ferreira R, Llesuy S, Milei J, Scordo D, Hourquebie H, Molteni L, de Palma C, and Boveris A (1988) Assessment of myocardial oxidative stress in patients after myocardial revascularization. Am Heart J 115: 307-312

Ferreira R, Burgos M, Milei J, Llesuy S, Molteni L, Hourquebie H, and Boveris A (1990) Effect of supplementing cardioplegic solution with deferoxamine on reperfused human myocardium. J Thorac Cardiovasc Surg 100: 708-714

Feuerstein G, Boyd LM, Ezra D, and Goldstein RE (1984) Effect of platelet-activating factor on coronary circulation of the domestic pig. Am J Physiol 246: H466-H471

Fibrinolytic Therapy Trialists' (FTT) Collaborative Group (1994) Indications for fibrinolytic therapy in suspected acute myocardial infarction: collaborative overview of early mortality and major morbidity results from all randomised trials of more than 1000 patients. Lancet 343: 311-322

Figueras J, Lidon R, and Cortadellas J (1991) Rebound myocardial ischaemia following abrupt interruption of intravenous nitroglycerin infusion in patients with unstable angina at rest. Eur Heart J 12: 405-411

Figueras J, Singh BN, Ganz W, Charuzi Y, and Swan HJ (1979) Mechanism of rest and nocturnal angina: observations during continuous hemodynamic and electrocardiographic monitoring. Circulation 59: 955-968

Fisch C (1992) Electrocardiography and vectorcardiography. In: Braunwald E (ed) Heart disease. W. B. Saunders Company, Philadelphia, pp 116-160

Fishbein MC, Y Rit J, Lando U, Kanmatsuse K, Mercier JC, and Ganz W (1980) The relationship of vascular injury and myocardial hemorrhage to necrosis after reperfusion. Circulation 62: 1274-1279

Fitzgerald DJ, Catella F, Roy L, and FitzGerald GA (1988) Marked platelet activation in vivo after intravenous streptokinase in patients with acute myocardial infarction. Circulation 77: 142-150

Fitzgerald AP, and Jarrett RJ (1992) Body weight and coronary heart disease mortality: an analysis in relation to age and smoking habit. 15 years follow-up data from the Whitehall Study. Int J Obes Relat Metab Disord 16: 119-123

Flaherty JT (1989) Nitrate tolerance. A review of the evidence. Drugs 37: 523-550

Flaherty JT, Pitt B, Gruber JW, Heuser RR, Rothbaum DA, Burwell LR, George BS, Kereiakes DJ, Deitchman D, Gustafson N, and et al (1994) Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction. Circulation 89: 1982-1991

Folts JD, Stamler J, and Loscalzo J (1991) Intravenous nitroglycerin infusion inhibits cyclic blood flow responses caused by periodic platelet thrombus formation in stenosed canine coronary arteries. Circulation 83: 2122-2127

Folts JD, Gallagher K, and Rowe GG (1982) Blood flow reductions in stenosed canine coronary arteries: vasospasm or platelet aggregation? Circulation 65: 248-255

Forman MB, Puett DW, Cates CU, McCroskey DE, Beckman JK, Greene HL, and Virmani R (1988) Glutathione redox pathway and reperfusion injury. Effect of N-acetylcysteine on infarct size and ventricular function. Circulation 78: 202-213

Forman R, Eng C, and Kirk ES (1983) Comparative effect of verapamil and nitroglycerin on collateral blood flow. Circulation 67: 1200-1204

Forman MB, Perry JM, Wilson BH, Verani MS, Kaplan PR, Shawl FA, and Friesinger GC (1991) Demonstration of myocardial reperfusion injury in humans: results of a pilot study utilizing acute coronary angioplasty with perfluorochemical in anterior myocardial infarction. J Am Coll Cardiol 18: 911-918

Forstermann U, Nakane M, Tracey WR, and Pollock JS (1993) Isoforms of nitric oxide synthase: functions in the cardiovascular system. Eur Heart J 14 Suppl I: 10-15

Frame LH, Lopez JA, Khaw BA, Fallon JT, Haber E, and Powell WJ, Jr. (1983) Early membrane damage during coronary reperfusion in dogs. Detection by radiolabeled anticardiac myosin (Fab')2. J Clin Invest 72: 535-544

Frankel EN, and Neff WE (1983) Formation of malondialdehyde from lipid oxidation products. Biochim Biophys Acta 754: 264-270

Freedman SB, Dunn RF, Bernstein L, Morris J, and Kelly DT (1985) Influence of coronary collateral blood flow on the development of exertional ischemia and Q wave infarction in patients with severe single-vessel disease. Circulation 71: 681-686

Frei B, England L, and Ames BN (1989) Ascorbate is an outstanding antioxidant in human blood plasma. Proc Natl Acad Sci U S A 86: 6377-6381

Frei B, Stocker R, England L, and Ames BN (1990) Ascorbate: the most effective antioxidant in human blood plasma. Adv Exp Med Biol 264: 155-163

Freye CJ, Wagner NB, Howe CM, Stack NC, Ideker RE, Selvester RH, and Wagner GS (1992) Evaluation of a QRS scoring system for estimating myocardial infarct size. VIII. Specificity in a control group with left ventricular hypertrophy and proposal of a new scoring system for use with this confounding factor. J Electrocardiol 25: 19-23

Fridovich I (1978) The biology of oxygen radicals. Science 201: 875-880

Gage JE, Hess OM, Murakami T, Ritter M, Grimm J, and Krayenbuehl HP (1986) Vasoconstriction of stenotic coronary arteries during dynamic exercise in patients with classic angina pectoris: reversibility by nitroglycerin. Circulation 73: 865-876

Ganote CE, and Kaltenbach JP (1979) Oxygen-induced enzyme release: early events and a proposed mechanism. J Mol Cell Cardiol 11: 389-406

Ganz W, Watanabe I, Kanamasa K, Yano J, Han DS, and Fishbein MC (1990) Does reperfusion extend necrosis? A study in a single territory of myocardial ischemia--half reperfused and half not reperfused. Circulation 82: 1020-1033

Ganz W, Buchbinder N, Marcus H, Mondkar A, Maddahi J, Charuzi Y, O'Connor L, Shell W, Fishbein MC, Kass R, Miyamoto A, and Swan HJ (1981) Intracoronary thrombolysis in evolving myocardial infarction. Am Heart J 101: 4-13

Ganz W, Tamura K, Marcus HS, Donoso R, Yoshida S, and Swan HJ (1971) Measurement of coronary sinus blood flow by continuous thermodilution in man. Circulation 44: 181-195

Garlick PB, Davies MJ, Hearse DJ, and Slater TF (1987) Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. Circ Res 61: 757-760

Gash AK, Spann JF, Sherry S, Belber AD, Carabello BA, McDonough MT, Mann RH, McCann WD, Gault JH, Gentzler RD, and et al (1986) Factors influencing reocclusion after coronary thrombolysis for acute myocardial infarction. Am J Cardiol 57: 175-177

Gellai M, Norton JM, and Detar R (1973) Evidence for direct control of coronary vascular tone by oxygen. Circ Res 32: 279-289

Geltman EM, Ehsani AA, Campbell MK, Schechtman K, Roberts R, and Sobel BE (1979) The influence of location and extent of myocardial infarction on long-term ventricular dysrhythmia and mortality. Circulation 60: 805-814

Gemmill JD, Hogg KJ, Douglas JT, Dunn FG, Lowe GD, Rae AP, and Hillis WS (1993) The incidence and mechanism of hypotension following thrombolytic therapy for acute myocardial infarction with streptokinase-containing agents--lack of relationship to pretreatment streptokinase resistance. Eur Heart J 14: 819-825

Geng Y, Hansson GK, and Holme E (1992) Interferon-gamma and tumor necrosis factor synergize to induce nitric oxide production and inhibit mitochondrial respiration in vascular smooth muscle cells. Circ Res 71: 1268-1276

Gertz EW, Wisneski JA, Neese R, Houser A, Korte R, and Bristow JD (1980) Myocardial lactate extraction: multi-determined metabolic function. Circulation 61: 256-261

Gertz EW, Wisneski JA, Neese R, Bristow JD, Searle GL, and Hanlon JT (1981) Myocardial lactate metabolism: evidence of lactate release during net chemical extraction in man. Circulation 63: 1273-1279

Gey KF, Puska P, Jordan P, and Moser UK (1991) Inverse correlation between plasma vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. Am J Clin Nutr 53: 326S-334S

Ghio S, de Servi S, Perotti R, Eleuteri E, Montemartini C, and Specchia G (1992) Different susceptibility to the development of nitroglycerin tolerance in the arterial and venous circulation in humans. Effects of N-acetylcysteine administration. Circulation 86: 798-802

Ghio S, Poli A, Ferrario M, Campana C, Diotallevi P, Eleuteri E, Mussini A, Specchia G, and Montemartini C (1994) Haemodynamic effects of glyceryl trinitrate during continuous 24 hour infusion in patients with heart failure. Br Heart J 72: 145-149

Giardina B, Penco M, Lazzarino G, Romano S, Tavazzi B, Fedele F, Di Pierro D, and Dagianti A (1993) Effectiveness of thrombolysis is associated with a time-dependent increase of malondialdehyde in peripheral blood of patients with acute myocardial infarction. Am J Cardiol 71: 788-793

Gibbons RJ, Holmes DR, Reeder GS, Bailey KR, Hopfenspirger MR, and Gersh BJ (1993) Immediate angioplasty compared with the administration of a thrombolytic agent followed by conservative treatment for myocardial infarction. The Mayo Coronary Care Unit and Catheterization Laboratory Groups. N Engl J Med 328: 685-691

Giroud D, Li JM, Urban P, Meier B, and Rutishauer W (1992) Relation of the site of acute myocardial infarction to the most severe coronary arterial stenosis at prior angiography. Am J Cardiol 69: 729-732

GISSI, and Gruppo Italiano Per Lo Studio Della Sopravvivenza Nell'Infarto miocardico. (1990) GISSI-2: A factorial randomisation trial of alteplase versus streptokinase and heparin versus no heparin among 12490 patients with acute myocardial infarction. Lancet 336: 65-71

Go LO, Murry CE, Richard VJ, Weischedel GR, Jennings RB, and Reimer KA (1988) Myocardial neutrophil accumulation during reperfusion after reversible or irreversible ischemic injury. Am J Physiol 255: H1188-98

Goldberg S, Greenspon AJ, Urban PL, Muza B, Berger B, Walinsky P, and Maroko PR (1983) Reperfusion arrhythmia: a marker or restoration of antegrade flow during intracoronary thrombolysis for acute myocardial infarction. Am Heart J 105: 26-32

Goldhaber JI, and Weiss JN (1992) Oxygen free radicals and cardiac reperfusion abnormalities. Hypertension 20: 118-127

Goldhaber JI, Ji S, Lamp ST, and Weiss JN (1989) Effects of exogenous free radicals on electromechanical function and metabolism in isolated rabbit and guinea pig ventricle. Implications for ischemia and reperfusion injury. J Clin Invest 83: 1800-1809

Golino P, Ashton JH, Glas Greenwalt P, McNatt J, Buja LM, and Willerson JT (1988) Mediation of reocclusion by thromboxane A2 and serotonin after thrombolysis with tissue-type plasminogen activator in a canine preparation of coronary thrombosis. Circulation 77: 678-684

Gordon JB, Ganz P, Nabel EG, Fish RD, Zebede J, Mudge GH, Alexander RW, and Selwyn AP (1989) Atherosclerosis influences the vasomotor response of epicardial coronary arteries to exercise. J Clin Invest 83: 1946-1952

Grech ED, Bellamy CM, Jackson MJ, Muirhead RA, Faragher EB, and Ramsdale DR (1994) Free-radical activity after primary coronary angioplasty in acute myocardial infarction. Am Heart J 127: 1443-1449

Grech ED, Dodd NJF, Bellamy CM, Perry RA, Morrison WL, and Ramsdale DR (1993) Free-radical generation during angioplasty reperfusion for acute myocardial infarction. Lancet 341: 990-991

Green MJ, and Hill HA (1984) Chemistry of dioxygen. Methods Enzymol 105: 3-22

Greenberg SM, and Frishman WH (1988) Coenzyme Q10: a new drug for myocardial ischemia? Med Clin North Am 72: 243-258

Gregg DE, and Patterson RE (1980) Functional importance of the coronary collaterals. N Engl J Med 303: 1404-1406

Gressier B, Cabanis A, Lebegue S, Brunet C, Dine T, Luyckx M, Cazin M, and Cazin JC (1993) Comparison of in vitro effects of two thiol-containing drugs on human neutrophils hydrogen peroxide production. Methods Find Exp Clin Pharmacol 15: 101-105

Gressier B, Cabanis A, Lebegue S, Brunet C, Dine T, Luyckx M, Cazin M, and Cazin JC (1994) Decrease of hypochlorous acid and hydroxyl radical generated by stimulated human neutrophils: comparison in vitro of some thiol-containing drugs. Methods Find Exp Clin Pharmacol 16: 9-13

Griendling KK, Minieri CA, Ollerenshaw JD, and Alexander RW (1994) Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res 74: 1141-1148

Grines CL, Bashore TM, Boudoulas H, Olson S, Shafer P, and Wooley CF (1989) Functional abnormalities in isolated left bundle branch block. The effect of interventricular asynchrony. Circulation 79: 845-853

Grines CL, Browne KF, Marco J, Rothbaum D, Stone GW, O'Keefe J, Overlie P, Donohue B, Chelliah N, Timmis GC, and et al (1993) A comparison of immediate angioplasty with thrombolytic therapy for acute myocardial infarction. The Primary Angioplasty in Myocardial Infarction Study Group. N Engl J Med 328: 673-679

Gross GJ, Farber NE, Hardman HF, and Warltier DC (1986) Beneficial actions of superoxide dismutase and catalase in stunned myocardium of dogs. Am J Physiol 250: H372-H377

Grossman W (1986) Evaluation of systolic and diastolic function of the myocardium. In: Grossman W (ed) Cardiac catheterisation and angiography. Lea & Febiger, Philadephia, pp 301-319

Grum CM, Gallagher KP, Kirsh MM, and Shlafer M (1989) Absence of detectable xanthine oxidase in human myocardium. J Mol Cell Cardiol 21: 263-267

Grum CM, Ragsale RA, Ketai LH, and Simon RH (1987) Plasma xanthine oxidase activity in patients with adult respiratory distress syndrome. J Crit Care 2: 22-26

Gruppo Italiano Per Lo Studio Della Streptochinasi Nell'infarto miocardico, and GISSI (1986) Effectiveness of intravenous thrombolytic treatment in acute myocardial infarction. Lancet 327: 397-401

Gryglewski RJ, Palmer RM, and Moncada S (1986) Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature 320: 454-456

Haan MN (1988) Job strain and ischaemic heart disease: an epidemiologic study of metal workers. Ann Clin Res 20: 143-145

Habib GB, Heibig J, Forman SA, Brown BG, Roberts R, Terrin ML, and Bolli R (1991) Influence of coronary collateral vessels on myocardial infarct size in humans. Results of phase I thrombolysis in myocardial infarction (TIMI) trial. The TIMI Investigators. Circulation 83: 739-746

Hackel DB, Reimer KA, Ideker RE, Mikat EM, Hartwell TD, Parker CB, Braunwald EB, Buja M, Gold HK, Jaffe AS, and et al (1984) Comparison of enzymatic and anatomic estimates of myocardial infarct size in man. Circulation 70: 824-835

Hackett D, Davies G, Chierchia S, and Maseri A (1987) Intermittent coronary occlusion in acute myocardial infarction. Value of combined thrombolytic and vasodilator therapy. N Engl J Med 317: 1055-1059

Hackett JG, Abboud FM, Mark AL, Schmid PG, and Heistad DD (1972) Coronary vascular responses to stimulation of chemoreceptors and baroreceptors: evidence for reflex activation of vagal cholinergic innervation. Circ Res 31: 8-17

Hackett D, McKenna W, Davies G, and Maseri A (1990) Reperfusion arrhythmias are rare during acute myocardial infarction and thrombolysis in man. Int J Cardiol 29: 205-213

