Chapter 1

INTRODUCTION
1.1. BACKGROUND

Cardiovascular disease is the leading cause of mortality in Australia and over half of these deaths are attributable to coronary heart disease (CHD) (Australian Institute of Health and Welfare (AIHW), 2001). While survival from an acute myocardial infarction (AMI) has improved since the 1960’s, it remains the major cause of death of patients with CHD.

Only recently are we beginning to understand the pathophysiology associated with coronary artery disease (CAD). Little was known of the coronary circulation’s functional significance until interest in this area was sparked by William Heberden’s presentation to the College of Physicians in London in 1768. It was in this paper that Heberden first described patients suffering from ‘angina pectoris’:

‘Those, who are afflicted with it, are seized, while they are walking, and more particularly when they walk soon after eating, with a painful and most disagreeable sensation in the breast, which seems as if it would take their life away, if it were to increase or continue: the moment they stand still, all this uneasiness vanishes’ (Fye, 1996).

Edward Jenner, who initially worked with Heberden, made the first direct link with angina pectoris and coronary atherosclerosis. At autopsy he discovered in these patients ‘a kind of firm fleshy tube, formed within the coronary vessel, with a considerable quantity of ossific matter dispersed irregularly through it’ (Fye, 1996).

It is even more recently, that the link between thrombotic occlusion of coronary arteries and AMI was made. While Vulpian and Osler (Osler, 1889) late in the 19th
century linked the occlusion of a coronary artery with an AMI, there was no distinct clinical syndrome that was thought to accompany this pathological finding. The first paper to acknowledge the clinical syndrome of acute coronary thrombosis published in German in 1910 (Obrastzow and Straschesko, 1910) attracted little attention until James Herrick’s landmark paper ‘Certain clinical features of sudden obstruction of the coronary arteries’ was published in English in 1912.

It is now understood that the pathophysiology of ACS involves atherosclerotic plaque disruption with associated thrombosis and vasoconstriction (Theroux and Fuster, 1998). Over the last 30 years some major advances have been made in our understanding of the pathogenesis of ACS. No contribution has been greater than the discovery of the link between vascular reactivity and endothelial function (Furchgott and Zawadzki, 1980). Once viewed largely as a semi-permeable barrier between the blood and tissues, the vascular endothelium is now known to have a pivotal role in the maintenance of vascular haemostasis including vasomotor effects, platelet function, thrombus formation and fibrinolysis, cell growth and inflammation predominantly through the effects of NO’ (Dillon and Vita, 2000). Nitric oxide also has important effects on myocardial contractility making this molecule one of the most pivotal in cardiovascular physiology. More importantly impaired bioavailability of NO’ through a decrease in NO’ production, an increase in NO’ inactivation or a decrease in NO’ sensitivity is present in not only atherosclerotic disease (Anderson et al., 1995; Freiman et al., 1986) but also in patients with known risk factors of CHD such as hypertension (Calver et al., 1992a; Panza et al., 1994), hypercholesterolaemia (Casino et al., 1993; Chowienczyk et al., 1992; Creager et al., 1990) and diabetes mellitus (DM) (McVeigh et al., 1992; Williams et al., 1996). Understanding the mechanisms behind impaired NO’ bioavailability, particularly in high risk patients,
will better enable us to tailor therapy to treat and possibly even prevent this phenomenon.

1.2 NITRIC OXIDE (PHYSIOLOGY)

Nitric oxide is a heterodiatomic free radical which at room temperature is a
colourless gas with a boiling point of –151°C at 1 Atm (Loscalzo, 2000a).
Comparable with molecular oxygen, NO– has a solubility of 1.9mM/atm (Armour,1974; Dean, 1985). However, its diffusion constant in aqueous solution, 3300µm²s⁻¹
at 37°C, is 1.4 times greater than that of oxygen (Kelm, 1999; Pryor et al., 1997).
Chemically, NO– can exist in three redox forms: nitrosonium (NO+), nitric oxide
(NO–) and nitroxyl anion (NO·). In the presence of O₂–, NO– forms peroxynitrite
(OONO–) in a diffusion limited reaction reducing NO– bioavailability. Peroxynitrite
can further react with tyrosyl residues in proteins to form 3-nitrotyrosyl residues and
with thiols (RSH) to form S-nitrosothiols (Loscalzo, 2001). Nitric oxide’s half-life
in-vivo is approximately 10 seconds (Moncada et al., 1991). In aqueous aerobic
solutions, NO– predominantly forms nitrite (NO₂–), while in the presence of
oxyhaemoglobin and oxymyoglobin, NO– is readily oxidized to nitrate (NO₃–)
(Ignarro, 1990; Ignarro et al., 1993). Oxidized derivatives of NO– with nitrosating
capacity such as N₂O₃, react with thiols to form S-nitrosothiols (Wink and Ford,
1995). S-nitrosothiols (RSNO) and S-nitrosation of other proteins such as albumin
offer a stable and available reservoir of NO– (Kelm, 1999; Stamler et al., 1992). By
reacting with heme iron, nitrosyl-heme charge-transfer complexes can form,
accounting for the biological activation of guanylyl cyclase by NO–. Lipid peroxides
(LOOH) can yield lipid peroxyl radicals (LOO·), which can react with NO– to form
lipid peroxynitrites (LOONO) and hydrogen peroxide (H$_2$O$_2$) can react with NO’ to form nitrous acid (HNO$_2$) and hydroxyl radical.

Nitric oxide’s predominant effects in the cardiovascular system result in cyclic guanosine 3’‚5’ monophosphate (cGMP) mediated effects. This results in vasodilation, inhibition of platelet aggregation and smooth muscle proliferation, anti-inflammatory properties, modulation of adhesion molecules as well as dose-dependent effects on the myocardium.

1. 2. 1. Synthesis

The free radical, NO’, is formed as a byproduct of the Nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen-dependent five electron oxidation of the terminal guanidino nitrogen of L-arginine to form L-citrulline via the intermediate NG-hydroxy-L-arginine (figure 1) (Ignarro, 1989). This reaction is catalyzed by a family of enzymes, nitric oxide synthase (NOS). Only a small amount is nonenzymatically produced from the chemical reduction of nitrite (Weitzberg and Lundberg, 1998). Although this process was initially described in vascular endothelial cells (Palmer et al., 1988) its effects are not limited to the cardiovascular system. It has subsequently been discovered to modulate the gastrointestinal, (Stark and Szurszewski, 1992) central nervous, (Garthwaite et al., 1988; Knowles et al., 1989) respiratory, (Gaston et al., 1993) genito-urinary (Burnett, 1995) and immunological systems (Albina and Reichner, 1995).
L-arginine (2-amino-5-guanidinovaleric acid) is a basic semi-essential amino acid that is the precursor of nitric oxide. Plasma L-arginine levels are regulated by dietary intake, protein turnover, arginine synthesis and metabolism.

L-arginine is metabolized via three main pathways, via NOS to NO̅, through the Krebs-Henseleit urea cycle via arginase to ornithine or via arginine decarboxylase to agmatine (Blantz et al., 2000). In a standard adult western diet, approximately 5.4 grams of L-arginine is absorbed each day, as each gram of dietary protein supplies approximately 54mg of L-arginine (Boger and Bode-Boger, 2001). The majority of plasma L-arginine is derived from the diet while small amounts are synthesized in the kidney from L-citrulline via the urea cycle (Dhanakoti et al., 1990). The hepatic system is capable of producing large amounts of L-arginine from argininosuccinate (Watford, 1991). The liver however contributes only minimal amounts to the systemic L-arginine pool, which is attributed to the low activity of the γ+ transporter of L-arginine in hepatocytes (Pardridge and Jefferson, 1975). L-arginine is rapidly
hydrolyzed to urea and ornithine by arginase. Indeed upregulation of either isoform of arginase (AI hepatic or AII extrahepatic) appears to limit NO\textsuperscript{•} production by limiting substrate for NOS (Buga et al., 1996; Chang et al., 1998; Hey et al., 1997; Que et al., 2002; Wang et al., 1993).

The intracellular transportation of L-arginine is mediated by several independent systems. The most important into endothelial cells is the \textit{y}+ transport system (Greene et al., 1993; Zharikov and Block, 1998). These high-affinity cationic arginine transporters (CAT1 and CAT2B) (Kavanaugh, 1993; Wang et al., 1991) at physiological L-arginine concentrations are responsible for 70% of its intracellular movement (Maxwell, 2002). Under normal physiological conditions this transport system is the rate limiting step for intracellular L-arginine availability, but not for NO\textsuperscript{•} synthesis. At higher (supra-physiological) concentrations of L-arginine (1mM), 70% of its transportation occurs by a nonsaturable component (Sobrevia et al., 1995).

A number of studies have assessed the pharmacokinetics of L-arginine either by intravenous or oral administration. Peak plasma levels are reached 20-30mins after intravenous infusion (Boger and Bode-Boger, 2001) and 60-90mins after oral administration (Bode-Boger et al., 1994; Tangphao et al., 1999). Orally administered L-arginine is extensively metabolized by enterocytes after being taken up by the intestinal brush border membrane (Blanchier et al., 1991). Uncertainty remains as to the oral bioavailability of L-arginine which ranges between 21% and 68% (Bode-Boger et al., 1994; Tangphao et al., 1999). There is no first pass metabolism of L-arginine due to the low activity of \textit{y}+ transporter in hepatocytes (White and Christensen, 1982). Its half-life is between 1.5-2hrs (Bode-Boger et al., 1994; Matera et al., 1993) and it is metabolized predominantly to urea and subsequently excreted in
the urine. Less than 1% of L-arginine is converted to NO⁻ (Castillo et al., 1993; Leaf et al., 1989).

Whilst L-arginine vasodilates through endothelium-derived NO⁻ production, it has other endothelium-independent vascular effects. This may occur through the guanidine group on L-arginine directly scavenging superoxide anions, known to inactivate NO⁻ (Wascher et al., 1997). Due to its highly basic nature, L-arginine also has significant hormone secreting effects. L-arginine stimulates the secretion of growth hormone (Merimee et al., 1967), insulin (Schmidt et al., 1992a), glucagons (Gerich et al., 1974) and prolactin (MacAllister et al., 1995). Insulin, growth hormone and glucagons are all vasodilators (Boger and Bode-Boger, 2001; Maxwell and Cooke, 2000) : the vasodilating properties of intravenous L-arginine can be attenuated by inhibiting the associated increased insulin secretion with octreotide. In the same study, performed in healthy subjects, vasodilation was restored by subsequent insulin infusion (Giugliano et al., 1997b). While L-arginine infusion induces an immediate insulin release, growth hormone release is delayed. Inhibiting both hormones with somatostatin inhibits only the late increase in NO⁻ production, implying that growth hormone contributes to the prolonged NO⁻-dependent vasodilation in high doses of intravenous L-arginine administration (Bode-Boger et al., 1999). Oral administration of L-arginine however does not appear to have the same effect on growth hormone secretion (Isidori et al., 1981).

An associated hormone rise is one of many hypotheses to explain the ‘arginine paradox’. This paradox fails to find a NO⁻ mediated explanation to the vasodilating properties of exogenously administered L-arginine, as the endogenous concentration of L-arginine is in the millimolar range (Gold et al., 1989) and the $K_m$ of endothelial NOS is in the micromolar range (Bredt and Snyder, 1990), thus ensuring under
physiological conditions ample available substrate for NOS. This ‘paradox’ clouds the data in relation to NO− dependent vasodilation properties of exogenous L-arginine particularly in healthy subjects. Intravenous L-arginine enhances endothelial-dependent vasodilation in normal subjects as measured by forearm plethysmography (Imaizumi et al., 1992; Panza et al., 1993) and is associated with increased urinary excretion of cGMP (Bode-Boger et al., 1994) suggesting an increase in NO− bioavailability. While oral L-arginine administration has been shown to improve NO− dependent vasodilation in patients with endothelial vasodilator dysfunction (Maxwell and Cooke, 2000), mixed results are seen in healthy human controls (Chin-Dusting et al., 1996; Imaizumi et al., 1992; Panza et al., 1993).

1.2.1.2. Nitric oxide synthases.

As previously stated NO− formation is the result of the catalytic conversion of L-arginine to L-citrulline by nitric oxide synthase. The nomenclature used in the classification of the different NOS isoforms has undergone a number of changes. The initial classification resulted in the observation that NO− synthesis was not characteristic of unactivated inflammatory cells, but could be produced upon immunoactivation. This inducible or ‘iNOS’ was thought to be different from the constitutive-type of NOS or ‘cNOS’ that was located in neuronal cells and the endothelium. This however served to be a confusing classification as it became apparent that iNOS may function as a constitutive enzyme under physiological conditions in some tissues (ie. both pulmonary and urogenital epithelium and the renal medulla) (Guo et al., 1995; Morrissey et al., 1994) and that endothelial and neuronal type NOS may be induced under certain physiological and pathophysiological conditions such as haemodynamic shear-stress and nerve injury (Feron and Michel, 2000; Michel and Feron, 1997). Therefore a widely accepted
classification (Moncada et al., 1997) reflects the initial tissue of origin for the initial protein, neuronal cells, immune activated ‘inducible’ macrophages and the vascular endothelium. Therefore the three NOS isoforms are nNOS, iNOS and eNOS. To add further complexity the human NOS genes are officially categorized in order of isolation and characterization and therefore the genes that encode nNOS, iNOS and eNOS are named NOS1, NOS2 and NOS3 respectively.

There is approximately a 55% homology of the amino acid sequence of the three NOS isoforms with a particularly strong conservation in the protein regions involving catalysis (Michel et al., 1995). The flavin containing ‘reductase domain’ or C-terminal domain comprises nearly half of the molecule and has a sequence similar to the mammalian cytochrome P450 reductase. The N-terminal domain, heme or oxygenase domain contains a site for binding the enzymes heme prosthetic group (Feron and Michel, 2000). As all NOS isoforms catalyse the same reaction it is understandable that they require the same cofactors and prosthetic groups. These include the reduced form of NADPH and molecular oxygen, along with the flavins adenine dinucleotide (FAD) and mononucleotide (FMN), a heme moiety and tetrohydrobiopterin (Fukuto and Mayer, 1996). Although not fully understood, it appears that the reductase domain binds NADPH and transfers electrons through FAD and FMN to the heme or oxygenase domain where bound molecular oxygen is reduced and activated. This ultimately results in the oxidation of L-arginine to NO\textsuperscript{\dot{}} (Fukuto and Mayer, 1996). This electron transfer however requires the presence of the Ca\textsuperscript{2+} binding protein calmodulin (Stuehr, 1997) in nNOS and eNOS but interestingly not with iNOS (Cho et al., 1992; Nathan and Xie, 1994a). While for many years the exact role of tetrohydrobiopterin was uncertain (Mayer and Werner, 1995), recent evidence suggests its role is in electron transfer (Wei et al., 2002).
1. 2. 1. 2. 1 nNOS

NOS1 is located on chromosome 12 (Kishimoto et al., 1992) and encodes a protein of 1434 amino acids with a predicted molecular mass of 160kDa, making this the largest and most complicated of all NOS genes (Bredt et al., 1991; Nakane et al., 1993). Variations in nNOS have been noted. Transcription initiation in exon 3 and 4, as distinct from exon 2, results in a truncated protein of 125kDa termed TnNOS (Feron and Michel, 2000), however to date no associated protein has been identified (Wang et al., 1997). An insertion of 102bp between exons 16 and 17 has resulted in a new nNOS transcript termed µNOS. While expression of µNOS has been detected in both rat and human (Magee et al., 1996; Silvagno et al., 1996) like TnNOS its functional significance remains uncertain (Feron and Michel, 2000).

As suggested by its name, nNOS was initially discovered in rat and porcine cerebellum (Bredt and Snyder, 1990; Mayer et al., 1990; Schmidt et al., 1991) and subsequently in human neuronal tissue (Chao et al., 1996). It has however also been located in non-neuronal tissue, such as the lung (Asano et al., 1994), myocytes (Nakane et al., 1993; Silvagno et al., 1996), testis (Wang et al., 1997), skin (Shimizu et al., 1997), neutrophils, platelets (Wallerath et al., 1997) as well as vascular media and adventitia (Schwarz et al., 1999). In the heart nNOS is located in cardiac sarcoplasmic reticulum (SR) (Xu et al., 1999) and in the conduction pathways (Schmidt et al., 1992b; Tanaka et al., 1993) where it is postulated to play a role in catecholamine release and reuptake (Kaye et al., 1995; Schwarz et al., 1995). NOS1 expression can be modified by a number of both physiological and pathological conditions. Physiological upregulation of NOS 1 occurs in the rat via altering environmental lighting conditions mediated through the pineal gland (Spessert et al., 1995). NOS 1 also appears to be osmoreponsive, with a neuronal upregulation
associated with chronic salt loading (Kadowaki et al., 1994) or water deprivation (O'Shea and Gundlach, 1996), yet interestingly in rat kidney this appears to be the reverse (Singh et al., 1996a; Tojo et al., 1995). It also appears to play a role in the reproductive system as NOS1 is upregulated with oestrogen (Ceccatelli et al., 1996), during pregnancy (Weiner et al., 1994; Xu et al., 1996) and by testosterone in the male reproductive system.