Hale SL, Lange R, Alker KJ, and Kloner RA (1984) Correlates of reperfusion ventricular fibrillation in dogs. Am J Cardiol 53: 1397-1400

Halliwell B, and Gutteridge JM (1990) Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 186: 1-85

Halliwell B, Gutteridge JMC, and Cross CE (1992) Free radicals, antioxidants, and human disease: where are we now? J Lab Clin Med 119: 598-620

Hanich RF, Levine JH, Prood C, Weiss JL, Callans DJ, Spear JF, and Moore EN (1993) Electrophysiologic recovery in postischemic, stunned myocardium despite persistent systolic dysfunction. Am Heart J 125: 23-32

Hansen PR (1995) Role of neutrophils in myocardial ischemia and reperfusion. Circulation 91: 1872-1885

Harrison DG, and Bates JN (1993) The nitrovasodilators. New ideas about old drugs. Circulation 87: 1461-1467

Headrick JP, Armiger LC, and Willis RJ (1990) Behaviour of energy metabolites and effect of allopurinol in the "stunned" isovolumic rat heart. J Mol Cell Cardiol 22: 1107-1116

Hearse DJ (1977) Reperfusion of the ischemic myocardium. J Mol Cell Cardiol 9: 605-616

Hearse DJ, and Tosaki A (1987) Free radicals and reperfusion-induced arrhythmias: protection by spin trap agent PBN in the rat heart. Circ Res 60: 375-383

Hearse DJ, Kusama Y, and Bernier M (1989) Rapid electrophysiological changes leading to arrhythmias in the aerobic rat heart. Photosensitization studies with rose bengal-derived reactive oxygen intermediates. Circ Res 65: 146-153

Hegstad AC, Ytrehus K, Myklebust R, and Jorgensen L (1994a) Ultrastructural changes in the myocardial myocytic mitochondria: crucial step in the development of oxygen radical-induced damage in isolated rat hearts? Basic Res Cardiol 89: 128-138

Hegstad AC, Strand H, and Ytrehus K (1994b) Phospholipid peroxidation after 60 min of global ischaemia and 10 min of reperfusion. A study in the isolated rat heart. J Mol Cell Cardiol 26: 569-578

Heikkila J, and Nieminen MS (1984) Effects of verapamil in patients with acute myocardial infarction: hemodynamics and function of normal and ischemic left ventricular myocardium. Am Heart J 107: 241-247

Hempel SL, Haycraft DL, Hoak JC, and Spector AA (1990) Reduced prostacyclin formation after reoxygenation of anoxic endothelium. Am J Physiol 259: C738-45

Henry PJ, Horowitz JD, and Louis WJ (1989b) Determinants of in vitro nitroglycerin tolerance induction and reversal: influence of dose regimen, nitrate-free period, and sulfhydryl supplementation. J Cardiovasc Pharmacol 14: 31-37

Henry PJ, Drummer OH, and Horowitz JD (1989a) S-nitrosothiols as vasodilators: implications regarding tolerance to nitric oxide-containing vasodilators. Br J Pharmacol 98: 757-766

Herlitz J, Elmfeldt D, Holmberg S, Malek I, Nyberg G, Pennert K, Ryden L, Swedberg K, Vedin A, Waagstein F, and et al (1984) Goteborg Metoprolol Trial: mortality and causes of death. Am J Cardiol 53: 9D-14D

Herlitz J, Hartford M, Aune S, and Karlsson T (1993) Occurrence of hypotension during streptokinase infusion in suspected acute myocardial infarction, and its relation to prognosis and metoprolol therapy. Am J Cardiol 71: 1021-1024

Hertog MG, Feskens EJ, Hollman PC, Katan MB, and Kromhout D (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342: 1007-1011

Hicks M, Wong LS, and Day RO (1993a) Identification of products from oxidation of uric acid induced by hydroxyl radicals. Free Radic Res Commun 18: 337-351

Hicks M, Wong LS, and Day RO (1993b) Suitability of reverse-phase columns from different sources for separation of uric acid from its oxidation products. Anal Biochem 210: 428-430

Higginbotham MB, Morris KG, Coleman RE, and Cobb FR (1986) Comparison of nifedipine alone with propranolol alone for stable angina pectoris including hemodynamics at rest and during exercise. Am J Cardiol 57: 1022-1028

Higgins CB, Vatner SF, and Braunwald E (1973) Parasympathetic control of the heart. Pharmacol Rev 25: 119-155

Higginson LA, White F, Heggtveit HA, Sanders TM, Bloor CM, and Covell JW (1982) Determinants of myocardial hemorrhage after coronary reperfusion in the anesthetized dog. Circulation 65: 62-69

Hildebrandt P, Torp Pedersen C, Joen T, Iversen E, Jensen G, Jeppesen D, Melchior T, Schytten HJ, Ringsdal V, Jensen J, and et al (1992) Reduced infarct size in nonreperfused myocardial infarction by combined infusion of isosorbide dinitrate and streptokinase. Am Heart J 124: 1139-1144

Hill KE, and Burk RF (1984) Influence of vitamin E and selenium on glutathione-dependent protection against microsomal lipid peroxidation. Biochem Pharmacol 33: 1065-1068

Hillis LD, and Braunwald E (1977) Myocardial ischemia (first of three parts). N Engl J Med 296: 971-978

Hindman NB, Schocken DD, Widmann M, Anderson WD, White RD, Leggett S, Ideker RE, Hinohara T, Selvester RH, and Wagner GS (1985) Evaluation of a QRS scoring system for estimating myocardial infarct size. V. Specificity and method of application of the complete system. Am J Cardiol 55: 1485-1490

Hirzel HO, Senn M, Nuesch K, Buettner C, Pfeiffer A, Hess OM, and Krayenbuehl HP (1984) Thallium-201 scintigraphy in complete left bundle branch block. Am J Cardiol 53: 764-769

Ho SW, and Beilin LJ (1983) Asthma associated with N-acetylcysteine infusion and paracetamol poisoning: report of two cases. Br Med J Clin Res Ed 287: 876-877

Hochstein P, Hatch L, and Sevanian A (1984) Uric acid: functions and determination. Methods Enzymol 105: 162-166

Hofmann M, Genth K, and Schaper W (1980) The influence of reperfusin on infarct size after experimental coronary artery occlusion. Basic Res Cardiol 75: 572-582

Hogan JC, Lewis MJ, and Henderson AH (1990) Chronic administration of N-acetylcysteine fails to prevent nitrate tolerance in patients with stable angina pectoris. Br J Clin Pharmacol 30: 573-577

Holdiness MR (1991) Clinical pharmacokinetics of N-acetylcysteine. Clin Pharmacokinet 20: 123-134

Holley AE, and Cheeseman KH (1993) Measuring free radical reactions in vivo. Br Med Bull 49: 494-505

Holley AE, Walker MK, Cheeseman KH, and Slater TF (1993) Measurement of n-alkanals and hydroxyalkenals in biological samples. Free Radic Biol Med 15: 281-289

Holman RL, McGill HC, Strong JP, and Geer JC (1958) The natural history of atherosclerosis. The early aortic lesions as seen in New Orleans in the middle of the 20th century. Am J Pathol 34: 209-235

Holman BL, Chisholm RJ, and Braunwald E (1978) The prognostic implications of acute myocardial infarct scintigraphy with 99mTc-pyrophosphate. Circulation 57: 320-326

Honan MB, Harrell FE, Reimer KA, Califf RM, Mark DB, Pryor DB, and Hlatky MA (1990) Cardiac rupture, mortality and the timing of thrombolytic therapy: a meta-analysis. J Am Coll Cardiol 16: 359-367

Horie M, Yasue H, Omote S, Takizawa A, Nagao M, Nishida S, and Kubota J (1986) A new approach for the enzymatic estimation of infarct size: serum peak creatine kinase and time to peak creatine kinase activity. Am J Cardiol 57: 76-81

Horowitz JD, Dynon MK, Woodward E, Sia ST, Macdonald PS, Morgan DJ, Goble AJ, and Louis WJ (1986a) Short-term myocardial uptake of lidocaine and mexiletine in patients with ischemic heart disease. Circulation 73: 987-996

Horowitz JD, Antman EM, Lorell BH, Barry WH, and Smith TW (1983) Potentiation of the cardiovascular effects of nitroglycerin by N-acetylcysteine. Circulation 68: 1247-1253

Horowitz JD, Sia ST, Macdonald PS, Goble AJ, and Louis WJ (1986b) Perhexiline maleate treatment for severe angina pectoris--correlations with pharmacokinetics. Int J Cardiol 13: 219-229

Horowitz JD, Henry CA, Syrjanen ML, Louis WJ, Fish RD, Smith TW, and Antman EM (1988) Combined use of nitroglycerin and N-acetylcysteine in the management of unstable angina pectoris. Circulation 77: 787-794

Horowitz JD (1991) Thiol-containing agents in the management of unstable angina pectoris and acute myocardial infarction. Am J Med 91 [suppl 3C]: 113S-117S

Horowitz JD, Button IK, Wing L (1995) Is perhexiline essential for the optimal management of angina pectoris? Aust N Z J Med (in press)

Horowitz JD, and Henry PJ (1987) Recent developments in nitrate therapy of ischaemic heart disease. Med J Aust 146: 93-96

Horowitz JD, and Mashford ML (1979) Perhexiline maleate in the treatment of severe angina pectoris. Med J Aust 1: 485-488

Horwitz LD, Fennessey PV, Shikes RH, and Kong Y (1994) Marked reduction in myocardial infarct size due to prolonged infusion of an antioxidant during reperfusion. Circulation 89: 1792-1801

Huber AR, Kunkel SL, Todd RF, 3d, and Weiss SJ (1991) Regulation of transendothelial neutrophil migration by endogenous interleukin-8 [published errata appear in Science 1991 Nov 1;254(5032):631 and 1991 Dec 6;254(5037):1435]. Science 254: 99-102

Hubert HB, Feinleib M, McNamara PM, and Castelli WP (1983) Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. Circulation 67: 968-977

Ideker RE, Wagner GS, Ruth WK, Alonso DR, Bishop SP, Bloor CM, Fallon JT, Gottlieb GJ, Hackel DB, Phillips HR, Reimer KA, Roark SF, Rogers WJ, Savage RM, White RD, and Selvester RH (1982) Evaluation of a QRS scoring system for estimating myocardial infarct size. II. Correlation with quantitative anatomic findings for anterior infarcts. Am J Cardiol 49: 1604-1614

Ignarro LJ, and Gruetter CA (1980) Requirement of thiols for activation of coronary arterial guanylate cyclase by glyceryl trinitrate and sodium nitrite: possible involvement of S-nitrosothiols. Biochim Biophys Acta 631: 221-231

Ingold KU, Bowry VW, Stocker R, and Walling C (1993) Autoxidation of lipids and antioxidation by alpha-tocopherol and ubiquinol in homogeneous solution and in aqueous dispersions of lipids: unrecognized consequences of lipid particle size as exemplified by oxidation of human low density lipoprotein. Proc Natl Acad Sci U S A 90: 45-49

Ingwall JS, Kramer MF, Fifer MA, Lorell BH, Shemin R, Grossman W, and Allen PD (1985) The creatine kinase system in normal and diseased human myocardium. N Engl J Med 313: 1050-1054

Isakov A, Shapira I, Burke M, and Almog C (1988) Serum myoglobin levels in patients with ischemic myocardial insult. Arch Intern Med 148: 1762-1765

Ishikawa T, and Sies H (1984) Cardiac transport of glutathione disulfide and S-conjugate. Studies with isolated perfused rat heart during hydroperoxide metabolism. J Biol Chem 259: 3838-3843

ISIS-2 (Second International Study of Infarct Survival) collaborative group (1988) Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17187 cases of suspected acute myocardial infarction. Lancet 332: 349-360

ISIS-3, and Third International Study of Infarct Survival Collaborative group. (1992) ISIS-3: A randomisation comparison of streptokinase vs tissue plasminogen activator vs anistreplase and of aspirin plus heparin vs aspirin alone among 41299 cases of suspected acute myocardial infarction. Lancet 339: 753-770

ISIS-1 Collaborative Group (1986) Randomised trial of intravenous atenolol among 16 027 cases of suspected acute myocardial infarction: ISIS-1. First International Study of Infarct Survival Collaborative Group. Lancet 2: 57-66

Isobe M, Nagai R, Yamaoki K, Nakaoka H, Takaku F, and Yazaki Y (1989) Quantification of myocardial infarct size after coronary reperfusion by serum cardiac myosin light chain II in conscious dogs. Circ Res 65: 684-694

Ito H, Tomooka T, Sakai N, Higashino Y, Fujii K, Katoh O, Masuyama T, Kitabatake A, and Minamino T (1993) Time course of functional improvement in stunned myocardium in risk area in patients with reperfused anterior infarction. Circulation 87: 355-362

Ito H, Tomooka T, Sakai N, Yu H, Higashino Y, Fujii K, Masuyama T, Kitabatake A, and Minamino T (1992) Lack of myocardial perfusion immediately after successful thrombolysis. A predictor of poor recovery of left ventricular function in anterior myocardial infarction. Circulation 85: 1699-1705

Iversen HK (1992) N-acetylcysteine enhances nitroglycerin-induced headache and cranial arterial responses. Clin Pharmacol Ther 52: 125-133

Jaffe AS, Serota H, Grace A, and Sobel BE (1986) Diagnostic changes in plasma creatine kinase isoforms early after the onset of acute myocardial infarction. Circulation 74: 105-109

Jandak J, Steiner M, and Richardson PD (1989) Alpha-tocopherol, an effective inhibitor of platelet adhesion. Blood 73: 141-149

Janoff A (1985) Elastase in tissue injury. Annu Rev Med 36: 207-216

Janssen M, Koster JF, Bos E, and de Jong JW (1993) Malondialdehyde and glutathione production in isolated perfused human and rat hearts. Circ Res 73: 681-688

Janzen EG (1984) Spin trapping. Methods Enzymol 105: 188-198

Jarasch ED, Bruder G, and Heid HW (1986) Significance of xanthine oxidase in capillary endothelial cells. Acta Physiol Scand Suppl 548: 39-46

Jennings RB, and Reimer KA (1981) Lethal myocardial ischemic injury. Am J Pathol 102: 241-255

Jennings RB, Murry CE, Steenbergen C, Jr., and Reimer KA (1990) Development of cell injury in sustained acute ischemia. Circulation 82: II2-12

Jensen T, Kharazmi A, Schiotz PO, Nielsen H, Stenvang Pedersen S, Stafanger G, Koch C, and Hoiby N (1988) Effect of oral N-acetylcysteine administration on human blood neutrophil and monocyte function. APMIS 96: 62-67

Jeremy RW, Links JM, and Becker LC (1990) Progressive failure of coronary flow during reperfusion of myocardial infarction: documentation of the no reflow phenomenon with positron emission tomography. J Am Coll Cardiol 16: 695-704

Ji LL, Fu RG, Waldrop TG, Liu KJ, and Swartz HM (1993) Myocardial response to regional ischemia and reperfusion in vivo in rat heart. Can J Physiol Pharmacol 71: 811-817

Johansson M, and Lenngren S (1988) Determination of cysteine, glutathione and N-acetylcysteine in plasma by ion-pair reversed-phase liquid chromatography and post-column derivatization. J Chromatogr 432: 65-74

Johnson G, Tsao P, and Lefer AM (1990) Synergism between superoxide dismutase and sodium nitrite in cardioprotection following ischemia and reperfusion. Am Heart J 119: 530-537

Johnson WB, Malone SA, Pantely GA, Anselone CG, and Bristow JD (1988) No reflow and extent of infarction during maximal vasodilation in the porcine heart. Circulation 78: 462-472

Johnson WD, Kayser KL, Brenowitz JB, and Saedi SF (1991) A randomized controlled trial of allopurinol in coronary bypass surgery. Am Heart J 121: 20-24

Jolly SR, Kane WJ, Bailie MB, Abrams GD, and Lucchesi BR (1984) Canine myocardial reperfusion injury. Its reduction by the combined administration of superoxide dismutase and catalase. Circ Res 54: 277-285

Joseph A, Ackerman D, Talley JD, Johnstone J, and Kupersmith J (1993) Manifestations of coronary atherosclerosis in young trauma victims--an autopsy study. J Am Coll Cardiol 22: 459-467

Jugdutt BI, and Warnica JW (1988) Intravenous nitroglycerin therapy to limit myocardial infarct size, expansion, and complications. Effect of timing, dosage, and infarct location [published erratum appears in Circulation 1989 May;79(5):1151]. Circulation 78: 906-919

Jugdutt BI (1991) Intravenous nitroglycerin unloading in acute myocardial infarction. Am J Cardiol 68: 52D-63D

Juilliere Y, Danchin N, Grentzinger A, Suty Selton C, Lethor JP, Courtalon T, Pernot C, and Cherrier F (1990) Role of previous angina pectoris and collateral flow to preserve left ventricular function in the presence or absence of myocardial infarction in isolated total occlusion of the left anterior descending coronary artery. Am J Cardiol 65: 277-281

Kaijser L, and Berglund B (1992) Myocardial lactate extraction and release at rest and during heavy exercise in healthy men. Acta Physiol Scand 144: 39-45

Kamp O, Beatt KJ, De Feyter PJ, van den Brand M, Suryapranata H, Luijten HE, and Serruys PW (1989) Short-, medium-, and long-term follow-up after percutaneous transluminal coronary angioplasty for stable and unstable angina pectoris. Am Heart J 117: 991-996

Karagounis L, Sorensen SG, Menlove RL, Moreno F, and Anderson JL (1992) Does thrombolysis in myocardial infarction (TIMI) perfusion grade 2 represent a mostly patent artery or a mostly occluded artery? Enzymatic and electrocardiographic evidence from the TEAM-2 study. Second Multicenter Thrombolysis Trial of Eminase in Acute Myocardial Infarction. J Am Coll Cardiol 19: 1-10

Keaney JF, Jr., Simon DI, Stamler JS, Jaraki O, Scharfstein J, Vita JA, and Loscalzo J (1993) NO forms an adduct with serum albumin that has endothelium-derived relaxing factor-like properties. J Clin Invest 91: 1582-1589

Keen JH, Habig WH, and Jakoby WB (1976) Mechanism for the several activities of the glutathione S-transferases. J Biol Chem 251: 6183-6188

Kenkare SR, Han C, and Benet LZ (1994) Correlation of the response to nitroglycerin in rabbit aorta with the activity of the mu class glutathione S-transferase. Biochem Pharmacol 48: 2231-2235

Khaja F, Walton JA, Jr., Brymer JF, Lo E, Osterberger L, O'Neill WW, Colfer HT, Weiss R, Lee T, Kurian T, Goldberg AD, Pitt B, and Goldstein S (1983) Intracoronary fibrinolytic therapy in acute myocardial infarction. Report of a prospective randomized trial. N Engl J Med 308: 1305-1311

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

Khurmi NS, and Raftery EB (1987) A comparison of nine calcium ion antagonists and propranolol: exercise tolerance, heart rate and ST-segment changes in patients with chronic stable angina pectoris. Eur J Clin Pharmacol 32: 539-548

King SB, and Schlumpf M (1993) Ten-year completed follow-up of percutaneous transluminal coronary angioplasty: the early Zurich experience. J Am Coll Cardiol 22: 353-360

Kingma JH, van Gilst WH, Peels CH, Dambrink JH, Verheugt FW, and Wielenga RP (1994) Acute intervention with captopril during thrombolysis in patients with first anterior myocardial infarction. Results from the Captopril and Thrombolysis Study (CATS). Eur Heart J 15: 898-907

Kingma JGJ, and Rouleau JR (1989) Effect of N-acetylcysteine on tissue necrosis during acute myocardial infarction in rabbits. Can J Cardiol 5: 321-326

Kirshenbaum LA, Thomas TP, Randhawa AK, and Singal PK (1992) Time-course of cardiac myocyte injury due to oxidative stress. Mol Cell Biochem 111: 25-31

Klebanoff SJ (1980) Oxygen metabolism and the toxic properties of phagocytes. Ann Intern Med 93: 480-489

Kleber AG, Janse MJ, van Capelle FJL, and Durrer D (1978) Mechanism and time course of S-T and T-Q segment changes during acute regional ischemia in the pig heart determined by extracellular and intracellular recordings. Circ Res 42: 603-613

Klein HH, Puschmann S, Schaper J, and Schaper W (1981) The mechanism of the tetrazolium reaction in identifying experimental myocardial infarction. Virchows Archiv A 393: 287-297

Klocke FJ, Braunwald E, and Ross J, Jr. (1966) Oxygen cost of electrical activation of the heart. Circ Res 18: 357-365

Kloner RA, Ganote CE, Whalen DA, Jr., and Jennings RB (1974b) Effect of a transient period of ischemia on myocardial cells. II. Fine structure during the first few minutes of reflow. Am J Pathol 74: 399-422

Kloner RA (1993) Does reperfusion injury exist in humans? J Am Coll Cardiol 21: 537-545

Kloner RA, Rude RE, Carlson N, Maroko PR, DeBoer LW, and Braunwald E (1980) Ultrastructural evidence of microvascular damage and myocardial cell injury after coronary artery occlusion: which comes first? Circulation 62: 945-952

Kloner RA, Ganote CE, and Jennings RB (1974a) The "no-reflow" phenomenon after temporary coronary occlusion in the dog. J Clin Invest 54: 1496-1508

Knight JA, Smith SE, Kinder VE, and Anstall HB (1987) Reference intervals for plasma lipoperoxides: age-, sex-, and specimen-related variations. Clin Chem 33: 2289-2291

Knutsson A, Akerstedt T, Jonsson BG, and Orth Gomer K (1986) Increased risk of ischaemic heart disease in shift workers. Lancet 2: 89-92

Koerner JE, Anderson BA, and Dage RC (1991) Protection against postischemic myocardial dysfunction in anesthetized rabbits with scavengers of oxygen-derived free radicals: superoxide dismutase plus catalase, N-2-mercaptopropionyl glycine and captopril. J Cardiovasc Pharmacol 17: 185-191

Kondo M, Tamura K, Tanio H, and Shimono Y (1993) Is ST segment re-elevation associated with reperfusion an indicator of marked myocardial damage after thrombolysis? J Am Coll Cardiol 21: 62-67

Koppenol WH, Moreno JJ, Pryor WA, Ischiropoulos H, and Beckman JS (1992) Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. Chem Res Toxicol 5: 834-842

Koppenol WH (1976) Reactions involving singlet oxygen and the superoxide anion. Nature 262: 420-421

Koren G, Weiss AT, Ben-David Y, Hasin Y, Luria MH, and Gotsman MS (1986) Bradycardia and hypotension following reperfusion with streptokinase (Bezold-Jarisch reflex): a sign of coronary thrombolysis and myocardial salvage. Am Heart J 112: 468-471

Kowaluk EA, Seth P, and Fung HL (1992) Metabolic activation of sodium nitroprusside to nitric oxide in vascular smooth muscle. J Pharmacol Exp Ther 262: 916-922

Kramer JH, Mak IT, and Weglicki WB (1984) Differential sensitivity of canine cardiac sarcolemmal and microsomal enzymes to inhibition by free radical-induced lipid peroxidation. Circ Res 55: 120-124

Krause SM, Jacobus WE, and Becker LC (1989) Alterations in cardiac sarcoplasmic reticulum calcium transport in the postischemic "stunned" myocardium. Circ Res 65: 526-530

Krien PM, Margou V, and Kermici M (1992) Electrochemical determination of fentomole amounts of free reduced and oxidized glutathione. Application to human hair follicles. J Chromatogr 576: 255-261

Krucoff MW, Croll MA, Pope JE, Granger CB, O'Connor CM, Sigmon KN, Wagner BL, Ryan JA, Lee KL, Kereiakes DJ, Samaha JK, Worley SJ, Ellis SG, Wall TC, Topol EJ, Califf RM, and TAMI 7 Study Group (1993a) Continuous 12-lead ST-segment recovery analysis in the TAMI 7 study. Performance of a non-invasive method for real-time detection of failed myocardial reperfusion. Circulation 88: 437-446

Krucoff MW, Green CE, Satler LF, Miller FC, Pallas RS, Kent KM, Del Negro AA, Pearle DL, Fletcher RD, and Rackley CE (1986) Noninvasive detection of coronary artery patency using continuous ST-segment monitoring. Am J Cardiol 57: 916-922

Krucoff MW, Croll MA, Pope JE, Pieper KS, Kanini PM, Granger CB, Veldkamp RF, Wagner BL, Sawchak ST, and Califf RM (1993b) Continuously updated 12-lead ST-segment recovery analysis for myocardial infarct artery patency assessment and its correlation with simultaneous early angiographic observations. Am J Cardiol 71: 145-151

Ku DD (1982) Coronary vascular reactivity after acute myocardial ischemia. Science 218: 576-578

Kubes P, Suzuki M, and Granger DN (1991) Nitric oxide: an endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci U S A 88: 4651-4655

Kubota I, Yamaki M, Shibata T, Ikeno E, Hosoya Y, and Tomoike H (1993) Role of ATP-sensitive K^+ channel on ECG ST segment elevation during a bout of myocardial ischemia. A study on epicardial mapping in dogs. Circulation 88: 1845-1851

Kukielka GL, Hawkins HK, Michael L, Manning AM, Youker K, Lane C, Entman ML, Smith CW, and Anderson DC (1993) Regulation of intercellular adhesion molecule-1 (ICAM-1) in ischemic and reperfused canine myocardium. J Clin Invest 92: 1504-1516

Kurose I, Wolf R, Grisham MB, and Granger DN (1994b) Modulation of ischemia/reperfusion-induced microvascular dysfunction by nitric oxide. Circ Res 74: 376-382

Kurose I, Anderson DC, Miyasaka M, Tamatani T, Paulson JC, Todd RF, Rusche JR, and Granger DN (1994a) Molecular determinants of reperfusion-induced leukocyte adhesion and vascular protein leakage. Circ Res 74: 336-343

Kusuoka H, and Marban E (1992) Cellular mechanisms of myocardial stunning. Annu Rev Physiol 54: 243-256

Kwon K, Freedman SB, Wilcox I, Allman K, Madden A, Carter GS, and Harris PJ (1991) The unstable ST segment early after thrombolysis for acute myocardial infarction and its usefulness as a marker of recurrent coronary occlusion. Am J Cardiol 67: 109-115

Kyriakides ZS, Kremastinos DT, Kolettis TM, Livanis E, Apostolou T, Michelakakis N, and Toutouzas P (1993) Short-term effects of atrial versus atrio-ventricular pacing on myocardial ischaemia in coronary artery disease patients. Eur Heart J 14: 607-613

Lakier JB (1992) Smoking and cardiovascular disease. Am J Med 93: 8S-12S

Lakka TA, Venalainen JM, Rauramaa R, Salonen R, Tuomilehto J, and Salonen JT (1994) Relation of leisure-time physical activity and cardiorespiratory fitness to the risk of acute myocardial infarction. N Engl J Med 330: 1549-1554

Lamas S, Marsden PA, Li GK, Tempst P, and Michel T (1992) Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proc Natl Acad Sci U S A 89: 6348-6352

Lang JK, Gohil K, and Packer L (1986) Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. Anal Biochem 157: 106-116

Lange RA, and Hillis LD (1993) Immediate angioplasty for acute myocardial infarction. N Engl J Med 328: 726-728

Largilliere C, and Melancon SB (1988) Free malondialdehyde determination in human plasma by high-performance liquid chromatography. Anal Biochem 170: 123-126

Lassila R (1993) Inflammation in atheroma: implications for plaque rupture and platelet-collagen interaction. Eur Heart J 14 Suppl K: 94-97

Laurindo FR, da Luz PL, Uint L, Rocha TF, Jaeger RG, and Lopes EA (1991) Evidence for superoxide radical-dependent coronary vasospasm after angioplasty in intact dogs. Circulation 83: 1705-1715

Lauterburg BH, Smith CV, Hughes H, and Mitchell JR (1984) Biliary excretion of glutathione and glutathione disulfide in the rat. Regulation and response to oxidative stress. J Clin Invest 73: 124-133

Lawrence GD, and Cohen G (1984) Concentrating ethane from breath to monitor lipid peroxidation in vivo. Methods Enzymol 105: 305-311

Lazzarino G, Di Pierro D, Tavazzi B, Cerroni L, and Giardina B (1991) Simultaneous separation of malondialdehyde, ascorbic acid, and adenine nucleotide derivatives from biological samples by ion-pairing high-performance liquid chromatography. Anal Biochem 197: 191-196

Lazzarino G, Raatikainen P, Nuutinen M, Nissinen J, Tavazzi B, Di Pierro D, Giardina B, and Peuhkurinen K (1994) Myocardial release of malondialdehyde and purine compounds during coronary bypass surgery. Circulation 90: 291-297

Lee KJ, Horowitz JD, McKay J, and Goble AJ (1988) Myocardial salvage with streptokinase combined with nitroglycerine and verapamil in acute myocardial infarction. Int J Cardiol 21: 279-291

Lee TH, and Goldman L (1986) Serum enzyme assays in the diagnosis of acute myocardial infarction. Recommendations based on a quantitative analysis. Ann Intern Med 105: 221-233

Lefer DJ, Nakanishi K, Johnston WE, and Vinten Johansen J (1993) Antineutrophil and myocardial protecting actions of a novel nitric oxide donor after acute myocardial ischemia and reperfusion of dogs. Circulation 88: 2337-2350

Lefer AM, Weyrich AS, and Buerke M (1994) Role of selectins, a new family of adhesion molecules, in ischaemia-reperfusion injury. Cardiovasc Res 28: 289-294

Lefer AM, and Lefer DJ (1993) Pharmacology of the endothelium in ischemia-reperfusion and circulatory shock. Annu Rev Pharmacol Toxicol 33: 71-90

Leger JO, Larue C, Ming T, Calzolari C, Gautier P, Mouton C, Grolleau R, Louisot P, Puech P, Peperstraete B, and et al (1990) Assay of serum cardiac myosin heavy chain fragments in patients with acute myocardial infarction: determination of infarct size and long-term follow-up. Am Heart J 120: 781-790

Lehr HA, Guhlmann A, Nolte D, Keppler D, and Messmer K (1991) Leukotrienes as mediators in ischemia-reperfusion injury in a microcirculation model in the hamster. J Clin Invest 87: 2036-2041

Lendon CL, Davies MJ, Born GV, and Richardson PD (1991) Atherosclerotic plaque caps are locally weakened when macrophages density is increased. Atherosclerosis 87: 87-90

Levin DC (1974) Pathways and functional significance of the coronary collateral circulation. Circulation 50: 831-837

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, and Stadtman ER (1990) Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 186: 464-478

Levy D, Wilson PW, Anderson KM, and Castelli WP (1990) Stratifying the patient at risk from coronary disease: new insights from the Framingham Heart Study. Am Heart J 119: 712-717

Lew AS, Laramee P, Cercek B, Shah P, and Ganz W (1985) The hypotensive effect of intravenous streptokinase in patients with acute myocardial infarction. Circulation 72: 1321-1326

Lewis HD, Jr., Davis JW, Archibald DG, Steinke WE, Smitherman TC, Doherty JE, 3d, Schnaper HW, LeWinter MM, Linares E, Pouget JM, Sabharwal SC, Chesler E, and DeMots H (1983) Protective effects of aspirin against acute myocardial infarction and death in men with unstable angina. Results of a Veterans Administration Cooperative Study. N Engl J Med 309: 396-403

Lim MJ, Gallagher MA, Ziadeh M, and Buda AJ (1991) Effect of coronary reocclusion after initial reperfusion on ventricular function and infarct size. J Am Coll Cardiol 18: 879-885

Lincoff AM, and Topol EJ (1993) Illusion of reperfusion. Does anyone achieve optimal reperfusion during acute myocardial infarction? Circulation 87: 1792-1805

Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, Loscalzo J, Singel DJ, and Stamler JS (1993) A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature 364: 626-632

Liu P, Houle S, Burns RJ, Kimball B, Warbick Cerone A, Johnston L, Gilday D, Weisel RD, and McLaughlin PR (1985) Effect of intracoronary nitroglycerin on myocardial blood flow and distribution in pacing-induced angina pectoris. Quantitative assessment by single-photon emission tomography. Am J Cardiol 55: 1270-1276

Loeper J, Goy J, Rozensztajn L, Bedu O, and Moisson P (1991) Lipid peroxidation and protective enzymes during myocardial infarction. Clin Chim Acta 196: 119-126

Lombardo TA, Rose L, Taeschler M, Tuluy S, and Bing RJ (1953) The effect of exercise on coronary blood flow, myocardial oxygen consumption and cardiac efficiency in man. Circulation 7: 71-78

Loop FD, Lytle BW, Cosgrove DM, Stewart RW, Goormastic M, Williams GW, Golding LA, Gill CC, Taylor PC, Sheldon WC, and et al (1986) Influence of the internal-mammary-artery graft on 10-year survival and other cardiac events. N Engl J Med 314: 1-6

Lopez-Farre A, Caramelo C, Esterban A, Alberola ML, Millas I, Monton M, and Casado S (1995) Effects of aspirin on platelet-neutrophil interactions. Role of nitric oxide and endothelin-1. Circulation 91: 2080-2088

Lorenz RL, Schacky CV, Weber M, Meister W, Kotzur J, Reichardt B, Theisen K, and Weber PC (1984) Improved aortocoronary bypass patency by low-dose aspirin (100 mg daily). Effects on platelet aggregation and thromboxane formation. Lancet 1: 1261-1264

Loscalzo J (1985) N-acetylcysteine potentiates inhibition of platelet aggregation by nitroglycerin. J Clin Invest 76: 703-708

Lowe JE, Reimer KA, and Jennings RB (1978) Experimental infarct size as a function of the amount of myocardium at risk. Am J Pathol 90: 363-379

Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, and Ganz P (1986) Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Engl J Med 315: 1046-1051

Luscher TF (1994) Endothelin, endothelin receptors, and endothelin antagonists. Curr Opin Nephrol Hypertens 3: 92-98

Ma XL, Tsao PS, and Lefer AM (1991) Antibody to CD-18 exerts endothelial and cardiac protective effects in myocardial ischemia and reperfusion. J Clin Invest 88: 1237-1243

Ma XL, Weyrich AS, Lefer DJ, and Lefer AM (1993) Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. Circ Res 72: 403-412

Mabin TA, Holmes DR, Jr., Smith HC, Vlietstra RE, Reeder GS, Bresnahan JF, Bove AA, Hammes LN, Elveback LR, and Orszulak TA (1985) Follow-up clinical results in patients undergoing percutaneous transluminal coronary angioplasty. Circulation 71: 754-760

MacIsaac AI, Thomas JD, and Topol EJ (1993) Toward the quiescent coronary plaque. J Am Coll Cardiol 22: 1228-1241

Madias JE, Venkataraman K, and Hood WB (1975) Precordial ST-segment mapping. 1. Clinical studies in the coronary care unit. Circulation 52: 799-809

Maggioni AP, Franzosi MG, Santoro E, White HD, Van de Werf F, Tognoni G, GISSI-2, and the International Study Group (1992) The risk of stoke in patients with acute myocardial infarction after thrombolytic and antithrombotic treatment. N Engl J Med 327: 1-6

Marban E, Kitakaze M, Koretsune Y, Yue DT, Chacko VP, and Pike MM (1990) Quantification of [Ca2+]i in perfused hearts. Critical evaluation of the 5F-BAPTA and nuclear magnetic resonance method as applied to the study of ischemia and reperfusion. Circ Res 66: 1255-1267

Marban E, Koretsune Y, Corretti M, Chacko VP, and Kusuoka H (1989) Calcium and its role in myocardial cell injury during ischemia and reperfusion. Circulation 80: IV17-22

Marcus ML, Chilian WM, Kanatsuka H, Dellsperger KC, Eastham CL, and Lamping KG (1990) Understanding the coronary circulation through studies at the microvascular level. Circulation 82: 1-7

Markis JE, Malagold M, Parker JA, Silverman KJ, Barry WH, Als AV, Paulin S, Grossman W, and Braunwald E (1981) Myocardial salvage after intracoronary thrombolysis with streptokinase in acute myocardial infarction. N Engl J Med 305: 777-782

Maseri A (1991) Coronary vasoconstriction: visible and invisible [editorial; comment]. N Engl J Med 325: 1579-1580

Matetzky S, Barabash GI, Shahar A, Rabinowitz B, Rath S, Zahav YH, Agranat O, Kaplinsky E, and Hod H (1994) Early T wave inversion after thrombolytic therapy predicts better coronary perfusion: clinical and angiographic study. J Am Coll Cardiol 24: 378-383

Maupoil V, and Rochette L (1988) Evaluation of free radical and lipid peroxide formation during global ischemia and reperfusion in isolated perfused rat heart. Cardiovasc Drugs Ther 2: 615-621

Maupoil V, Rochette L, Tabard A, Clauser P, and Harpey C (1990) Evolution of free radical formation during low-flow ischemia and reperfusion in isolated rat heart. Cardiovasc Drugs Ther 4: 791-796

May DC, Popma JJ, Black WH, Schaefer S, Lee HR, Levine BD, and Hillis LD (1987) In vivo induction and reversal of nitroglycerin tolerance in human coronary arteries. N Engl J Med 317: 805-809

Maza SR, and Frishman WH (1988) Therapeutic options to minimize free radical damage and thrombogenicity in ischemic/reperfused myocardium. Med Clin North Am 72: 227-242

McCord JM (1985) Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 312: 159-163

McKenzie JE, Steffen RP, and Haddy FJ (1982) Relationships between adenosine and coronary resistance in conscious exercising dogs. Am J Physiol 242: H24-9

McMurray J, Chopra M, Abdullah I, Smith WE, and Dargie HJ (1992) Evidence for oxidative stress in unstable angina. Br Heart J 68: 454-457

McMurray J, Chopra M, Abdullah I, Smith WE, and Dargie HJ (1993) Evidence of oxidative stress in chronic heart failure in humans. Eur Heart J 14: 1493-1498

Mehra A, Shotan A, Ostrzega E, Hsueh W, Vasquez Johnson J, and Elkayam U (1994) Potentiation of isosorbide dinitrate effects with N-acetylcysteine in patients with chronic heart failure. Circulation 89: 2595-2600

Mehta JL, Nichols WW, and Mehta P (1988) Neutrophils as potential participants in acute myocardial ischemia: relevance to reperfusion. J Am Coll Cardiol 11: 1309-1316

Meier B, Luethy P, Finci L, Steffenino GD, and Rutishauser W (1987) Coronary wedge pressure in relation to spontaneously visible and recruitable collaterals. Circulation 75: 906-913

Meisel SR, Kutz I, Dayan KI, Pauzner H, Chetboun I, Arbel Y, and David D (1991) Effect of Iraqi missile war on incidence of acute myocardial infarction and sudden death in Israeli civilians. Lancet 338: 660-661

Meister A, and Anderson ME (1983) Glutathione. Annu Rev Biochem 52: 711-760

Meister A (1985) Methods for the selective modification of glutathione metabolism and study of glutathione transport. Methods Enzymol 113: 571-585

Menasche P, Grousset C, Gauduel Y, Mouas C, and Piwnica A (1992) Maintenance of the myocardial thiol pool by N-acetylcysteine. An effective means of improving cardioplegic protection. J Thorac Cardiovasc Surg 103: 936-944

Menasche P, Grousset C, Gauduel Y, Mouas C, and Piwnica A (1986) Enhancement of cardioplegic protection with the free-radical scavenger peroxidase. Circulation 74: III138-44

Menasche P, Pasquier C, Bellucci S, Lorente P, Jaillon P, and Piwnica A (1988) Deferoxamine reduces neutrophil-mediated free radical production during cardiopulmonary bypass in man. J Thorac Cardiovasc Surg 96: 582-589

Meredith IT, Alison JF, Zhang FM, Horowitz JD, and Harper RW (1993) Captopril potentiates the effects of nitroglycerin in the coronary vascular bed. J Am Coll Cardiol 22: 581-587

Meydani M, Evans WJ, Handelman G, Biddle L, Fielding RA, Meydani SN, Burrill J, Fiatarone MA, Blumberg JB, and Cannon JG (1993) Protective effect of vitamin E on exercise-induced oxidative damage in young and older adults. Am J Physiol 264: R992-R998

Michiels C, and Remacle J (1991) Cytotoxicity of linoleic acid peroxide, malondialdehyde and 4-hydroxynonenal towards human fibroblasts. Toxicology 66: 225-234

Miki T, Miura T, Shimamoto K, Urabe K, Sakamoto J, and Iimura O (1993) Do angiotensin converting enzyme inhibitors limit myocardial infarct size? Clin Exp Pharmacol Physiol 20: 429-434

Milei J, Ferreira R, Llesuy S, Forcada P, Covarrubias J, and Boveris A (1992) Reduction of reperfusion injury with preoperative rapid intravenous infusion of taurine during myocardial revascularization. Am Heart J 123: 339-345

Miura T (1990) Does reperfusion induce myocardial necrosis? [comment]. Circulation 82: 1070-1072

Mohri M, Tomoike H, Noma M, Inoue T, Hisano K, and Nakamura M (1989) Duration of ischemia is vital for collateral development: repeated brief coronary artery occlusions in conscious dogs. Circ Res 64: 287-296

Moise A, Lesperance J, Theroux P, Taeymans Y, Goulet C, and Bourassa MG (1984) Clinical and angiographic predictors of new total coronary occlusion in coronary artery disease: analysis of 313 nonoperated patients. Am J Cardiol 54: 1176-1181

Moldeus P, Cotgreave IA, and Berggren M (1986) Lung protection by a thiol-containing antioxidant: N-acetylcysteine. Respiration 50 Suppl 1: 31-42

Moncada S, and Higgs A (1993) The L-arginine-nitric oxide pathway. N Engl J Med 329: 2002-2012

Montrucchio G, Bergerone S, Bussolino F, Alloatti G, Silvestro L, Lupia E, Cravetto A, Di Leo M, Emanuelli G, and Camussi G (1993b) Streptokinase induces intravascular release of platelet-activating factor in patients with acute myocardial infarction and stimulates its synthesis by cultured human endothelial cells. Circulation 88: 1476-1483

Montrucchio G, Alloatti G, Mariano F, Lupia E, Lucchina PG, Musso E, Emanuelli G, and Camussi G (1993a) Role of platelet-activating factor in hypotension and platelet activation induced by infusion of thrombolytic agents in rabbits. Circ Res 72: 658-670

Montrucchio G, Alloatti G, Tetta C, De Luca R, Saunders RN, Emanuelli G, and Camussi G (1989) Release of platelet-activating factor from ischemic-reperfused rabbit heart. Am J Physiol 256: H1236-46

Moro MA, Darley Usmar VM, Goodwin DA, Read NG, Zamora Pino R, Feelisch M, Radomski MW, and Moncada S (1994) Paradoxical fate and biological action of peroxynitrite on human platelets. Proc Natl Acad Sci U S A 91: 6702-6706

Muller JE, Tofler GH, and Stone PH (1989) Circadian variation and triggers of onset of acute cardiovascular disease. Circulation 79: 733-743

Muller JE, Morrison J, Stone PH, Rude RE, Rosner B, Roberts R, Pearle DL, Turi ZG, Schneider JF, Serfas DH, and et al (1984a) Nifedipine therapy for patients with threatened and acute myocardial infarction: a randomized, double-blind, placebo-controlled comparison. Circulation 69: 740-747

Muller JE, Turi ZG, Pearle DL, Schneider JF, Serfas DH, Morrison J, Stone PH, Rude RE, Rosner B, Sobel BE, and et al (1984b) Nifedipine and conventional therapy for unstable angina pectoris: a randomized, double-blind comparison. Circulation 69: 728-739

Munzel T, Sayegh H, Freeman BA, Tarpey MM, and Harrison DG (1995) Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. J Clin Invest 95: 187-194

Munzel T, Holtz J, Mulsch A, Stewart DJ, and Bassenge E (1989) Nitrate tolerance in epicardial arteries or in the venous system is not reversed by N-acetylcysteine in vivo, but tolerance-independent interactions exist. Circulation 79: 188-197

Murohara Y, Yui Y, Hattori R, and Kawai C (1991) Effects of superoxide dismutase on reperfusion arrhythmias and left ventricular function in patients undergoing thrombolysis for anterior wall acute myocardial infarction. Am J Cardiol 67: 765-767

Myers PR, Minor RLJ, Guerra RJ, Bates JN, and Harrison DG (1990) Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. Nature 345: 161-163

Myers ML, Bolli R, Lekich RF, Hartley CJ, and Roberts R (1985) Enhancement of recovery of myocardial function by oxygen free-radical scavengers after reversible regional ischemia. Circulation 72: 915-921

Myers ML, Bolli R, Lekich RF, Hartley CJ, and Roberts R (1986) N-2-mercaptopropionylglycine improves recovery of myocardial function after reversible regional ischemia. J Am Coll Cardiol 8: 1161-1168

Nabel EG, Selwyn AP, and Ganz P (1990) Large coronary arteries in humans are responsive to changing blood flow: an endothelium-dependent mechanism that fails in patients with atherosclerosis. J Am Coll Cardiol 16: 349-356

Nabel EG, Topol EJ, Galeana A, Ellis SG, Bates ER, Werns SW, Walton JA, Muller DW, Schwaiger M, and Pitt B (1991) A randomized placebo-controlled trial of combined early intravenous captopril and recombinant tissue-type plasminogen activator therapy in acute myocardial infarction. J Am Coll Cardiol 17: 467-473

Nabel EG, Ganz P, Gordon JB, Alexander RW, and Selwyn AP (1988) Dilation of normal and constriction of atherosclerotic coronary arteries caused by the cold pressor test. Circulation 77: 43-52

Naslund U, Haggmark S, Johansson G, Marklund SL, and Reiz S (1990) Limitation of myocardial infarct size by superoxide dismutase as an adjunct to reperfusion after different durations of coronary occlusion in the pig [published erratum appears in Circ Res 1990 Sep;67(3):786]. Circ Res 66: 1294-1301

National Heart Foundation of Australia (1992) Heart facts report 1992. 1-61

Nayler WG, and Elz JS (1986) Reperfusion injury: laboratory artifact or clinical dilemma? Circulation 74: 215-221

Needleman P, Jakschik B, and Johnson EM, Jr. (1973) Sulfhydryl requirement for relaxation of vascular smooth muscle. J Pharmacol Exp Ther 187: 324-331

Newman PE (1981) The coronary collateral circulation: determinants and functional significance in ischemic heart disease. Am Heart J 102: 431-445

Neya K, Takamoto S, Morita K, Matsuda T, Kyo S, Yokote Y, and Omoto R (1993) Reduction of reperfusion injury by recombinant human superoxide dismutase administered intravenously just prior to reperfusion. Cardiology 82: 354-364

Nichols CG, Ripoll C, and Lederer WJ (1991) ATP-sensitive potassium channel modulation of the guinea pig ventricular action potential and contraction. Circ Res 68: 280-287

Nicolini FA, Ferrini D, Ottani F, Galvani M, Ronchi A, Behrens PH, Rusticali F, and Mehta JL (1994) Concurrent nitroglycerin therapy impairs tissue-type plasminogen activator-induced thrombolysis in patients with acute myocardial infarction. Am J Cardiol 74: 662-666

Niu XF, Smith CW, and Kubes P (1994) Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. Circ Res 74: 1133-1140

Nohara R, Myears DW, Sobel BE, and Abendschein DR (1989) Optimal criteria for rapid detection of myocardial reperfusion by creatine kinase MM isoforms in the presence of residual high grade coronary stenosis. J Am Coll Cardiol 14: 1067-1073