1. 2. 1. 2 iNOS

NOS2 is located on chromosome 17 and encodes iNOS, a protein of 1153 amino acids with a predicted molecular mass of 130kDa (Feron and Michel, 2000; Marsden et al., 1994). Initially characterized in murine macrophages (Lyons et al., 1992; Xie et al., 1992) it subsequently has been expressed in human hepatocytes along with macrophages, monocytes, granulocytes, epithelial and endothelial cells, astrocytes, fibroblasts, keratinocytes and osteoblasts (Geller and Billiar, 1998). In the heart, NOS2 has been detected in the endocardial endothelium, infiltrating inflammatory cells, vascular smooth muscle, fibroblasts, microvascular endothelium and in cardiac myocytes (Kelly et al., 1996). One of the main differences between iNOS and the predominantly constitutive nNOS and eNOS is in its activation. As already briefly mentioned, eNOS and nNOS require an increase in resting intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]) for their binding of calmodulin and subsequent full activation (Michel and Feron, 1997). However, iNOS is able to bind calmodulin with extremely high affinity even at the low [Ca\(^{2+}\)] characteristic of resting cells and therefore its activation is not regulated by alterations in intracellular calcium concentration (Nathan and Xie, 1994b).
While there is evidence of some constitutive function of iNOS, its expression remains predominantly inducible. Immunoactivation of iNOS can occur as a result of infection and/or inflammation as is mediated through lipopolysaccharides (LPS) and cytokines such as interferon γ (IFN-γ), tumour necrosis factor-alpha (TNFα) and interleukin-1 (IL-1) (Kroncke et al., 1998). In cardiac myocytes, as in other tissues, induction of the NOS2 gene by IFN-γ and IL-1β appears to be preceded by activation of mitogen-activated protein kinase (MAPK) and signal transducers and activators of transcription 1α (STAT1α) phosphorylation (Chu et al., 1995; Singh et al., 1996b). Both human and rodent NOS2 promoters contain activation protein (AP-1) and gamma activating sequence (GAS) which are consistent with MAPK and STAT-mediated signalling (Chu et al., 1995; Spink et al., 1995), while LPS induces NOS2 in myocytes by a protein kinase C (PKC) dependent pathway (McKenna et al., 1995). A sequence homology in the promoter region for iNOS for human hypoxia-responsive element (HRE) (Semenza and Wang, 1994), results in induction of iNOS at decreased oxygen tension in IFN-γ stimulated macrophages. The requirement for IFN-γ costimulation for iNOS induction is thought to limit effects to inflammatory sites with hypoxia (Kroncke et al., 1998). With this in mind, it is no surprise to find evidence for iNOS expression in a wide variety of predominantly chronic inflammatory conditions including multiple sclerosis, pulmonary tuberculosis and sarcoidosis, asthma, inflammatory bowel disease and rheumatoid arthritis (Nathan, 1997).

Due to its predominantly inducible nature its role in normal physiological homeostasis is less certain. The human iNOS gene does however contain a shear-stress responsive element (GAGACC) which is identical to that in human eNOS (Nunokawa et al., 1994). Induction of NO⁺ by this shear-stress element seems to be a key mediator for protection of cardiovascular diseases via inhibition of leucocyte...
adhesion, platelet aggregation, and vascular smooth muscle cell proliferation
(Kroncke et al., 1998). The pulmonary epithelium also contains a significant amount
of constitutively expressed iNOS. Its exact role in this system is uncertain, but its
postulated actions range from regulation of pulmonary endothelial fluid, maintainance
or even regulation of arteriolar diameter and thus flow, predetermined by alveolar
oxygen thus maximizing gas transfer (Jia et al., 1996; Nathan, 1997). Normal uterine
contractions during delivery are also influenced by iNOS. In the nongravid uterus
iNOS is undetectable, yet in the cytokine rich enviroment of child-birth, iNOS is
expressed in myometrial myocytes only to decline again postpartum (Bansal et al.,
1997).

1. 2. 1. 2. 3 eNOS

NOS3 is located on chromosome 7 (Marsden et al., 1993) and encodes the 135kDa
eNOS protein. Initially identified and isolated from bovine aortic endothelial cells
(BAEC) (Förstermann et al., 1991; Pollock et al., 1991), eNOS is the dominant
constitutive source of NO˙ in the human vasculature and is located in the endothelial
cell layer. Its predominant function in the vasculature is NO˙ mediated vasodilation
along with inhibition of platelet aggregation, leukocyte adhesion, smooth muscle
proliferation and inflammation. Endothelial derived NOS is therefore ideally
positioned to mount a potent defence against atherogenesis and its acute thrombotic
complications. As with the other NOS isoforms, eNOS has also been located in other
tissues. These include bone (Helfrich et al., 1997) along with the gastrointestinal
(Teng et al., 1998), central nervous (Dinerman et al., 1994), genitourinary (Tracey et
al., 1994; Tseng et al., 1996; Zini et al., 1996), respiratory (Shaul et al., 1994) and the
endocrine (Colin et al., 1997) systems. More importantly for the cardiovascular
system however, it has also been located in cardiac myocytes (Balligand et al., 1995),
coronary and endocardial endothelium (Smith et al., 1991), cardiac conduction tissue (sinoatrial and atrioventricular nodal tissue) (Han et al., 1996a), platelets and megakaryocytes (Sase and Michel, 1995; Wallerath et al., 1997) and neutrophils (de Frutos et al., 2001).

A number of physiological stimuli have been associated with upregulation of eNOS expression. Shear-stress is one such potent stimulus (Tuttle et al., 2001). The mechanisms behind this are complex. Tyrosine kinase c-Src plays a central role in both enhancing NOS3 transcription and stabilizing eNOS mRNA while a more complex kinase cascade involving Raf, Ras, MEK 1/2 and ERK 1/2 result in increased eNOS transcription (Davies et al., 2001). Increased shear-stress and associated increase in e-NOS expression is seen with chronic exercise (Sessa et al., 1994) and at arterial side branches (Poppa et al., 1998) (ie intercostal branches of the aorta) secondary to either increased or turbulent blood flow. Endothelial cell proliferation also enhances eNOS expression, predominantly through certain growth factors (Li et al., 2002). Growing versus resting BAEC show both a three to sixfold increase in eNOS mRNA expression and associated increase in NO\(^\cdot\) production (Arnal et al., 1994). This may serve as a protective mechanism as denuded or damaged endothelium during regeneration also increases eNOS expression (Poppa et al., 1998). These findings may be mediated through growth factors such as transforming growth factor \(\beta\) (TGF-\(\beta\)) (Inoue et al., 1995), fibroblast growth factor (FGF) (Cuevas et al., 1991), vascular endothelial growth factor (VEGF) (Kroll and Waltenberger, 1998) or platelet-derived growth factor (PDGF) (Guillot et al., 1999)all of which have shown to increase eNOS expression.

Certain hormones, such as oestrogen, have also been shown to enhance eNOS expression (Kleinert et al., 1998). Of particular relevance to this thesis is the
stimulating effect of insulin on eNOS expression. Pharmacological doses of insulin result in increased NO\(^{-}\) production from human umbilical vein endothelial cells (HUEVC), with an EC\(_{50}\) of 500nM (Zeng and Quon, 1996). The effect on eNOS expression was not only seen after chronic exposure of insulin (6-7 days) (Alijada and Dandona, 2000; Ding et al., 2000) but was also seen with physiological concentrations (0.1-100nM) of insulin as early as 2-8 hours after exposure (Kuboki et al., 2000). This effect appears to be mediated by PI3K activation as insulin-induced eNOS expression is blocked by PI3K inhibitors (Kuboki et al., 2000). Both potent constrictors endothelin-1 and angiotensin II have also been shown to upregulate eNOS expression, a phenomenon which is mediated through their respective receptors (Li et al., 1999; Marsen et al., 1999).