Norris RM, Whitlock RM, Barratt Boyes C, and Small CW (1975) Clinical measurement of myocardial infarct size. Modification of a method for the estimation of total creatine phosphokinase release after myocardial infarction. Circulation 51: 614-620

O'Rouke M, Baron D, Keogh A, Kelly R, Nelson G, Barnes C, Raftos J, Graham K, Hillman K, Newman H, Healey J, Woolridge J, Rivers J, White H, Whitlock R, and Norris R (1988) Limitation of myocardial infarction by early infusion of recombinant tissue-type plasminogen activator. Circulation 77: 1311-1315

Ohman L, Dahlgren C, Follin P, Lew D, and Stendahl O (1992) N-acetylcysteine enhances receptor-mediated phagocytosis by human neutrophils. Agents Actions 36: 271-277

Ohman EM, Califf RM, Topol EJ, Candela R, Abbottsmith C, Ellis S, Sigmon KN, Kereiakes D, George B, and Stack R (1990) Consequences of reocclusion after successful reperfusion therapy in acute myocardial infarction. TAMI Study Group. Circulation 82: 781-791

Oldroyd KG, Paterson JR, Rumley AG, Eteiba H, Rae AP, Shepherd J, Cobbe SM, and Hutton I (1992) Coronary venous lipid peroxide concentrations after coronary angioplasty: correlation with biochemical and electrocardiographic evidence of myocardial ischaemia [published erratum appears in Br Heart J 1992 Nov;68(5):534]. Br Heart J 68: 43-47

Oldroyd KG, Chopra M, Rankin AC, Belch JJ, and Cobbe SM (1990) Lipid peroxidation during myocardial ischaemia induced by pacing. Br Heart J 63: 88-92

Olivieri D, Marsico SA, and Del Donno M (1985) Improvement of mucociliary transport in smokers by mucolytics. Eur J Respir Dis Suppl 139: 142-145

Olsson B, Johansson M, Gabrielsson J, and Bolme P (1988) Pharmacokinetics and bioavailability of reduced and oxidized N-acetylcysteine. Eur J Clin Pharmacol 34: 77-82

Ono S, Nohara R, Kambara H, Okuda K, and Kawai C (1992) Regional myocardial perfusion and glucose metabolism in experimental left bundle branch block. Circulation 85: 1125-1131

Onodera T, Takemura G, Oguro T, and Ashraf M (1992) Effect of exogenous hydrogen peroxide on myocardial function and structure in isolated rat heart. Can J Cardiol 8: 989-997

Opie LH (1989) Reperfusion injury and its pharmacologic modification. Circulation 80: 1049-1062

Opie LH (1993) The mechanism of myocyte death in ischaemia. Eur Heart J 14 Suppl G: 31-33

Opie LH (1990) Clinical use of calcium channel antagonist drugs. Kluwer Academic Publishers, Boston, pp 1-326

Ovize M, de Lorgeril M, Ovize A, Ciavatti M, Delaye J, and Renaud S (1991) U74006F, a novel 21-aminosteroid, inhibits in vivo lipid peroxidation but fails to limit infarct size in a canine model of myocardial ischemia reperfusion. Am Heart J 122: 681-689

Owen J, Friedman KD, Grossman BA, Wilkins C, Berke AD, and Powers ER (1988) Thrombolytic therapy with tissue plasminogen activator or streptokinase induces transient thrombin activity. Blood 72: 616-620

Packer M, Lee WH, Kessler PD, Gottlieb SS, Medina N, and Yushak M (1987) Prevention and reversal of nitrate tolerance in patients with congestive heart failure. N Engl J Med 317: 799-804

Pallandi RT, Perry MA, and Campbell TJ (1987) Proarrhythmic effects of an oxygen-derived free radical generating system on action potentials recorded from guinea pig ventricular myocardium: a possible cause of reperfusion-induced arrhythmias. Circ Res 61: 50-54

Palmer RM, Ashton DS, and Moncada S (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 333: 664-666

Parente JE, Walsh MP, Girard PR, Kuo JF, Ng DS, and Wong K (1989) Effects of gold coordination complexes on neutrophil function are mediated via inhibition of protein kinase C. Mol Pharmacol 35: 26-33

Parfitt VJ, Rubba P, Bolton C, Marotta G, Hartog M, and Mancini M (1994) A comparison of antioxidant status and free radical peroxidation of plasma lipoproteins in healthy young persons from Naples and Bristol. Eur Heart J 15: 871-876

Parinandi NL, Weis BK, Natarajan V, and Schmid HHO (1990) Peroxidative modification of phospholipids in myocardial membranes. Arch Biochem Biophys 280: 45-52

Parisi AF, Folland ED, and Hartigan P (1992) A comparison of angioplasty with medical therapy in the treatment of single-vessel coronary artery disease. Veterans Affairs ACME Investigators. N Engl J Med 326: 10-16

Parker JO, Farrell B, Lahey KA, and Rose BF (1987) Nitrate tolerance: the lack of effect of N-acetylcysteine. Circulation 76: 572-576

Pasternak RC, Braunwald E, Sobel BE (1992) Acute myocardial infarction. In: Braunwald E (ed) Heart disease. W. B. Saunders Company, Philadelphia, pp 1200-1291

Patterson RE, Jones Collins BA, Aamodt R, and Ro YM (1983) Differences in collateral myocardial blood flow following gradual vs abrupt coronary occlusion. Cardiovasc Res 17: 207-213

Patterson RE, and Kirk ES (1983) Coronary steal mechanisms in dogs with one-vessel occlusion and other arteries normal. Circulation 67: 1009-1015

Paulsen O, and Forsgren A (1989) Effects of N-acetylcysteine on human polymorphonuclear leukocytes. APMIS 97: 115-119

Pedersen TR (1983) The Norwegian Multicenter Study of Timolol after Myocardial Infarction. Circulation 67: I49-53

Pedersen AK, and FitzGerald GA (1984) Dose-related kinetics of aspirin. Presystemic acetylation of platelet cyclooxygenase. N Engl J Med 311: 1206-1211

Peter T, Heng MK, Singh BN, Ambler P, Nisbet H, Elliot R, and Norris RM (1978) Failure of high doses of propranolol to reduce experimental myocardial ischemic damage. Circulation 57: 534-540

Pogwizd SM, and Corr PB (1987) Electrophysiologic mechanisms underlying arrhythmias due to reperfusion of ischemic myocardium. Circulation 76: 404-426

Poole Wilson PA, Harding DP, Bourdillon PD, and Tones MA (1984) Calcium out of control. J Mol Cell Cardiol 16: 175-187

Porter NA (1984) Chemistry of lipid peroxidation. Methods Enzymol 105: 273-282

Prasad K, Kalra J, Bharadwaj B, and Chaudhary AK (1992) Increased oxygen free radical activity in patients on cardiopulmonary bypass undergoing aortocoronary bypass surgery. Am Heart J 123: 37-45

Prasad K, Kalra J, Chaudhary AK, and Debnath D (1990) Effect of polymorphonuclear leukocyte-derived oxygen free radicals and hypochlorous acid on cardiac function and some biochemical parameters. Am Heart J 119: 538-550

Prescott LF (1983) Paracetamol overdosage. Pharmacological considerations and clinical management. Drugs 25: 290-314

Preuss KC, Gross GJ, Brooks HL, and Warltier DC (1987) Time course of recovery of "stunned" myocardium following variable periods of ischemia in conscious and anesthetized dogs. Am Heart J 114: 696-703

Probst P, Zangl W, and Pachinger O (1985) Relation of coronary arterial occlusion pressure during percutaneous transluminal coronary angioplasty to presence of collaterals. Am J Cardiol 55: 1264-1269

Pryor WA, and Castle L (1984) Chemical methods for the detection of lipid hydroperoxides. Methods Enzymol 105: 293-299

Pryor WA, and Stanley JP (1975) Letter: A suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation. J Org Chem 40: 3615-3617

Przyklenk K, and Kloner RA (1986) Superoxide dismutase plus catalase improve contractile function in the canine model of the "stunned myocardium". Circ Res 58: 148-156

Puleo PR, Guadagno PA, Roberts R, Scheel MV, Marian AJ, Churchill D, and Perryman MB (1990) Early diagnosis of acute myocardial infarction based on assay for subforms of creatine kinase-MB. Circulation 82: 759-764

Puleo PR, Perryman MB, Bresser MA, Rokey R, Pratt CM, and Roberts R (1987) Creatine kinase isoform analysis in the detection and assessment of thrombolysis in man. Circulation 75: 1162-1169

Qiu Y, Bernier M, and Hearse DJ (1990) The influence of N-acetylcysteine on cardiac function and rhythm disorders during ischemia and reperfusion. Cardioscience 1: 65-74

Rao PS, and Mueller HS (1983) Lipid peroxidation and acute myocardial infarction. Adv Exp Med Biol 161: 347-363

Rashid MA, and William Olsson G (1991) Influence of allopurinol on cardiac complications in open heart operations. Ann Thorac Surg 52: 127-130

Ravkilde J, Nissen H, Horder M, and Thygesen K (1995) Independent prognostic value of serum creatine kinase isoenzyme MB mass, cardiac troponin T and myosin light chain levels in suspected acute myocardial infarction. Analysis of 28 months of follow-up in 196 patients. J Am Coll Cardiol 25: 574-581

Reddy D, Lancaster JR, Jr., and Cornforth DP (1983) Nitrite inhibition of Clostridium botulinum: electron spin resonance detection of iron-nitric oxide complexes. Science 221: 769-770

Reimer KA, and Jennings RB (1985) Failure of the xanthine oxidase inhibitor allopurinol to limit infarct size after ischemia and reperfusion in dogs. Circulation 71: 1069-1075

Reimer KA, Hill ML, and Jennings RB (1981) Prolonged depletion of ATP and of the adenine nucleotide pool due to delayed resynthesis of adenine nucleotides following reversible myocardial ischemic injury in dogs. J Mol Cell Cardiol 13: 229-239

Reimer KA, Lowe JE, Rasmussen MM, and Jennings RB (1977) The wavefront phenomenon of ischemic cell death. 1. Myocardial infarct size vs duration of coronary occlusion in dogs. Circulation 56: 786-794

Res JC, Simoons ML, van der Wall EE, van Eenige MJ, Vermeer F, Verheugt FW, Wijns W, Braat S, Remme WJ, Serruys PW, and et al (1986) Long term improvement in global left ventricular function after early thrombolytic treatment in acute myocardial infarction. Report of a randomised multicentre trial of intracoronary streptokinase in acute myocardial infarction. Br Heart J 56: 414-421

Reynard K, Riley A, and Walker BE (1992) Respiratory arrest after N-acetylcysteine for paracetamol overdose [letter]. Lancet 340: 675

Richard VJ, Murry CE, Jennings RB, and Reimer KA (1988) Therapy to reduce free radicals during early reperfusion does not limit the size of myocardial infarcts caused by 90 minutes of ischemia in dogs. Circulation 78: 473-480

Richardson SG, Morton P, Murtagh JG, Scott ME, and O'Keeffe DB (1988) Relation of coronary arterial patency and left ventricular function to electrocardiographic changes after streptokinase treatment during acute myocardial infarction. Am J Cardiol 61: 961-965

Richie JP, Jr., and Lang CA (1987) The determination of glutathione, cysteine, and other thiols and disulfides in biological samples using high-performance liquid chromatography with dual electrochemical detection. Anal Biochem 163: 9-15

Roark SF, Ideker RE, Wagner GS, Alonso DR, Bishop SP, Bloor CM, Bramlet DA, Edwards JE, Fallon JT, Gottlieb GJ, Hackel DB, Phillips HR, Reimer KA, Rogers WJ, Ruth WK, Savage RM, White RD, and Selvester RH (1983) Evaluation of a QRS scoring system for estimating myocardial infarct size. III. Correlation with quantitative anatomic findings for inferior infarcts. Am J Cardiol 51: 382-389

Roberts AJ, Spies SM, Meyers SN, Moran JM, Sanders JH, Jr., Lichtenthal PR, and Michaelis LL (1980) Early and long-term improvement in left ventricular performance following coronary bypass surgery. Surgery 88: 467-475

Roberts MJ, Young IS, Trouton TG, Trimble ER, Khan MM, Webb SW, Wilson CM, Patterson GC, and Adgey AA (1990) Transient release of lipid peroxides after coronary artery balloon angioplasty. Lancet 336: 143-145

Roberts R, Croft C, Gold HK, Hartwell TD, Jaffe AS, Muller JE, Mullin SM, Parker C, Passamani ER, Poole WK, and et al (1984) Effect of propranolol on myocardial-infarct size in a randomized blinded multicenter trial. N Engl J Med 311: 218-225

Roberts R (1987) Reperfusion and the plasma isoforms of creatine kinase isoenzymes: a clinical perspective. J Am Coll Cardiol 9: 464-466

Roberts R, and Sobel BE (1973) Ediortial: Isoenzymes of creatine phosphokinase and diagnosis of myocardial infarction. Ann Intern Med 79: 741-743

Rogers WJ, McDaniel HG, Smith LR, Mantle JA, Russel RO, Jr., and Rackley CE (1977) Correlation of angiographic estimates of myocardial infarct size and accumulated release of creatine kinase MB isoenzyme in man. Circulation 56: 199-205

Romson JL, Hook BG, Kunkel SL, Abrams GD, Schork MA, and Lucchesi BR (1983) Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circulation 67: 1016-1023

Roncaglioni MC, Santoro L, D'Avanzo B, Negri E, Nobili A, Ledda A, Pietropaolo F, Franzosi MG, La Vecchia C, Feruglio GA, and et al (1992) Role of family history in patients with myocardial infarction. An Italian case-control study. GISSI-EFRIM Investigators. Circulation 85: 2065-2072

Rooke GA, and Feigl EO (1982) Work as a correlate of canine left ventricular oxygen consumption, and the problem of catecholamine oxygen wasting. Circ Res 50: 273-286

Rosen GM, and Rauckman EJ (1984) Spin trapping of superoxide and hydroxyl radicals. Methods Enzymol 105: 198-209

Rosengren A, Anderson K, and Wilhelmsen L (1991) Risk of coronary heart disease in middle-aged male bus and tram drivers compared to men in other occupations: a prospective study. Int J Epidemiol 20: 82-87

Ross R (1992) The pathogenesis of atherosclerosis. In: Braunwald E (ed) Heart disease. W. B. Saunders Company, Philadelphia, pp 1106-1124

Ross R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362: 801-809

Rossen RD, Swain JL, Michael LH, Weakley S, Giannini E, and Entman ML (1985) Selective accumulation of the first component of complement and leukocytes in ischemic canine heart muscle. A possible initiator of an extra myocardial mechanism of ischemic injury. Circ Res 57: 119-130

Rossen RD, Michael LH, Kagiyama A, Savage HE, Hanson G, Reisberg MA, Moake JN, Kim SH, Self D, and Weakley S (1988) Mechanism of complement activation after coronary artery occlusion: evidence that myocardial ischemia in dogs causes release of constituents of myocardial subcellular origin that complex with human C1q in vivo. Circ Res 62: 572-584

Rovetto MJ, Lamberton WF, and Neely JR (1975) Mechanisms of glycolytic inhibition in ischemic rat hearts. Circ Res 37: 742-751

Royston D, Fleming JS, Desai JB, Westaby S, and Taylor KM (1986) Increased production of peroxidation products associated with cardiac operations. Evidence for free radical generation. J Thorac Cardiovasc Surg 91: 759-766

Rubio R, and Berne RM (1975) Regulation of coronary blood flow. Prog Cardiovasc Dis 18: 105-122

Ruffmann R, and Wendel A (1991) GSH rescue by N-acetylcysteine. Klin Wochenschr 69: 857-862

Rutherford JD, Braunwald E (1992) Chronic ischaemic heart disease. In: Braunwald E (ed) Heart disease. W. B. Saunders Company, Philadelphia, pp 1292-1364

Sabri MN, DiSciascio G, Cowley MJ, Alpert D, and Vetrovec GW (1991) Coronary collateral recruitment: functional significance and relation to rate of vessel closure. Am Heart J 121: 876-880

Sardinas A, Miller JW, and Hansen H (1986) Ischemic heart disease mortality of firemen and policemen. Am J Public Health 76: 1140-1141