Cytokines and LPS, potent upregulators of iNOS expression, appear to have the opposite effect on eNOS (Li et al., 2002). TNF\(\alpha\), for example, downregulates eNOS expression in BAEC, HUVEC and in human neutrophils (de Frutos et al., 2001; Lamas et al., 1992; Nishida et al., 1992; Yoshizumi et al., 1993). In high concentrations, NO\(^{-}\) itself works in a negative feedback mechanism to inhibit both activity and expression of eNOS (Abu Soud et al., 2000; Griscavage et al., 1995; Ma et al., 1996). Examined in both cultured human coronary artery endothelial cells and Sprague Dawley rats the NO\(^{-}\) donor S-nitroso-N-acetylpenicillamine (SNAP) significantly depressed eNOS expression, whereas oxyhaemoglobin (a NO\(^{-}\) trapping agent) had the opposite effect. The phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX) and cGMP analogue 8-bromo-cGMP downregulated, while 1H-[1,2,4]oxadiazolo-[4,3-2]quinoxalin-1-one (ODQ, a guanylate cyclase inhibitor), upregulated eNOS expression indicating, that the basis of feed-back was related to cGMP concentrations. Furthermore the downregulatory action of SNAP was completely overcome by ODQ and the upregulatory action of oxyhaemoglobin was
aborated by 8-bromo-cGMP, thus confirming the pivotal role of cGMP on the negative feedback of NO’ on eNOS expression (Vaziri and Wang, 1999).

1. 2. 1. 2. 4 Location

The evidence supporting the subcellular location of NOS is conflicting in nature. While it is possible that NOS is ubiquitous, its subcellular location appears to vary depending on the isoform and tissue involved. The location of nNOS in neurons is both soluble and particulate (Fürstermann et al., 1998). While initially thought to be predominantly soluble, it is now known that the particulate fraction of nNOS ranges from between 30-60% (Arbones et al., 1996; Hecker et al., 1994; Rodrigo et al., 1997) In neurons the particulate localization of nNOS is probably due to the PDZ/GLGF motif found in the NH₂-terminal sequence which binds to the postsynaptic density protein PSD-95 (Brenman et al., 1996), consistent with the enzyme’s role in neurotransmission. In neurons the particulate fraction of nNOS is predominantly associated with rough endoplasmic reticulum and within specialized electron-dense synaptic membrane structures (Xia and Bredt, 1996a). In skeletal muscle, the muscle specific isoform µNOS is predominantly particulate (Fürstermann et al., 1998). The PDZ/GLGF motif is also involved in attachment of µNOS, which in skeletal muscle is mainly to α₁-synthrophin (Brenman et al., 1995).

Similar to nNOS, iNOS has also been located in both soluble and particulate fraction. In macrophages, iNOS has been located in intracellular vesicles, which possibly reflects a role for NO’-dependent killing of opsonized intracellular microorganisms (Vodovotz et al., 1995). While the mitochondria appear to have their own functional NOS (mtNOS) (Giulivi et al., 1998; Tatoyan and Giulivi, 1998), it has a number of attributes similar to iNOS. Mitochondrial NOS shows strong crossreactivity with
monoclonal antibodies against iNOS and exhibits kinetic parameters, molecular weight and the requirement of tightly bound calmodulin similar to iNOS (Feron and Michel, 2000).

The Golgi apparatus and plasmalemmal caveolae are the predominant location of eNOS. Plasmalemmal caveolae are invaginations of the plasma membrane and can be identified by a biochemical marker for these unusual organelles, caveolin (Feron et al., 1996). However, eNOS contains no hydrophobic transmembrane domain, therefore to be located in the plasmalemmal caveolae it requires not only caveolin but also myristylation, palmitoylation, and tyrosine phosphorylation (Förstermann et al., 1998). A caveolar complex has been described, involving the arginine transporter CAT1 and eNOS, therefore maximizing the delivery of substrate for NO\(^\cdot\) production (McDonald et al., 1997). As the binding of caveolin and calmodulin are mutually exclusive, it therefore is unlikely that iNOS is located in the plasmalemmal caveolae (Feron and Michel, 2000). In the resting state, caveolin suppresses NOS activity (Kinlay et al., 2001)(figure 2). The Golgi apparatus has also been found to be a target of eNOS (Liu et al., 1997a; Sessa et al., 1995). Even though it has been postulated that transport of eNOS from the plasmalemma to intracellular organelles such as the golgi apparatus is part of a physiological cycle highly sensitive to the state of cell activation, the exact role of golgi apparatus located eNOS remains uncertain.
1. 2. 2. Signalling

Traditionally, the effects of NO\textsuperscript{−} were thought to be exclusively mediated through stimulation of soluble guanylyl cyclase, inducing an increase in intracellular cGMP levels and in turn activation of cGMP- dependent kinase (protein kinase G [PKG]). While this remains the predominant pathway, a number of other signalling mechanisms are important for NO\textsuperscript{−} to exert its tissue specific effects.

1. 2. 2. 1. Vasculature

The vasodilator effects of NO\textsuperscript{−} were those initially identified. The initial effects on the vasculature of exogenous NO\textsuperscript{−} and its associated activation of guanylate cyclase in bovine coronary arteries was made in 1979 (Gruetter et al., 1979). A year later, Furchgott discovered that the endothelium played an active role in vasomotor regulation (Furchgott and Zawadzki, 1980). Prior to this, the endothelium was thought to be nothing more than a passive lining of the vasculature. The definitive
link between Furgott’s ‘endothelium-derived relaxing factor’ and NO\(^-\) was not made however for a further seven years (Ignarro et al., 1987; Palmer et al., 1987).

Shear-stress is one of the main stimuli for physiological NO\(^-\) production. This is mediated by an increase in endothelial intracellular Ca\(^{2+}\) levels, binding to calmodulin and resultant eNOS activation. A number of processes appear to be involved in shear-stress induced NO\(^-\) liberation. The mechanical triggers that induce shear-stress related changes are unknown, but are thought to come from alterations in the cellular cytoskeleton. In knock-out mice lacking the structural protein vimentin, flow-mediated dilatation (FMD) is greatly reduced (Henrion et al., 1997). During shear-stress, potassium channels are activated in the endothelium, resulting in hyperpolarisation (Cooke et al., 1991; Olesen et al., 1988). This results in an increase in Ca\(^{2+}\) influx (Cooke et al., 1991; Luckhoff and Busse, 1990) and subsequent eNOS mediated NO\(^-\) production (Jacobs et al., 1995). The initial Ca\(^{2+}\)-dependent endothelial release is only transient. The prolonged phase is Ca\(^{2+}\)-independent and is thought to be mediated by tyrosine phosphorylation-signalling cascades, such as the tyrosine phosphorylation of eNOS (Ayajiki et al., 1996; Fleming et al., 1997; Fleming et al., 1998).

Shear-stress or ‘flow-mediated’ dilatation is the mechanism widely used to assess in vivo endothelial function utilizing impedance plethysmography (Hokanson et al., 1975) or brachial ultrasound (Celermajer et al., 1992) techniques. These effects however are not exclusive to NO\(^-\), as shear-stress also liberates PGI\(_2\) (Cohen, 2000) and PDGF (Hsieh et al., 1992), as well as inhibiting endothelin release (Malek and Izumo, 1992) from the endothelium. The flow-mediated vasodilating effects of NO\(^-\) are thought to be most physiologically important in exercising muscle, where blood flow may vary by more than 100-fold (Cohen, 2000).
While shear-stress remains the main stimulus for physiological release of endothelial derived NO\(^\cdot\), certain humoral factors can also stimulate its production. Bradykinin and acetylcholine were the first substances noted to stimulate endothelial release of NO\(^\cdot\). The in vivo physiological significance of this finding however still remains uncertain. Humoral stimulation of NO\(^\cdot\) production appears mediated through a phospholipase C (PLC), inositol 1, 4, 5-trisphosphate (IP\(_3\)) mediated release of calcium and resultant activation of eNOS (Frey et al., 1989). An intact and functional endothelium ingeniously responds to many substances released by degranulated platelets (adenosine diphosphate [ADP], serotonin, adrenaline and vasopressin) (Cohen et al., 1983; Lopez et al., 1989) by releasing NO\(^\cdot\). The release of similar substances by leukocytes also stimulates endothelial NO\(^\cdot\) release (Kaul et al., 1994; Mugge et al., 1993). The problem arises when a ‘dysfunctional’ or denuded endothelium is present, as seen in atherosclerotic vessels, where NO\(^\cdot\) is not produced in response to degranulation of platelets or leukocytes. In this situation a potent vasoconstrictive response results.

Free intracellular calcium concentration [Ca\(^{2+}\)] exerts pivotal control in vascular smooth muscle contraction/relaxation. Alterations in [Ca\(^{2+}\)] are modulated by net flux through both the plasma membrane and SR, with increasing Ca\(^{2+}\) levels resulting in vasoconstriction. The SR’s handling of Ca\(^{2+}\) is determined by its release through the IP\(_3\) receptor and its uptake via Ca\(^{2+}\) ATPase. Phospholipase C catalyzes the hydrolysis of phosphatidylinositol 4,5-diphosphate (PIP\(_2\)) to form IP\(_3\) and therefore plays an important role in the liberation of Ca\(^{2+}\) from the SR (Ganong, 1993). Of the three PLC isoforms (β,γ,δ), PLC β is critical in vascular smooth muscle contraction (Heydrick, 2000).
There is little or no effect of NO• on resting smooth muscle. The principal action, therefore, of NO• is to prevent a rise in intracellular Ca^{2+} or to reduce an already raised level (Cohen, 2000; Heydrick, 2000; McDaniel et al., 1992). Nitric oxide activates the sarcolemmal Ca^{2+} adenosine triphosphatase (ATPase) and blunts intracellular Ca^{2+} entry through the L-type voltage-dependent Ca^{2+} channels resulting in a reduction of cytosolic Ca^{2+} concentration. The activation of sarcolemmal Ca^{2+} ATPase is mediated by PKG. This is consistent with the finding that little NO• mediated vasodilation is noted in PKG knock-out mice (Vrolix et al., 1988). Nitric oxide also inhibits both and the IP_3 receptor by phosphorylation and PLCβ, thus reducing the intracellular effect of IP_3. Protein kinase G ultimately influences the above reduction in intracellular Ca^{2+} levels, predominantly by phosphorylation of the calcium regulatory proteins. A second mechanism by which NO• mediates vasodilation is via hyperpolarization. This effect likewise is the result of an outward potassium current through both calcium-dependent (Archer et al., 1994; Bolotina et al., 1994; Robertson et al., 1993) and voltage-dependent (Carrier et al., 1997) potassium channels in the plasma membrane.

Nitric oxide is also a potent inhibitor of vascular smooth muscle cell growth, which likewise is predominantly mediated through cGMP-dependent effects on platelet derived growth factor (PDGF), MAPK, PLC and PKC (Heydrick, 2000). The NO• mediated inhibition of vascular smooth muscle cell growth via cGMP-independent mechanism is less studied but may result from inhibition of ribonucleotide reductase and prolongation of the G1 and S phases of cell growth (Sciorati et al., 1997).

Nitric oxide also has significant autocrine and paracrine effects on endothelial cells. In the endothelial layer, basal release of NO• is obviously dependent on an increase in Ca^{2+} levels with subsequent binding to calmodulin and associated eNOS activation
(Schmidt et al., 1992c). Cyclic GMP mediated effects of NO$^-$ are responsible for reduced production of prostacyclin (PGI$_2$) and endothelin (in the presence of elevated intracellular Ca$^{2+}$ levels) by the endothelium (Heydrick, 2000). In contrast, NO$^-$ has stimulatory effects on PGI$_2$ production in the absence of calcium mobilizing agents (Alanko et al., 1995). There appears to be no feedback mechanism on its own production, as sodium nitroprusside (SNP, a direct NO$^-$ donor) and associated increases in cGMP have little or no effect on NO$^-$ synthesis in endothelial cells (Kuhn et al., 1991).

Nitric oxide is also involved in maintenance of the endothelial barrier function. The effect of increased endothelial permeability by thrombin (Draijer et al., 1995), histamine (Huang and Yuan, 1997) and VEGF (Wu et al., 1996a) is attenuated by NO$^-$. This is mediated through one of two mechanisms:- firstly, increased PKA activation, through cGMP mediated inhibition of PDE III with a subsequent increase in cAMP (Lugnier and Komas, 1993), or secondly a decrease in intracellular Ca$^{2+}$ via a PKG-dependent inhibition of PLC (Draijer et al., 1995).

1.2. Platelets

For the unstimulated ‘innocent’ circulating platelet to play a pivotal role in a potential life-threatening vascular event a number of morphological changes need to occur. These changes are generally divided into five phases: (i) platelet adhesion and spread, (ii) platelet activation and secretion, (iii) platelet aggregation, (iv) platelet coagulant activity, and (v) clot retraction (Wu, 1996b).

Platelets adhere to the damaged subendothelial matrix via the binding of glycoprotein (GP) Ib-IX to von Willebrand factor (vWF). GPIb-IX is constitutively expressed on
the surface of platelets and only binds to vWF after it has interacted with the subcellular matrix. GPIb-IX is a transmembranous complex and the cytosolic portion of GPIb is coupled to submembranous actin-binding proteins (Fox, 1985). Binding of vWF to the membrane GPIb-IX results in actin activation which leads to platelet contraction and pseudopod formation with resultant spreading of the platelets at the damaged vessel wall. Signals are also generated to activate GPIIb-IIIa (Chow et al., 1992; Savage et al., 1992).

Once adhered to the subcellular matrix, the platelet is activated by collagen via the receptors GPIa-IIa (integrin α2β1), GPVI and GPIb-V-IX via vWF as a bridging molecule (Clemetson and Clemetson, 2001) as well as by thrombin. Thrombin binds to its receptor and activates the platelet by several independent and interactive signal transduction pathways (Van Obberghen-Schilling and Pouyssegur, 1993). At least two types of G proteins coupled to the thrombin receptor have been identified. The Gp protein coupled to thrombin receptor transduces the signal via PLC activation, which hydrolyses PIP₂ to produce two potent messengers: (i) IP₃, which triggers the release of calcium from the dense tubular system into the cytosol, and (ii) diacylglycerol, which activates PKC. Increased cytosolic calcium is involved in several key activation-related cellular processes in the platelet including thromboxane A₂ (TXA₂) biosynthesis, cell contraction and spreading. Elevated cytosolic Ca²⁺ triggered by IP₃ and PKC-mediated protein phosphorylation are two fundamental signals essential for platelet activation. Thrombin receptor is coupled to the Gi protein which leads to inhibition of adenylate cyclase activity. The Gi protein is also stimulated through the recently identified ADP receptor P2Y12 (Hollopeter et al., 2001; Savi et al., 2001). Cytosolic Ca²⁺ elevation and PKC activation are responsible for activating phospholipase A, which catalyses the cleavage of arachidonic acid from phospholipids in the activated platelet. Arachidonic acid is
converted to TXA$_2$ (Ohashi et al., 1992) via prostaglandin G$_2$ and prostaglandin H$_2$ formation.

Platelet aggregation is initiated by TXA$_2$ and ADP with subsequent release of, dense granules containing ADP, serotonin, ATP pyrophosphate and Ca$^{2+}$ followed by alpha granules containing fibrinogen, factor V, PDGF, platelet factor 4, beta thromboglobulin, albumin, fibronectin and thrombospondin (Kamath et al., 2001). A conformational change in GPIIb-IIIa results in the activated form becoming a high affinity receptor for fibrinogen. Fibrinogen binds GPIIb-IIIa from two separate platelets and thus forms platelet aggregates. Activation-induced GPIIb-III conformational changes and resultant binding of fibrinogen is the fundamental mechanism underlying platelet aggregation induced by all physiological agonists (Wu, 1996b).

Platelet activation and aggregation alters the platelet membrane to provide a procoagulant surface for clot formation. Activation of factor X and prothrombin occurs on the platelet membrane surface. Evidence to date implies that binding of IXa is promoted by factor VIIIa on activated platelets. Following binding of VIIIa to the activated membrane, factor IXa, factor X and calcium form a complex with factor VIIIa. This facilitates catalytic conversion of factor X to Xa by factor IXa. Similarly, it has been suggested that factor Va binds to a specific receptor on activated platelets. Factor Xa and calcium form a complex with Va, called prothrombinase, which catalyses the conversion of prothrombin to thrombin. Thrombin has several important roles in haemostasis and thrombosis. It catalyses the conversion of fibrinogen to fibrin; it catalyses the formation of activated factor VII (VIIa), a key enzyme in the initiation of coagulation cascade; it also converts factors V and VIII from inactive forms to active cofactors. As previously discussed,
thrombin is also a potent platelet activator and aggregant involved not only in initiating but also in recruiting and amplifying platelet aggregate formation. Its dual roles in coagulation reaction and platelet activation coordinate and amplify the concurrent formation of platelet and fibrin masses in the haemostatic and thrombotic plugs (Wu, 1996b).