Sawa Y, Schaper J, Roth M, Nagasawa K, Ballagi G, Bleese N, and Schaper W (1994) Platelet-activating factor plays an important role in reperfusion injury in myocardium. Efficacy of platelet-activating factor receptor antagonist (CV-3988) as compared with leukocyte-depleted reperfusion. J Thorac Cardiovasc Surg 108: 953-959

Schaper W, Sharma HS, Quinkler W, Markert T, Wunsch M, and Schaper J (1990) Molecular biologic concepts of coronary anastomoses. J Am Coll Cardiol 15: 513-518

Schaper W, Hofmann M, Muller KD, Genth K, and Carl M (1979) Experimental occlusion of two small coronary arteries in the same heart. A new validation method for infarct size manipulation. Basic Res Cardiol 74: 224-229

Scharfstein JS, Keaney JF, Jr., Slivka A, Welch GN, Vita JA, Stamler JS, and Loscalzo J (1994) In vivo transfer of nitric oxide between a plasma protein-bound reservoir and low molecular weight thiols. J Clin Invest 94: 1432-1439

Schoneich C, Dillinger U, von Bruchhausen F, and Asmus KD (1992) Oxidation of polyunsaturated fatty acids and lipids through thiyl and sulfonyl radicals: reaction kinetics, and influence of oxygen and structure of thiyl radicals. Arch Biochem Biophys 292: 456-467

Schroder R, Dissmann R, Bruggemann T, Wegscheider K, Linderer T, Tebbe U, and Neuhaus KL (1994) Extent of early ST segment elevation resolution: a simple but strong predictor of outcome in patients with acute myocardial infarction. J Am Coll Cardiol 24: 384-391

Schror K, Woditsch I, and Forster S (1991) Generation of nitric oxide from organic nitrovasodilators during passage through the coronary vascular bed and its role in coronary vasodilation and nitrate tolerance. Blood Vessels 28: 62-66

Schwartz H, Leiboff RH, Bren GB, Wasserman AG, Katz RJ, Varghese PJ, Sokil AB, and Ross AM (1984) Temporal evolution of the human coronary collateral circulation after myocardial infarction. J Am Coll Cardiol 4: 1088-1093

Sellke FW, Tomanek RJ, and Harrison DG (1991) L-cysteine selectively potentiates nitroglycerin-induced dilation of small coronary microvessels. J Pharmacol Exp Ther 258: 365-369

Sellke FW, Myers PR, Bates JN, and Harrison DG (1990) Influence of vessel size on the sensitivity of porcine coronary microvessels to nitroglycerin. Am J Physiol 258: H515-20

Semb AG, Ytrehus K, Vaage J, Myklebust R, and Mjos OD (1989) Functional impairment in isolated rat hearts induced by activated leukocytes: protective effect of oxygen free radical scavengers. J Mol Cell Cardiol 21: 877-887

Senes-Ferrari S (1994) Statistics on causes of death 1993. (personal communication). National Heart Foundation of Australia.

Seo B, Oemar BS, Siebenmann R, von Segesser L, and Luscher TF (1994) Both ETA and ETB receptors mediate contraction to endothelin-1 in human blood vessels. Circulation 89: 1203-1208

Serruys PW, Wijns W, van den Brand M, Meij S, Slager C, Schuurbiers JC, Hugenholtz PG, and Brower RW (1984) Left ventricular performance, regional blood flow, wall motion, and lactate metabolism during transluminal angioplasty. Circulation 70: 25-36

Seth P, and Fung HL (1993) Biochemical characterization of a membrane-bound enzyme responsible for generating nitric oxide from nitroglycerin in vascular smooth muscle cells. Biochem Pharmacol 46: 1481-1486

Sevilla DC, Wagner NB, Pegues R, Peck SL, Mikat EM, Ideker RE, Hutchins G, Reimer KA, Hackel DB, Selvester RH, and et al (1992) Correlation of the complete version of the Selvester QRS scoring system with quantitative anatomic findings for multiple left ventricular myocardial infarcts. Am J Cardiol 69: 465-469

Shah PK, Cercek B, Lew AS, and Ganz W (1993) Angiographic validation of bedside markers of reperfusion. J Am Coll Cardiol 21: 55-61

Shandelya SM, Kuppusamy P, Weisfeldt ML, and Zweier JL (1993) Evaluation of the role of polymorphonuclear leukocytes on contractile function in myocardial reperfusion injury. Evidence for plasma-mediated leukocyte activation. Circulation 87: 536-546

Sharma B, Behrens TW, Erlein D, Hodges M, Asinger RW, and Francis GS (1983a) Left ventricular diastolic properties and filling characteristics during spontaneous angina pectoris at rest. Am J Cardiol 52: 704-709

Sharma AD, Saffitz JE, Lee BI, Sobel BE, and Corr PB (1983b) Alpha adrenergic-mediated accumulation of calcium in reperfused myocardium. J Clin Invest 72: 802-818

Shechter M, Rabinowitz B, Beker B, Motro M, Barbash G, Kaplinsky E, and Hod H (1992) Additional ST segment elevation during the first hour of thrombolytic therapy: an electrocardiographic sign predicting a favorable clinical outcome. J Am Coll Cardiol 20: 1460-1464

Sheehan FH, Doerr R, Schmidt WG, Bulson EL, Uebis R, Von Essen R, Effert S, and Dodge HT (1988) Early recovery of left ventricular function after thrombolytic therapy for acute myocardial infarction: an important determinant of survival. J Am Coll Cardiol 12: 289-300

Shell WE, Kjekshus JK, and Sobel BE (1971) Quantitative assessment of the extent of myocardial infarction in the conscious dog by means of analysis of serial changes in serum creatine phosphokinase activity. J Clin Invest 50: 2614-2625

Shirato C, Miura T, Ooiwa H, Toyofuku T, Wilborn WH, and Downey JM (1989) Tetrazolium artifactually indicates superoxide dismutase-induced salvage in reperfused rabbit heart. J Mol Cell Cardiol 21: 1187-1193

Sies H, Stahl W, and Sundquist AR (1992) Antioxidant functions of vitamins. Vitamins E and C, beta-carotene, and other carotenoids. Ann N Y Acad Sci 669: 7-20

Sies H (1991) Oxidative stress: from basic research to clinical application. Am J Med 91 [suppl 3C]: 31S-38S

Silver PJ (1984) Perhexiline directly inhibits arterial myosin phosphorylation and stimulates cardiac myofibrillar ATPase activity. Eur J Pharmacol 106: 217-218

Simonson MS, and Dunn MJ (1990) Endothelin. Pathways of transmembrane signaling. Hypertension 15(suppl I): I-5-I-12

Simoons ML, Arnold AE, Betriu A, de Bono DP, Col J, Dougherty FC, Von Essen R, Lambertz H, Lubsen J, Meier B, and et al (1988) Thrombolysis with tissue plasminogen activator in acute myocardial infarction: no additional benefit from immediate percutaneous coronary angioplasty. Lancet 1: 197-203

Simpson PJ, Todd RF, 3d, Mickelson JK, Fantone JC, Gallagher KP, Lee KA, Tamura Y, Cronin M, and Lucchesi BR (1990) Sustained limitation of myocardial reperfusion injury by a monoclonal antibody that alters leukocyte function. Circulation 81: 226-237

Sirnes PA, Overskeid K, Pedersen TR, Bathen J, Drivenes A, Froland GS, Kjekshus JK, Landmark K, Rokseth R, Sirnes KE, and et al (1984) Evolution of infarct size during the early use of nifedipine in patients with acute myocardial infarction: the Norwegian Nifedipine Multicenter Trial. Circulation 70: 638-644

Slater TF (1984) Overview of methods used for detecting lipid peroxidation. Methods Enzymol 105: 283-293

Smith P, Arnesen H, and Holme I (1990) The effect of warfarin on mortality and reinfarction after myocardial infarction. N Engl J Med 323: 147-152

Sobel BE, Markham J, Karlsberg RP, and Roberts R (1977) The nature of disappearance of creatine kinase from the circulation and its influence on enzymatic estimation of infarct size. Circ Res 41: 836-844

Sobey CG, Dusting GJ, Grossman HJ, and Woodman OL (1990) Impaired vasodilatation of epicardial coronary arteries and resistance vessels following myocardial ischemia and reperfusion in anethetized dogs. Coron Artery Dis 1: 363-374

Sochman J, Kolc J, Vrana M, and Fabian J (1990) Cardioprotective effects of N-acetylcysteine: the reduction in the extent of infarction and occurrence of reperfusion arrhythmias in the dog. Int J Cardiol 28: 191-196

Sochman J, and Peregrin JH (1992) Total recovery of left ventricular function after acute myocardial infarction: comprehensive therapy with streptokinase, N-acetylcysteine and percutaneous transluminal coronary angioplasty. Int J Cardiol 35: 116-118

Springer TA (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76: 301-314

St Goar FG, and Stone GW (1994) Interventional strategies for managing acute myocardial infarction. Cardiol Clin 12: 559-571

Stamler J, Cunningham M, and Loscalzo J (1988) Reduced thiols and the effect of intravenous nitroglycerin on platelet aggregation. Am J Cardiol 62: 377-380

Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, Singel D, Valeri CR, and Loscalzo J (1992a) Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. Proc Natl Acad Sci U S A 89: 7674-7677

Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ, and Loscalzo J (1992b) S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. Proc Natl Acad Sci U S A 89: 444-448

Stammen F, Piessens J, Vrolix M, Glazier JJ, De Geest H, and Willems JL (1991) Immediate and short-term results of a 1988-1989 coronary angioplasty registry. Am J Cardiol 67: 253-258

Stark WS, Miller GV, and Itoku KA (1984) Calibration of microspectrophotometers as it applies to the detection of lipofuscin and the blue- and yellow-emitting fluorophores in situ. Methods Enzymol 105: 341-347

Stary HC (1989) Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. Arteriosclerosis 9: I19-32(Abstract)

Steering Committee of the Physician's Health Study Research Group (1989) Final report on the aspirin component of the ongoing Physicians' Health Study. Steering Committee of the Physicians' Health Study Research Group. N Engl J Med 321: 129-135

Steffenino G, Meier B, Finci L, and Rutishauser W (1987) Follow up results of treatment of unstable angina by coronary angioplasty. Br Heart J 57: 416-419

Stein PD, Marzilli M, Sabbah HN, and Lee T (1980) Systolic and diastolic pressure gradients within the left ventricular wall. Am J Physiol 238: H625-30

Stern MD, Chien AM, Capogrossi MC, Pelto DJ, and Lakatta EG (1985) Direct observation of the "oxygen paradox" in single rat ventricular myocytes. Circ Res 56: 899-903

Stocker R, Bowry VW, and Frei B (1991) Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does alpha-tocopherol. Proc Natl Acad Sci U S A 88: 1646-1650

Stone DL, Stephens JD, and Banim SO (1983) Coronary haemodynamic effects of nifedipine. Comparison with glyceryl trinitrate. Br Heart J 49: 442-446

Straehl P, and Galeazzi RL (1985) Isosorbide dinitrate bioavailability, kinetics, and metabolism. Clin Pharmacol Ther 38: 140-149

Strieter RM, Kunkel SL, Showell HJ, Remick DG, Phan SH, Ward PA, and Marks RM (1989) Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-alpha, LPS, and IL-1 beta. Science 243: 1467-1469

Strong JP, and McGill HC (1961) The natural history of coronary atherosclerosis. Am J Pathol 40: 37-49

Stuehr DJ, Cho HJ, Kwon NS, Weise MF, and Nathan CF (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. Proc Natl Acad Sci U S A 88: 7773-7777

Subramanian R, Volovsek A, and Ho YS (1993) Lack of change in MnSOD during ischemia/reperfusion of isolated rat heart. J Mol Cell Cardiol 25: 1179-1186

Suga H, Goto Y, Yamada O, and Igarashi Y (1984) Independence of myocardial oxygen consumption from pressure-volume trajectory during diastole in canine left ventricle. Circ Res 55: 734-739

Suga H, Hisano R, Goto Y, Yamada O, and Igarashi Y (1983) Effect of positive inotropic agents on the relation between oxygen consumption and systolic pressure volume area in canine left ventricle. Circ Res 53: 306-318

Svardal AM, Mansoor MA, Ueland PM (1990) Determination of reduced, oxidized, and protein-bound glutathione in human plasma with precolumn derivatization with monobromobimane and liquid chromatography. Anal.Biochem 184:338-346

SWIFT Trial Study Group (1991) SWIFT trial of delayed elective intervention v conservative treatment after thrombolysis with anistreplase in acute myocardial infarction. SWIFT (Should We Intervene Following Thrombolysis?) Trial Study Group. BMJ 302: 555-560

Tabayashi K, Suzuki Y, Nagamine S, Ito Y, Sekino Y, and Mohri H (1991) A clinical trial of allopurinol (Zyloric) for myocardial protection. J Thorac Cardiovasc Surg 101: 713-718

Tamura H, and Shibamoto T (1991) Gas chromatographic analysis of malonaldehyde and 4-hydroxy-2-(E)-nonenal produced from arachidonic acid and linoleic acid in a lipid peroxidation model system. Lipids 26: 170-173

Tanaka M, Brooks SE, Richard VJ, FitzHarris GP, Stoler RC, Jennings RB, Arfors KE, and Reimer KA (1993) Effect of anti-CD18 antibody on myocardial neutrophil accumulation and infarct size after ischemia and reperfusion in dogs. Circulation 87: 526-535

Tang LD, Sun JZ, Wu K, Sun CP, and Tang ZM (1991) Beneficial effects of N-acetylcysteine and cysteine in stunned myocardium in perfused rat heart. Br J Pharmacol 102: 601-606

Tavazzi B, Lazzarino G, Di Pierro D, and Giardina B (1992a) Malondialdehyde production and ascorbate decrease are associated to the reperfusion of the isolated postischemic rat heart. Free Rad Biol Med 13:

Teplick R, Haas GS, Trautman E, Titus J, Geffin G, and Daggett WM (1986) Time dependence of the oxygen cost of force development during systole in the canine left ventricle. Circ Res 59: 27-38

Terada LS, Beehler CJ, Banerjee A, Brown JM, Grosso MA, Harken AH, McCord JM, and Repine JE (1988) Hyperoxia and self- or neutrophil-generated O2 metabolites inactivate xanthine oxidase. J Appl Physiol 65: 2349-2353

The GUSTO Investigators (1993) An international randomisation trial comparing four thrombolytic strategies for acute myocardial infarction. N Engl J Med 329: 673-682

The HINT Research Group (1986) Early treatment of unstable angina in the coronary care unit: a randomised, double blind, placebo controlled comparison of recurrent ischaemia in patients treated with nifedipine or metoprolol or both. Report of The Holland Interuniversity Nifedipine/Metoprolol Trial (HINT) Research Group. Br Heart J 56: 400-413

The Veterans Administration Coronary Artery Bypass Surgery Cooperative Study Group (1984) Eleven-year survival in the Veterans Administration randomized trial of coronary bypass surgery for stable angina. The Veterans Administration Coronary Artery Bypass Surgery Cooperative Study Group. N Engl J Med 311: 1333-1339

The TIMI Study Group (1989) Comparison of invasive and conservative strategies after treatment with intravenous tissue plasminogen activator in acute myocardial infarction. Results of the thrombolysis in myocardial infarction (TIMI) phase II trial. The TIMI Study Group. N Engl J Med 320: 618-627

The TIMI Research Group (1988) Immediate vs delayed catheterization and angioplasty following thrombolytic therapy for acute myocardial infarction. TIMI II A results. The TIMI Research Group. JAMA 260: 2849-2858

The Multicenter Diltiazem Postinfarction Trial Research Group (1988) The effect of diltiazem on mortality and reinfarction after myocardial infarction. The Multicenter Diltiazem Postinfarction Trial Research Group. N Engl J Med 319: 385-392

The Multicenter Postinfarction Research Group (1983) Risk stratification and survival after myocardial infarction. N Engl J Med 309: 331-336