Nitric oxide modulates platelet function by inhibiting adhesion (Radomski et al., 1987a; Radomski et al., 1987b; Wu et al., 1997) and aggregation (Hampton et al., 1967; Mellion et al., 1981), as well as enhancing disaggregation (Chirkov et al., 1991; Chirkov et al., 1992; Chirkov et al., 1993a). L-arginine enters the platelet by a single, saturable, sodium-independent transporter system (Vasta et al., 1995) as substrate for NOS isoforms that have been characterized in platelets. A constitutive isoform of NOS (platelet eNOS) has been located in human platelets and megakaryocytes (Pigazzi et al., 1995; Sase and Michel, 1995), requiring similar cofactors as eNOS itself. Inducible NOS has also been located in platelets (Lelchuk et al., 1992). As the platelet is anucleate and iNOS induction dependent on cytokine exposure, iNOS expression likely reflects the immediate environment of the prior megakaryocyte. While endothelial derived NO\textsuperscript{•} is obviously important in modulation of platelet function, platelet-derived NO\textsuperscript{•} only modestly modulates platelet aggregation, but importantly markedly inhibits platelet recruitment to the growing platelet thrombus (Loscalzo, 2001). Evidence to support these findings are found in the NOS3 knock-out mouse, confirming a reduction in bleeding times compared to its wild-type counterpart (Freedman et al., 1999). Ex-vivo flow cytometry studies in the same animal model also confirm the importance of platelet-derived NO\textsuperscript{•} in attenuating platelet recruitment (Freedman et al., 1999).
Nitric oxide stimulates intra-platelet guanylyl cyclase, resulting in an increase in cGMP and activation of PKG (Mellion et al., 1981). L-arginine stimulates cGMP mediated anti-platelet effects (Radomski et al., 1990) and an increase in platelet aggregation, observed with inhibition of cGMP formation by ODQ (Moro et al., 1996) supports this finding. Cyclic GMP also has effects by regulation of the intra-platelet PDE. In the platelet, three have been identified PDE II, PDEIII and cGMP specific PDE V (Haslam et al., 1999; Wallis et al., 1999). One mechanism by which cGMP mediates its platelet effects is through inhibiting PDE III (Fisch et al., 1995; Grunberg et al., 1995). Nitric oxide, as in other tissues, inhibits PLC activation via a PKG-dependent mechanism. This in turn inhibits the hydrolysis of PIP$_2$, and therefore reducing the levels of IP$_3$ and diacylglycerol, thus reduces intracellular Ca$^{2+}$ and PKC activity (Geiger et al., 1992; Nakashima et al., 1986; Waldmann and Walter, 1989).

Other cGMP-dependent mechanisms have also been implicated, such as phosphorylation of the signal molecule rap 1b (Reep and Lapetina, 1996) and the TXA$_2$ receptor (Wang et al., 1998) as well as inhibition of the Gi protein linked P2Y12 receptor (Aktas et al., 2002), resulting in reduced platelet aggregation.

1.2.2.3. Myocardium

Cardiac excitation-contraction coupling, the process from electrical excitation of the myocyte to contraction of the heart, is critically dependent on the second messenger Ca$^{2+}$ (Bers, 2002a). Indeed, myocyte mishandling of Ca$^{2+}$ is a central cause of contractile dysfunction (Pogwizd et al., 2001). During the cardiac action potential, Ca$^{2+}$ enters the cell through depolarization-activated Ca$^{2+}$ channels as inward Ca$^{2+}$ current (I$_{Ca}$), which contributes to the action potential plateau. Ca$^{2+}$ entry triggers Ca$^{2+}$ release from the SR (via ryanodine receptors). The combination of Ca$^{2+}$ influx and release raises the free [Ca$^{2+}$], allowing Ca$^{2+}$ to bind to the myofilament protein
troponin C, which then switches on the contractile machinery. For relaxation to occur, $[\text{Ca}^{2+}]_{i}$ must decline, allowing Ca$^{2+}$ to dissociate from troponin. This requires Ca$^{2+}$ transport out of the cytosol by four pathways involving SR Ca$^{2+}$-ATPase, sarcolemmal Na$^{+}$/Ca$^{2+}$ exchange, sarcolemmal Ca$^{2+}$-ATPase or mitochondrial Ca$^{2+}$ uniport (Bers, 2002a).

The signalling mechanisms behind NO’ effects on cardiomyocytes are complex. This may be due in part to the dose-dependent effects of NO’ on contractility. As will be discussed in detail later, NO’ is positively inotropic in low doses, while negatively inotropic with high doses (Kojda and Kottenberg, 1999; Mohan et al., 1996). Neuronal NOS is ideally located on the cardiac SR to effect Ca$^{2+}$ flux and inotropy (Xu et al., 1999). Nitric oxide stimulation of SR Ca$^{2+}$ release via the ryanodine receptors in vitro (Eu et al., 2000; Xu et al., 1998) suggests that NOS1 has a positive inotropic effect. This is supported by the evidence that NOS1 knock-out mice have an impaired inotropic response to β adrenoceptor agonists (Barouch et al., 2002). The most extensively examined intracellular signal transduction pathways for NOS2 induction in ventricular myocytes and microvascular endothelial cells are through IL-1β and IFNγ. IL-1β activates MAPK (ERK1/ERK2) while IFNγ initiates STAT1 alpha phosphorylation and translocation to the nucleus in endothelial cells and cardiomyocytes (Kelly et al., 1996). Induction of NOS2 has also been shown in ventricular myocytes by a LPS mediated activation of a PKC-dependent pathway (McKenna et al., 1995). The production of NO’ from constitutive NOS is in the nanomolar range, while iNOS produces NO’ in the micromolar range, further complicating the differential effects these isoforms have on intropy (Kojda and Kottenberg, 1999). Endothelial NOS is localized in the cardiomyocyte to the caveolae where compartmentalization with β adrenoceptors and L-type Ca$^{2+}$ channels results in NO’ mediated inhibition in β adrenoceptor stimulated inotropy (Hare et al., 1998;
Hare et al., 1995). The eNOS knock-out mice have an enhanced contractile response to β adrenoceptor stimulation (Barouch et al., 2002; Gyurko et al., 2000; Varghese et al., 2000). Indeed it is not surprising to find that β adrenergic increases in intracellular Ca^{2+} (mediated through cAMP) also activate the calcium sensitive eNOS (Balligand et al., 1993; Keaney et al., 1996). Confirmation of the importance of endothelial (both vascular and endocardial) production of NO on contractility is seen with the negative inotropic response noted with its removal (Brutsaert et al., 1988a; Li et al., 1993).

Muscarinic cholinergic receptor agonists can also affect contractility by elevating cGMP concentration as well as inhibiting cAMP mediated positive inotropy. This phenomenon known as ‘accentuated antagonism’, is thought to link the inotropic effects of muscarinic cholinergic receptor agonists with NO (Balligand et al., 1993). This is supported by failure to lower intracellular Ca^{2+} by muscarinic cholinergic receptor stimulation (in the presence of β adrenergic stimulation) in cardiomyocytes exposed to a NOS inhibitor (Balligand et al., 1995).

As discussed, the contractile effects of NO vary from the intracellular site of formation and the isoform involved. Conflicting data also exist from different species investigated and even between different myocardial tissues from the same species (atrial, ventricular, trabeculae, papillary) (Kelly et al., 1996), further complicating the issue. The negative inotropic effects of NO are mediated through cGMP, which decreases myocyte L-type calcium current and contraction through activation of cGMP-stimulated cAMP PDE II (Balligand and Cannon, 1997). This reduces intracellular cAMP levels and associated activity of protein kinase A (PKA) which in turn alters the phosphorylation state of several target proteins, including the α-subunit of the L-type calcium channel. Higher concentrations of NO activate PKG.
which also decreases calcium current intensity and contractility. This is predominantly observed on background of an increase in cAMP levels (Han et al., 1996b; Kojda et al., 1996; Mery et al., 1991; Sumii and Sperelakis, 1995; Wahler and Dollinger, 1995). Protein kinase G activation may also decrease cardiac myocyte contraction through a desensitization of cardiac myofilaments for Ca\(^{2+}\) which is possibly related to phosphorylation of troponin I (Herbertson et al., 1996).

Conversely, low doses of NO\(^{–}\) may potentiate the adrenergic effect of cAMP on contractility and associated Ca\(^{2+}\) influx through PDE III and subsequent increases in cAMP levels (Kirstein et al., 1995; Kojda et al., 1996; Mery et al., 1993). Others have also demonstrated that intracellular cGMP could activate Ca\(^{2+}\) release from the ryanodine channel through activation of ADP ribosyl cyclase and subsequent increases in cyclic ADP ribose (Galione et al., 1991).

Cyclic GMP independent effects on contractility are speculative and not extensively studied. Peroxynitrite inhibits enzymes involved in the citric acid cycle and potentially limiting available energy for contraction (Hausladen and Fridovich, 1994). By S-nitrosylating the glycolytic enzyme glyceraldehydes 3-phosphate dehydrogenase (GAPDH), NO\(^{–}\) increases its auto-ADP-ribosylation and decreases its activity, thereby impeding glycolysis (Dimmler et al., 1992; MacDonald and Moss, 1993). Oxygen consumption has been reduced in skeletal muscle strips exposed to NO\(^{–}\), presumably by inhibiting mitochondrial electron transfer (Shen et al., 1995; Shen et al., 1994). Finally, studies of isolated, perfused hearts by nuclear magnetic resonance spectroscopy have suggested that NO\(^{–}\) released from S-nitroso-acetylcysteine (an NO\(^{–}\) donor) impaired augmentation of contractile performance in response to increased Ca\(^{2+}\), possibly by nitrosylating creatine kinase (CK) and impeding phosphoryl transfer from creatine phosphate to ATP (Gross et al., 1996).
1. 2. 2. 4. Cell-Cell Interactions.

The interaction between the cellular components of the blood with each other and the endothelium play a critical role in cardiovascular pathophysiology. This is mediated through cellular adhesion molecules (CAMs). There are three major groups of CAMs, selectins, integrins and the Ig super-gene family (Krieglstein and Granger, 2001).

Platelets contain all three major groups of CAMs. Whilst there is no interaction between platelets and the healthy endothelium, exposure of the subendothelial matrix, importantly collagen and vWF as previously discussed, leads to a cascade of platelet adherence, activation and aggregation. Nitric oxide has been shown to attenuate platelet-endothelial adhesion (Provost and Merhi, 1997) which is mediated via a guanylate cyclase mediated mechanism (Cerwinka et al., 2002). The final pathway of platelet aggregation mediated through GPIIb/IIIa is modulated by NO'. The expression of GP IIb/IIIa (Pigazzi et al., 1999) and its binding to fibrinogen is down regulated by NO' (Mendelsohn et al., 1990; Michelson et al., 1996).

P-selectin plays a significant role in platelet-leukocyte and leukocyte-endothelial interactions. P-selectin is a transmembrane protein that is localized in the membrane of platelet alpha granules as well as part of the membrane of the Weibel-Palade bodies in endothelial cells (Furie et al., 2001; Gurney et al., 2002). Upon platelet activation and subsequent fusion of the alpha granule with the plasma membrane, P-selectin becomes exposed to the outer surface of the platelet. Nitric oxide synthase inhibitors have been shown to induce P-selectin expression on the endothelial cell surface (Murohara et al., 1995), while NO' donors downregulate its expression (Gries et al., 1998; Michelson et al., 1996), possibly via inhibition of PKC (Murohara
et al., 1995). S-nitroso-N-acetylcysteine also inhibits both thrombin and TXA₂-induced expression of P-selectin (Michelson et al., 1996).

Conversely, NOS inhibitors have been shown to promote the formation of platelet-leukocyte aggregates within postcapillary venules (Kurose et al., 1993; Kurose et al., 1995). NOS inhibitors have been shown to increase leukocyte adherence to post capillary and mesenteric venules (Kubes et al., 1991; Kurose et al., 1995; Lopez-Belmonte and Whittle, 1995) and porcine aorta (Provost and Merhi, 1997). The integrin CD11/CD18 on isolated neutrophils, which is specific for ICAM (Krieglstein and Granger, 2001), is upregulated by the NOS inhibitor L-N⁵G-monomethyl-L-arginine (L-NMMA) (Kubes et al., 1991). Nitric oxide also downregulates VCAM-1 expression in HUVEC (Takahashi et al., 1996), an effect that may be mediated through an inhibitory action on the nuclear transcription factor, NFκB (Khan et al., 1996). Monocyte adhesion mediated by V-CAM-1, I-CAM-1, E-selectin and MHC II antigen on human saphenous vein endothelial cells is also inhibited by NO⁻ donors. (Zampolli et al., 2000). These findings are particularly important in ischaemia-reperfusion (I-R) which is associated with high oxidative stress.

Nitric oxide, through formation of peroxynitrite reduces superoxide levels. Superoxide is known to promote leukocyte adhesion (Cerwinka and Granger, 2000). Indeed peroxynitrite itself may also inhibit leukocyte-endothelial cell adhesion (Lefer et al., 1997). In vitro studies also confirm that NO⁻ donors inhibit monocyte-endothelial adhesion (De Caterina et al., 1995) as well as monocyte chemotaxis (Bath et al., 1991; Belenky et al., 1993; Zeiher et al., 1995).
1. 2. 3. Nitric Oxide and Platelet Physiology

As previously stated both endothelial and platelet derived NO have potent anti-aggregatory effects. Platelet derived NO appears to predominantly inhibit platelet recruitment, whilst only modestly inhibiting platelet activation (Freedman et al., 1997). Factors that enhance platelet-derived NO synthesis include α-tocopherol, by inhibiting protein kinase C (Freedman et al., 1996; Freedman et al., 2000) and HMG CoA reductase inhibitors (‘statins’) by increasing expression of platelet-derived NOS (Laufs et al., 1998; Laufs et al., 2000). By reducing intracellular Ca\(^{2+}\) flux required for the activation of platelet NOS, cyclooxygenase inhibitors can reduce platelet-derived NO\(^{\cdot}\) generation (Chen et al., 1997) as can the effects of hypertension and cigarette smoking (Ichiki et al., 1996; Ikeda et al., 2000). This offers a possible mechanistic explanation to the increase in thrombotic complications associated with these factors (Bombardier et al., 2000; Doyle et al., 1964; MacMahon et al., 1990). Further evidence to support its role in thrombotic events is that patients with unstable angina produce significantly less platelet-derived NO\(^{\cdot}\) than patients with stable angina (Freedman et al., 1998).

It has been known for over 30 years that both indirect (Hampton et al., 1967; Schafer et al., 1980; Synek et al., 1970) and direct (Glusa et al., 1974; Pfleider, 1972; Saxon and Kattlove, 1976) NO\(^{\cdot}\) donors (ie. SNP) have potent anti-aggregatory effects, mediated predominantly through cGMP (Mellion et al., 1981). Other NO\(^{\cdot}\) donating compounds such as the S-nitrosothiols also have similar effects (Mellion et al., 1981). One such example is the anti-aggregatory effects of the S-nitroso-protein S-nitroso-albumin (Keaney et al., 1993). The mechanism behind this effect however is complex. The S-nitrosothiols appear to be a more stable source of NO\(^{\cdot}\) minimizing its inactivation by superoxide. Nitrosation of N-acetylcysteine to S-Nitroso-N-acetylcysteine decreases the intracellular Ca\(^{2+}\) flux in response to ADP stimulation.
leading to inhibition of fibrinogen binding in activated platelets (Mendelsohn et al., 1990) as well as potentiating the anti-platelet effects of nitroglycerin (Loscalzo, 1985; Stamler et al., 1988). S-nitoso-N-acetylcysteine also inhibits both thrombin and TXA$_2$-induced expression of P-selectin and active configuration of GPIIb/IIIa (Michelson et al., 1996). S-nitrosothiols can also be stored in platelets (Hirayama et al., 1999) and the platelet surface is able to facilitate the release of NO$^-$ from S-nitrosothiol by transnitrosation reactions (Simon et al., 1993). The effect of N-acetylcysteine however is not limited to its S-nitrosothiol forming effect (Chirkov et al., 1993b): N-acetylcysteine has antioxidant effects and therefore may also minimize NO$^-$ inactivation (Aruoma et al., 1989), as well as potentially accelerating the bioconversion process involved in liberating NO$^-$ from organic nitrates (Horowitz, 2000; Sage et al., 2000).