The Israeli SPRINT Study Group (1988) Secondary prevention reinfarction Israeli nifedipine trial (SPRINT). A randomized intervention trial of nifedipine in patients with acute myocardial infarction. The Israeli Sprint Study Group. Eur Heart J 9: 354-364

The International Collaborative Study Group (1984) Reduction of infarct size with the early use of timolol in acute myocardial infarction. N Engl J Med 310: 9-15

The I.S.A.M.Study Group. (1986) A prospective trial of intravenous streptokinase in acute myocardial infarction (I.S.A.M.). Mortality, morbidity, and infarct size at 21 days. The I.S.A.M. Study Group. N Engl J Med 314: 1465-1471

The DAVIT Study Group (1984) Verapamil in acute myocardial infarction. The Danish Study Group on Verapamil in Myocardial Infarction. Eur Heart J 5: 516-528

Theroux P, Ouimet H, McCans J, Latour JG, Joly P, Levy G, Pelletier E, Juneau M, Stasiak J, deGuise P, and et al (1988) Aspirin, heparin, or both to treat acute unstable angina. N Engl J Med 319: 1105-1111

Theroux P, Taeymans Y, Morissette D, Bosch X, Pelletier GB, and Waters DD (1985) A randomized study comparing propranolol and diltiazem in the treatment of unstable angina. J Am Coll Cardiol 5: 717-722

Thomassen A, Bagger JP, Nielsen TT, and Henningsen P (1988) Altered global myocardial substrate preference at rest and during pacing in coronary artery disease with stable angina pectoris. Am J Cardiol 62: 686-693

Thompson JA, and Hess ML (1986) The oxygen free radical system: a fundamental mechanism in the production of myocardial necrosis. Prog Cardiovasc Dis 28: 449-462

TIMI Study Group (1985) The Thrombolysis in Myocardial Infarction (TIMI) trial. Phase I findings. TIMI Study Group. N Engl J Med 312: 932-936

Tosaki A, and Braquet P (1990) DMPO and reperfusion injury: arrhythmia, heart function, electron spin resonance, and nuclear magnetic resonance studies in isolated working guinea pig hearts. Am Heart J 120: 819-830

Tosaki A, and Das DK (1994) Reperfusion induced arrhythmias are caused by generation of free radicals. Cardiovasc Res 28: 422-432

Traber J, Suter M, Walter P, and Richter C (1992) In vivo modulation of total and mitochondrial glutathione in rat liver. Depletion by phorone and rescue by N-acetylcysteine. Biochem Pharmacol 43: 961-964

Tranum Jensen J, Janse MJ, Fiolet WT, Krieger WJ, D'Alnoncourt CN, and Durrer D (1981) Tissue osmolality, cell swelling, and reperfusion in acute regional myocardial ischemia in the isolated porcine heart. Circ Res 49: 364-381

Tsao PS, Ma XL, and Lefer AM (1992) Activated neutrophils aggravate endothelial dysfunction after reperfusion of the ischemic feline myocardium. Am Heart J 123: 1464-1471

Tsao PS, Aoki N, Lefer DJ, Johnson G, 3d, and Lefer AM (1990) Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. Circulation 82: 1402-1412

Tsujino M, Hirata Y, Imai T, Kanno K, Eguchi S, Ito H, and Marumo F (1994) Induction of nitric oxide synthase gene by interleukin-1 beta in cultured rat cardiocytes. Circulation 90: 375-383

Tsuneyoshi H, Akatsuka N, Ohno M, Hara K, Ochiai M, and Moroi M (1989) Inhibition of development of tolerance to nitroglycerin by preventive administration of N-acetylcysteine in rats. Jpn Heart J 30: 733-741

Tubau JF, Wikman Coffelt J, Massie BM, Sievers R, and Parmley WW (1987) Improved myocardial efficiency in the working perfused heart of the spontaneously hypertensive rat. Hypertension 10: 396-403

Turner JJ, Rice Evans CA, Davies MJ, and Newman ES (1991) The formation of free radicals by cardiac myocytes under oxidative stress and the effects of electron-donating drugs. Biochem J 277: 833-837

Underwood MJ, and More RS (1994) The aspirin papers [editorial; comment]. BMJ 308: 71-72

Vallance P, Collier J, and Moncada S (1989) Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. Lancet 2: 997-1000

van der Wal AC, Becker AE, van der Loos CM, and Das PK (1994) Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation 89: 36-44

VanBenthuysen KM, McMurtry IF, and Horwitz LD (1987) Reperfusion after acute coronary occlusion in dogs impairs endothelium-dependent relaxation to acetylcholine and augments contractile reactivity in vitro. J Clin Invest 79: 265-274

Vane JR, Anggard EE, and Botting RM (1990) Regulatory functions of the vascular endothelium. N Engl J Med 323: 27-36

Varnauskas E (1988) Twelve-year follow-up of survival in the randomized European Coronary Surgery Study. N Engl J Med 319: 332-337

Vatner SF, Franklin D, Van Citters RL, and Braunwald E (1970) Effects of carotid sinus nerve stimulation on blood-flow distribution in conscious dogs at rest and during exercise. Circ Res 27: 495-503

Vatner DE, Knight DR, Homcy CJ, Vatner SF, and Young MA (1986) Subtypes of beta-adrenergic receptors in bovine coronary arteries. Circ Res 59: 463-473

Vekshtein VI, Yeung AC, Vita JA, Fish RD, Antman EM, Selwyn AP, Ganz P (1990) N-acetylcysteine potentiates coronary artery dilation by nitroglycerin. J Am Coll Cardiol 15: 159A(Abstract)

Veldkamp RF, Green CL, Wilkins ML, Pope JE, Sawchak ST, Ryan JA, Califf RM, Wagner GS, and Krucoff MW (1994) Comparison of continuous ST-segment recovery analysis with methods using static electrocardiograms for noninvasive patency assessment during acute myocardial infarction. Thrombolysis and Angioplasty in Myocardial Infarction (TAMI) 7 Study Group. Am J Cardiol 73: 1069-1074

Vena JE, Violanti JM, Marshall J, and Fiedler RC (1986) Mortality of a municipal worker cohort: III. Police officers. Am J Ind Med 10: 383-397

Verbunt RJAM, Vandockum WG, Bastiaanse EML, Egas JM, and Vanderlaarse A (1995) Glutathione disulfide as an index of oxidative stress during postischemic reperfsuion in isolated rat hearts. Mol Cell Biochem 144: 85-93

Vik Mo H, and Mjos OD (1981) Influence of free fatty acids on myocardial oxygen consumption and ischemic injury. Am J Cardiol 48: 361-365

Villa LM, Salas E, Darley Usmar VM, Radomski MW, and Moncada S (1994) Peroxynitrite induces both vasodilatation and impaired vascular relaxation in the isolated perfused rat heart. Proc Natl Acad Sci U S A 91: 12383-12387

Villari B, Ambrosio G, Golino P, Ragni M, Focaccio A, Tritto I, Salvatore M, and Chiariello M (1993) The effects of calcium channel antagonist treatment and oxygen radical scavenging on infarct size and the no-reflow phenomenon in reperfused hearts. Am Heart J 125: 11-23

Vincent J, Kongpatanakul S, Blaschke TF, and Hoffman BB (1992) Desensitization of nitrate-induced venodilation: reversal with oral N-acetylcysteine in humans. J Cardiovasc Pharmacol 20: 907-912

Vincent GM, Abildskov JA, and Burgess MJ (1977) Mechanisms of ischemic ST-segment displacement. Evaluation by direct current recordings. Circulation 56: 559-566

Virmani R, Kolodgie FD, Forman MB, Farb A, and Jones RM (1992) Reperfusion injury in the ischemic myocardium. Cardiovasc Pathol 1: 117-129

Vyska K, Meyer W, Stremmel W, Notohamiprodjo G, Minami K, Machulla HJ, Gleichmann U, Meyer H, and Korfer R (1991) Fatty acid uptake in normal human myocardium. Circ Res 69: 857-870

Wade CR, Jackson PG, and Van Rij AM (1985) Quantitation of malondialdehyde (MDA) in plasma, by ion-pairing reverse phase high performance liquid chromatography. Biochem Med 33: 291-296

Wagner GS, Freye CJ, Palmeri ST, Roark SF, Stack NC, Ideker RE, Harrell FEJ, and Selvester RH (1982) Evaluation of a QRS scoring system for estimating myocardial infarct size. I. Specificity and observer agreement. Circulation 65: 342-347

Wajner M, and Harkness RA (1989) Distribution of xanthine dehydrogenase and oxidase activities in human and rabbit tissues. Biochim Biophys Acta 991: 79-84

Wall TC, Califf RM, Blankenship J, Talley JD, Tannenbaum M, Schwaiger M, Gacioch G, Cohen MD, Sanz M, Leimberger JD, and et al (1994) Intravenous Fluosol in the treatment of acute myocardial infarction. Results of the Thrombolysis and Angioplasty in Myocardial Infarction 9 Trial. TAMI 9 Research Group. Circulation 90: 114-120

Wall TC, Mark DB, Califf RM, Collins G, Burgess R, Skelton TN, Hinohara T, Kong DF, Mantell S, Aronson L, and et al (1989) Prediction of early recurrent myocardial ischemia and coronary reocclusion after successful thrombolysis: a qualitative and quantitative angiographic study. Am J Cardiol 63: 423-428

Ward RM, White RD, Ideker RE, Hindman NB, Alonso DR, Bishop SP, Bloor CM, Fallon JT, Gottlieb GJ, Hackel DB, and et al (1984) Evaluation of a QRS scoring system for estimating myocardial infarct size. IV. Correlation with quantitative anatomic findings for posterolateral infarcts. Am J Cardiol 53: 706-714

Warltier DC, Gross GJ, and Brooks HL (1980) Coronary steal-induced increase in myocardial infarct size after pharmacologic coronary vasodilation. Am J Cardiol 46: 83-90

Warren JS, Ward PA, Johnson KJ (1989) Oxygen radicals as mediators of inflammation. In: Henson PM, Murphy RC (eds) Mediators of the inflammatory process. Elsevier Science Publishers B.V. (Biomedical Division), Amsterdam, pp 159-176

Weisel RD, Mickle DA, Finkle CD, Tumiati LC, Madonik MM, Ivanov J, Burton GW, and Ingold KU (1989) Myocardial free-radical injury after cardioplegia. Circulation 80: III14-III18

Weiss SJ (1989) Tissue destruction by neutrophils. N Engl J Med 320: 365-376

Weiss SJ, and Peppin GJ (1986) Collagenolytic metalloenzymes of the human neutrophil. Characteristics, regulation and potential function in vivo. Biochem Pharmacol 35: 3189-3197

Welch G, and Loscalzo J (1994) Nitric oxide and the cardiovascular system. J Card Surg 9: 361-371

Werns SW, Shea MJ, Driscoll EM, Cohen C, Abrams GD, Pitt B, and Lucchesi BR (1985) The independent effects of oxygen radical scavengers on canine infarct size. Reduction by superoxide dismutase but not catalase. Circ Res 56: 895-898

Werns SW, Shea MJ, and Lucchesi BR (1986) Free radicals and myocardial injury: pharmacologic implications. Circulation 74: 1-5

Werns SW, Simpson PJ, Mickelson JK, Shea MJ, Pitt B, and Lucchesi BR (1988) Sustained limitation by superoxide dismutase of canine myocardial injury due to regional ischemia followed by reperfusion. J Cardiovasc Pharmacol 11: 36-44

Werns SW, and Lucchesi BR (1990) Free radicals and ischemic tissue injury. Trends Pharmacol Sci 11: 161-166

Westlin W, and Mullane KM (1989) Alleviation of myocardial stunning by leukocyte and platelet depletion. Circulation 80: 1828-1836

Westlin W, and Mullane KM (1988) Does captopril attenuate reperfusion-induced myocardial dysfunction by scavenging free radicals? Circulation 77 [suppl I]: I-30-I-39

Weyrich AS, Ma XL, and Lefer AM (1992) The role of L-arginine in ameliorating reperfusion injury after myocardial ischemia in the cat. Circulation 86: 279-288

Wheatley RM, Dockery SP, Kurz MA, Sayegh HS, and Harrison DG (1994) Interactions of nitroglycerin and sulfhydryl-donating compounds in coronary microvessels. Am J Physiol 266: H291-H297

White HD, Norris RM, Brown MA, Takayama M, Maslowski A, Bass NM, Ormiston JA, and Whitlock T (1987) Effect of intravenous streptokinase on left ventricular function and early survival after acute myocardial infarction. N Engl J Med 317: 850-855

White HD, Rivers JT, Maslowski AH, Ormiston JA, Takayama M, Hart HH, Sharpe DN, Whitlock RML, and Norris RM (1989) Effect of intravenous streptokinase as compared with that of tissue plasminogen activator on left ventricular function after first myocardial infaction. N Engl J Med 320: 817-821

White DG, Cannon TR, Garratt H, Mundin JW, Sumner MJ, and Watts IS (1993) Endothelin ETA and ETB receptors mediate vascular smooth-muscle contraction. J Cardiovasc Pharmacol 22 Suppl 8: S144-8

Wijns W, Serruys PW, Slager CJ, Grimm J, Krayenbuehl HP, Hugenholtz PG, and Hess OM (1986) Effect of coronary occlusion during percutaneous transluminal angioplasty in humans on left ventricular chamber stiffness and regional diastolic pressure-radius relations. J Am Coll Cardiol 7: 455-463

Williamson JR, Schaffer SW, Ford C, and Safer B (1976) Contribution of tissue acidosis to ischemic injury in the perfused rat heart. Circulation 53: 3-14

Willich SN, Lewis M, Lowel H, Arntz HR, Schubert F, and Schroder R (1993) Physical exertion as a trigger of acute myocardial infarction. Triggers and Mechanisms of Myocardial Infarction Study Group. N Engl J Med 329: 1684-1690

Wilson RF, Laughlin DE, Ackell PH, Chilian WM, Holida MD, Hartley CJ, Armstrong ML, Marcus ML, and White CW (1985) Transluminal, subselective measurement of coronary artery blood flow velocity and vasodilator reserve in man. Circulation 72: 82-92

Winder WW, Arogyasami J, Barton RJ, Elayan IM, and Vehrs PR (1989) Muscle malonyl-CoA decreases during exercise. J Appl Physiol 67: 2230-2233

Wink DA, Darbyshire JF, Nims RW, Saavedra JE, and Ford PC (1993) Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O2 reaction. Chem Res Toxicol 6: 23-27

Winniford MD, Kennedy PL, Wells PJ, and Hillis LD (1986) Potentiation of nitroglycerin-induced coronary dilatation by N-acetylcysteine. Circulation 73: 138-142

Wisneski JA, Gertz EW, Neese RA, Gruenke LD, Morris DL, and Craig JC (1985) Metabolic fate of extracted glucose in normal human myocardium. J Clin Invest 76: 1819-1827

Wisneski JA, Gertz EW, Neese RA, and Mayr M (1987) Myocardial metabolism of free fatty acids. Studies with 14C-labeled substrates in humans. J Clin Invest 79: 359-366

Wong SH, Kjight JA, Hopfer SM, Zaharia O, Leach CN, and Sunderman FW (1987) Lipoperoxides in plasma as measured by liquid-chromatographic separation of malondialdehyde-thiobarbituric acid adduct. Clin Chem 33: 214-220

Woodman OL, and Vatner SF (1987) Coronary vasoconstriction mediated by alpha 1- and alpha 2-adrenoceptors in conscious dogs. Am J Physiol 253: H388-93

Wozniak A, Betts WH, Murphy GA, and Rokicinski M (1993) Interleukin-8 primes human neutrophils for enhanced superoxide anion production. Immunology 79: 608-615

Yagi K (1984) Assay for blood plasma or serum. Methods Enzymol 105: 328-331

Yeung AC, Vekshtein VI, Krantz DS, Vita JA, Ryan TJ, Jr., Ganz P, and Selwyn AP (1991) The effect of atherosclerosis on the vasomotor response of coronary arteries to mental stress. N Engl J Med 325: 1551-1556

Yoshida S, Ganz W, Donoso R, Marcus HS, and Swan HJ (1971) Coronary hemodynamics during successive elevation of heart rate by pacing in subjects with angina pectoris. Circulation 44: 1062-1071

Young IS, Purvis JA, Lightbody JH, Adgey AAJ, and Trimble ER (1993) Lipid peroxidation and antioxidant status following thrombolytic therapy for acute myocardial infarction. Eur Heart J 14: 1027-1033

Yusuf S, Collins R, MacMahon S, and Peto R (1988) Effect of intravenous nitrates on mortality in acute myocardial infarction: an overview of the randomised trials. Lancet 1: 1088-1092

Zamorano J, Erbel R, Ge J, Gorge G, Kearney P, Koch L, Scholte A, and Meyer J (1994) Spontaneous plaque rupture visualized by intravascular ultrasound. Eur Heart J 15: 131-133

Zijlstra F, de Boer MJ, Hoorntje JC, Reiffers S, Reiber JH, and Suryapranata H (1993) A comparison of immediate coronary angioplasty with intravenous streptokinase in acute myocardial infarction. N Engl J Med 328: 680-684

Zimmerman BJ, and Granger DN (1990) Reperfusion-induced leukocyte infiltration: role of elastase. Am J Physiol 259: H390-4

Zucchi R, Ronca-Testoni S, Yu G, Galbani P, Ronca G, and Mariani M (1994) Effect of ischemia and reperfusion on cardiac ryanodine receptors-sarcoplasmic reticulum Ca^{2+} channels. Circ Res 74: 271-280

APPENDIX

Addendum and Corrections

Rewritten Summary (page xii)

SUMMARY

Previous studies have shown that prolonged myocardial ischaemia resulted in depletion of endogenous tissue antioxidant mechanisms. Where such ischaemic tissue was subsequently reperfused either pharmacologically or mechanically, many models have consistently reported an associated immediate and sometimes prolonged release of oxygen-derived radical species and oxidants. This oxidative stress may be the primary event that resulted in further exacerbation of cellular injury, and had been designated the term "reperfusion injury." Previous investigators believed that the overall effect of myocardial reperfusion injury was manifested as the phenomenon of myocardial "stunning," reperfusion arrhythmias and possibly further necrosis after reperfusion. Strategies aimed at limiting oxidative stress in humans have met with little success to date. However, thiol-containing drugs have shown some promise, perhaps based upon extensive tissue penetration, multiple pharmacological effects and minimal toxicity. Of such agents, N-acetylcysteine (NAC) is already in clinical use as adjunct to glyceryl trinitrate (GTN) therapy in patients with ischaemic heart disease. Furthermore, NAC has been shown *in vitro* both to limit free radical release from neutrophils and to increase clearance of ("scavenge") a variety of free radicals and oxidants.

A variety of plasma biochemical markers of the effects of oxidative stress are frequently used to assess the extent of such changes. Two such markers in this series of studies measured both changes in the global or regional redox state of glutathione and the release of the lipid peroxidation product, malondialdehyde (MDA). A sensitive and specific assay for malondialdehyde in plasma was developed.

Three models of myocardial ischaemia and reperfusion were developed and used to assess the mechanism of action and potential utility of NAC beyond that understood in the literature:

1. An isolated Krebs-perfused rat heart, measuring cardiac haemodynamics and MDA concentrations in the coronary effluent,

2. An *in vivo* human model of pacing-induced myocardial ischaemia in patients with stable angina pectoris and significant coronary stenoses in the left coronary system, measuring cardiac and coronary haemodynamics and the trans-coronary gradients of lactate, MDA utilising coronary sinus catheterisation,

3. An *in vivo* human model of patients receiving treatment with intravenous streptokinase for evolving acute myocardial infarction.

In all models the effect of the use of NAC either alone or in combination with GTN were assessed.

In the isolated Krebs-perfused rat heart model, total global ischaemia for 30 minutes followed by reperfusion was not associated with a marked or sustained release of myocardial MDA into the coronary effluent during reperfusion. However, this protocol resulted in marked LV dysfunction after 30 minutes of reperfusion. A small nonsustained incremental increase in myocardial MDA release in the early reperfusion period was noted only if either metabolic or oxidative stress was used in combination with ischaemia. Protocols that produced incremental stress over and above ischaemia included depletion of glucose before ischaemia and infusion of activated human neutrophils before and after ischaemia. In parallel, LV dysfunction was more severe as compared with ischaemia alone. Perfusion of the rat heart with 200 μmol/L NAC, either throughout the protocol or at reperfusion, failed to protect the heart from ischaemia/reperfusion induced left ventricular dysfunction. Also, NAC in this protocol failed to change the pattern of myocardial MDA release into the coronary effluent.

Right ventricular pacing in patients with (n = 12) or without (n = 2) significant left coronary artery disease at 140 bpm for three minutes was associated with a significant myocardial release of MDA. This result suggested the occurrence of oxidative stress during ventricular tachycardia. In contrast, no significant release of MDA from the nonischaemic femoral vascular bed (n = 2) was detectable after mild exercise. Furthermore, myocardial MDA release was not detectable during rapid atrial pacing in patients (n = 5) with significant left coronary artery disease. Intravenous NAC infusion (5g over 10 minutes before the onset of ischaemia, n = 7) decreased myocardial lactate flux but myocardial MDA flux did not significantly decrease in this small study. Furthermore, the addition of 5 μg/min intravenous glyceryl trinitrate (GTN) did not potentiate NAC effect.

Developmental studies were carried out to assess the technical validity of blood sampling from the coronary sinus for myocardial metabolic assessment. It was noted that plasma lactate concentration artefactually decreased when sampled through a 90 cm coronary sinus catheter due to extraction of lactate by the catheter. Because of this *ex vivo* extraction, the metabolic extent of myocardial ischaemia during rapid pacing would be underestimated.

Prior to reperfusion of an acutely occluded coronary artery resulting in evolving infarction, significant aggravation of myocardial ischaemia occurred within the first 20 minutes after initiation of intravenous streptokinase infusion (n = 20). Aggravation of myocardial ischaemia was manifested both by increased S-T segment elevation and increased slope of the S-T segment versus time curve during continuous 12 lead electrocardiographic (ECG) monitoring. Aggravated S-T segment elevation was temporally, but not quantitatively associated with transient hypotension in most patients. Therefore, it was likely that streptokinase aggravated ischaemia before reperfusion, possibly via an unidentified

mechanism common to both phenomena. Systemic hypotension *per se* was unlikely to be the causative mechanism by which ischaemia was aggravated because of the lack of a quantitative correlation. This data suggested that better protection of the ischaemic myocardium before reperfusion may improve overall myocardial salvage. It was also possible that this effect contributes to the "early hazard" of thrombolytic therapy.

A study was performed of patients with evolving acute myocardial infarction comparing the treatment protocols of streptokinase in combination with intravenous NAC and GTN (n = 20) with streptokinase and intravenous GTN (n=7). The results of this study showed a statistically significant reduction in oxidative stress for NAC-treated patients as compared with patients managed without NAC. Plasma MDA concentration was lower over the first four hours and plasma GSH:GSSG ratio higher at four and 24 hours in the NAC-treated patients. Plasma concentration of GSH was directly proportional to the plasma concentration of NAC. Treatment with the combination of NAC, GTN and streptokinase was associated with a non-significant trend toward more rapid reperfusion of the occluded infarct related artery and better cardiac haemodynamics in the early post-infarction period.

Therefore, NAC in combination with GTN appeared to decrease oxidative stress in human models of myocardial ischaemia and reperfusion with no significant adverse effects. Furthermore, this combination may have improved the speed of reperfusion and increased myocardial salvage in evolving acute myocardial infarction. The mechanism of this reduction in oxidative stress could not be explained from these studies. Whether the reduction in oxidative stress is secondary to the antioxidant effect of NAC by potentiation of the anti-ischaemic and anti-platelet effects of GTN is unclear. However, these compelling results require further investigation in larger clinical trials to

assess the haemodynamic and clinical effects of the reduction of oxidative stress during myocardial ischaemia and infarction. Furthermore, determination of the mechanisms of GTN and/or NAC effect on myocardial ischaemia, cytoprotection and possible synergism with streptokinase during reperfusion requires further investigation.

An appendage to Chapter 1

1.7.3.2 Animal studies

(paragraph 3, page 147)

The potentiation of the anti-ischaemic and antiplatelet effect of GTN by NAC may also be another mechanism by which NAC may reduce oxidative stress. As discussed in 1.5.2.1 the extent of oxidative stress was directly correlated to the length and extent of myocardial ischaemia. With the potentiation of GTN effect by NAC (see 1.7.2.2), overall ischaemia and consequently oxidative stress would be reduced. This hypothesis was examined indirectly by Lefer *et al* {368}. They demonstrated a cytoprotective effect from the infusion of a sulphydryl-containing organic nitrate (SPM-5185) in an open-chest dog model of one hour of myocardial ischaemia followed by 4.5 hours reperfusion. Infusion of SPM-5185 from the time of reperfusion was associated with decreased infarct size and LV dysfunction.

Furthermore, NO has been shown to decrease neutrophil adhesion during reperfusion after myocardial ischaemia (see 1.5.2.3). Potentially GTN, as a NO donor may also have an anti-neutrophil effect. This has not been confirmed in the literature. However, if this were the case, NAC would further limit neutrophil adhesion and oxidant release during reperfusion after ischaemia. Therefore, NAC in combination with GTN may have a variety of possible mechanisms by which they exert antioxidant effects. Conclusive evidence of these putative mechanisms, and an indication of the relative significance of each is not available in the literature. It is likely that multiple mechanisms will apply *in vivo*.

Changes to Chapter 3

TABLE 3.1 (page 205) Summary of haemodynamic effects and release of MDA by the isolated rat heart after a variety of myocardial stresses. (results mean ± SD)

Experimental protocol	No. of hearts	MDA release (AUC)	Haemodynamic Parameters After 30 minutes Reperfusion		
			Recovery of LVDP (% baseline)	δLVDias (mmHg)	Perfusion Pressure (% baseline)
30 min anoxia	2	246 ± 90	75 ± 9	15 ± 14	131 ± 29
15 min total global ischaemia	3	127 ± 172	69 ± 36	25 ± 14	145 ± 49
30 min total global ischaemia	12	86 ± 53	40 ± 18	25 ± 14	109 ± 14
60 min total global ischaemia	2	120 ±25	0 ± 0	55 ± 37	129 ± 38
10 min no glucose + 30 min total global ischaemia	6	180 ± 146*	12 ± 18†	42 ± 23‡‡	144 ± 27††
PMN/plasma + 30 min total global ischaemia	2	64 ± 17	42 ± 45	18 ± 14	132 ± 10
Activated PMN + 30 min total global ischaemia	6	148 ± 98‡	16 ± 12¥	45 ± 25#	186 ± 72**

Legend: AUC, area under the curve; δLVDias, change in left ventricular diastolic pressure from baseline; LVDP, left ventricular developed pressure; MDA, malondialdehyde; min, minutes; mmHg, millimetres of mercury; No., number; PMN, polymorphonuclear neutrophils; U, units. (P=0.056, ‡p=0.095, †p=0.007, ¥p=0.01, ‡‡p=0.07, #p=0.0.048, ††p=0.006, **p=0.002 versus 30 minutes total global ischaemia)

3.1.5.2 Myocardial MDA release during myocardial ischaemia and reperfusion

(paragraph 2, page 217)

Inflicting more oxidant stress on the heart beyond total global ischaemia produced a larger release of MDA from the myocardium. In particular, 10 minutes of hypoglycaemia before ischaemia was associated with an increased release of MDA (180 ± 146 versus 86 ± 53, p = 0.056). The infusion of activated PMN before and after reperfusion was also associated with a similar trend, again not reaching statistical significance (148 ± 98 versus 86 ± 53, p = 0.095). This trend toward increased release of myocardial MDA during reperfusion was associated with significantly increased left ventricular dysfunction. These results demonstrated that release of myocardial MDA from the isolated Krebs-perfused rat heart gave a small signal of short duration. Consequently, analyses of changes in this signal were limited.

3.1.6 Discussion

(paragraph 3, page 218)

A main purpose of this series of experiments was to determine the conditions required for the rat myocardium to release the lipid peroxide product MDA. This putative marker of oxidative stress could then have been used to assess the effect of pharmacological interventions aimed at reducing oxidative stress. These experiments demonstrated that the isolated rat heart released a small amount of MDA under normal conditions. However, this model did not result in a sustained, elevated release of myocardial MDA after a variety of manoeuvres previously shown to induce oxidative stress (Maupoil and Rochette, 1988; Garlick et al. 1987; Maupoil et al. 1990). In contrast, LV performance was impaired to a variable but marked extent. Only after significant preceding combined ischaemic and metabolic or oxidative stress was any trend toward significant lipid peroxidation as measured by myocardial MDA release seen. These results suggested that in the rat heart model used for these studies there was little lipid peroxidation making it an insensitive and/or late biochemical marker of oxidative stress.

TABLE 3.3 (page 227) Summary of the effect on haemodynamic function and coronary effluent MDA concentration of 200 μmol/l NAC infusion in isolated rat hearts subjected to 30 minutes total global ischaemia and 30 minutes reperfusion.

Treatment Group	No. of hearts	MDA Release (AUC)	Haemodynamics at 30 minutes Reperfusion		
			LVDP (% baseline)	δLVDias (mmHg)	Perfusion Pressure (% baseline)
control	12	86±53	40±18	25±15	109±14
NAC throughout	6	110±47	32±22	20±15	135±65
NAC at reperfusion	6	82±68	44±21	32±32	150±27
ANOVA		p, NS	p, NS	p, NS	p, NS

Legend: ANOVA, analysis of variance; AUC, area under the curve; LVDP, left ventricular developed pressure; δLVDias, change in left ventricular diastolic pressure from baseline; MDA, malondialdehyde; mmHg, millimetres of mercury; NAC, N-acetylcysteine; No., number; NS, not significant; p, probability.

Changes to Chapter 6

6.5 Discussion

(paragraph 8, page 323)

The precise mechanism of biochemical and haemodynamic effects of the NAC/GTN/streptokinase treatment regimen cannot be deduced from this study. As discussed in 1.7.3, it remains unclear as to the extent of antioxidant effect attributable to NAC alone compared with the reduction of ischaemia and thus oxidative stress by the combination of NAC/GTN. Indeed, it is uncertain that NAC must be administered prior to reperfusion to exert a beneficial effect. However, the current regimen and previous animal model of myocardial ischaemia/reperfusion (Ceconi et al. 1988) are concordant with the hypothesised major mechanism of effect. The timing of therapy, and the examination of components of NAC/GTN or NAC/streptokinase interaction versus direct NAC effect would require future larger studies, comparing NAC/GTN, NAC and GTN with streptokinase monotherapy both before and after reperfusion with streptokinase. Furthermore, the safety and effect of other thrombolytic agents with NAC/GTN cannot be determined (Nicolini et al. 1994).

Changes to Chapter 7

(paragraph 3, page 326)

The studies in Chapter 3 used a Langendorff-perfused rat heart model. Only severe metabolic stress in the form of a combination of hypoglycaemia and myocardial ischaemia showed evidence of an appreciable concentration of coronary effluent MDA during early reperfusion. However, this release of myocardial MDA was not sustained for more than two minutes into the reperfusion period and represented an inadequate biochemical marker of oxidative stress. In contrast, 30 minutes of total global ischaemia followed by 30 minutes of reperfusion resulted in marked impairment of left ventricular function. The addition of 200 μmol/L NAC into the perfusate, either before the onset of ischaemia or at reperfusion was not associated with a significant recovery of left ventricular function as compared with Krebs solution alone.

(Paragraph 8, page 328)

An important observation was the difference between the human *in vivo* and rat *in vitro* heart regarding extent of myocardial lipid peroxidation in response to oxidative stress. The human heart readily released MDA in response to oxidative stress as compared with the small, nonsustained response of the Langendorff-perfused rat heart. The reason for the discrepancy is unclear from these studies. However, from this thesis it can be concluded that the rat heart is not necessarily an appropriate model for of the circumstances within the human heart.