Other molecules produced by the endothelium work synergistically with NO$^-$ to enhance its antiplatelet effect including PGI$_2$, PGD$_2$ and tissue plasminogen activator (t-PA) (Loscalzo and Vaughan, 1987; Radomski and Moncada, 1991; Stamler et al., 1989a). Interestingly the synergistic effect of PGI$_2$ and NO$^-$ in limiting aggregation and enhancing disaggregation (Maurice and Haslam, 1990; Stamler et al., 1989b) is mediated through cGMP responses, rather than through the usual PGI$_2$ secondary messenger of cAMP (Battinelli and Loscalzo, 2000).
1. 2. 4. Nitric Oxide and Myocardial Physiology

1. 2. 4.1. Inotropy

Since the first report in 1991 of a role for NO\textsuperscript{\textminus} in modulating myocardial contractility, a number of conflicting studies have been published in this area (Smith et al., 1991). This is in part due to the obvious variation in experimental protocols as well as the presence/absence of β adrenoceptor stimulation. As is summarized in Table 1 and 2, both positive and negative effects of NO\textsuperscript{\textminus} on contractility have been documented. These results are difficult to compare, reflecting the different species, tissue, NO\textsuperscript{\textminus} donor and dose used. Further more others have reported no inotropic effect in rat, cat or rabbit papillary muscle (Ishibashi et al., 1993; Lefer and Murohara, 1995). A review of these articles suggest a biphasic effect of NO\textsuperscript{\textminus} donors on myocardial contractility, with high dose inducing negative inotropy and low doses, positive inotropy. With high doses of NO\textsuperscript{\textminus} donors, the negative inotropic effects are more consistently seen on the background of β-adrenoceptor stimulation, as shown in isolated rat cardiomyocytes (Sandirasegarane and Diamond, 1999), Langendorff-perfused rat hearts (Weiss et al., 1997) and isolated rat and cat papillary muscles (Weyrich et al., 1994).

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<th>Article</th>
<th>Species</th>
<th>NO\textsuperscript{\textminus} Donor</th>
<th>Results</th>
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<tr>
<td>(Kojda et al., 1995)</td>
<td>rat cardiomyocytes and Langendorff prep</td>
<td>SPM 3672</td>
<td>Myocyte contraction amplitude increased by 75%, ( \text{dP/dt} ) increased by 18%, at dose of 100µM of SPM 3672.</td>
</tr>
<tr>
<td>(Kojda et al., 1996)</td>
<td>rat cardiomyocytes</td>
<td>SNAP and DEA/NO</td>
<td>With 1µmol/L. of SNAP and DEA/NO, contractile response improved by 42%.</td>
</tr>
<tr>
<td>(Mohan et al., 1996)</td>
<td>RV cat papillary muscle</td>
<td>SNP, SIN-1, SNAP</td>
<td>Biphasic response, positive inotropy with low doses, negative with high.</td>
</tr>
<tr>
<td>(Kojda et al., 1997)</td>
<td>rat Langendorff</td>
<td>DEA/NO</td>
<td>At doses of (0.01µM-0.1µM) increase ( \text{dP/dt} ).</td>
</tr>
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(Preckel et al., 1997) open-chest dogs GTN,SNAP,DEA/NO All NO\textsuperscript{\textminus} donors positive inotropy.
Similarly, even with background β-adrenoceptor stimulation, a positive inotropic response is seen with low dose NO• donors (Kojda et al., 1996) and a biphasic response seen with increasing concentrations (Kirstein et al., 1995; Ono and Trautwein, 1991).

The effect of ‘physiological doses’ or low dose NO• resulting in enhanced contractility is further supported by experiments using NOS inhibitors. A number of these studies in rat heart in vitro (Klabunde et al., 1992; Kojda et al., 1997) and in

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<th>Article</th>
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<tr>
<td>(Smith et al., 1991)</td>
<td>ferret RV papillary muscle</td>
<td>SNP</td>
<td>Negative inotropy at 1µM of SNP</td>
</tr>
<tr>
<td>(Fort and Lewis, 1991)</td>
<td>perfused ferret heart</td>
<td>SNP</td>
<td>A reduction in peak LV pressure by 5-6% with SNP (10µmol/L)</td>
</tr>
<tr>
<td>(Brady et al., 1993)</td>
<td>guinea pig cardiomyocytes</td>
<td>SNP</td>
<td>23% reduction in contraction amplitude at SNP (3x10⁻⁴M)</td>
</tr>
<tr>
<td>(Wyeth et al., 1996)</td>
<td>rat/left atrial and LV papillary muscle</td>
<td>SIN-1 and NO gas</td>
<td>Negative inotropy only at NO conc 10⁻⁴M</td>
</tr>
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Table 1. Summary of studies showing a positive inotropic effect of NO• donors.

<table>
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<tr>
<th>Species and LV papillary muscle</th>
<th>NO• Donor</th>
<th>Results</th>
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<tr>
<td></td>
<td>SIN-1</td>
<td>Reduction in isometric force of contraction at SNP (100µmol/L)</td>
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</table>

Table 2. Summary of studies showing a negative inotropic effects of NO• donors.
vivo (Gardiner et al., 1991) and in the dog heart in vivo (Klabunde et al., 1991; Lechevalier et al., 1994; Zappellini et al., 1997) have shown impaired contractility with these agents. However, there are conflicting results in rat cardiomyocytes (Kaye et al., 1996).

1. 2. 4. 2. Chronotropy

The variable effect of NO$^-$ on inotropy is also seen with its effects on chronotropy. It is further complicated by chronotropic implications of NO$^-$ vasodilating properties. While rat cardiomyocytes (Balligand et al., 1993) and atrial tissue (Kennedy et al., 1994) showed no chronotropic effect, the isolated heart preparation showed a positive chronotropic effect to NO$^-$, by approximately 20% (Pabla and Curtis, 1995a). It is quite possible, as with its inotropic effects, that NO$^-$ has a biphasic response on chronotropy. In the isolated guinea pig sinus node, NO$^-$ donors showed a positive chronotropic effect with small doses, an effect which declined at higher concentrations (Musialek et al., 1997). The most convincing evidence for the chronotropic effects of NO$^-$ comes from a number of experiments with NOS inhibitors, where baroceptor activation has been abolished. As NOS inhibitors can increase blood pressure and block muscarinic receptors it is important these experiments are undertaken with autonomic reflexes ablated (Pagliaro, 1999). Both in the dog (Pagliaro et al., 1996) and rabbit model (Ward and Angus, 1993), with surgical and chemical sympathectomy respectively, NOS inhibitors have negative chronotropic effects. Prior to re-innervation in heart transplant recipients, an infusion of SNP also increases basal heart rate (Kojda and Kottenberg, 1999).

1. 2. 4. 3. Frank-Starling Response/ Left Ventricular Relaxation
More consistent effects of NO\(^-\) donors on contractility have been observed in alterations of diastolic function. The initial evidence for a lusitropic action of NO\(^-\) was observed in ferret papillary muscle (Smith et al., 1991) with a relaxation-hastening effect. This has been duplicated in cat and human papillary muscle with SNP or via stimulating NO\(^-\) release with substance P or 5-HT (Flesch et al., 1997; Mohan et al., 1996; Mohan et al., 1995a; Shah et al., 1989). Interestingly, these effects may be modulated through eNOS activation of β3 adrenoceptor activation (Gauthier et al., 1998). Similar effects have been observed in working heart studies (Grocott-Mason et al., 1994a; Grocott-Mason et al., 1994b) and isolated cardiomyocytes, mediated through cGMP (Ito et al., 1997; Shah et al., 1994; Vila-Petrof et al., 1998).

These NO\(^-\) effects therefore obviously have implications on the Frank-Starling response which is influenced by ventricular diastolic properties. This has been seen in human in vivo experiments with a bicoronal infusion of SNP (Paulus et al., 1994). In this study the effects of a low dose of SNP were an earlier onset of LV relaxation, a reduced peak and end-systolic LVP (with no change in dP/dt), a reduction in LVEDP, an increase in LVEDV and a down and rightward displacement of the LV diastolic pressure-volume relation. Whilst Paulus et.al. were not the first to find such results in humans (Brodie et al., 1977; Carroll et al., 1986; Kingma et al., 1986) these findings had previously been attributed to right ventricular unloading and biventricular interaction secondary to its vasodilator effect. Paulus et.al. refuted these claims by using small doses of SNP (2-4µg/min), finding no effect with right atrial infusion of SNP as well as observing that a fall in LVEDP and heart rate (small) with an increase in LVEDV is incompatible with a vasodilator effect of SNP. These findings have been duplicated with intracoronary administration of substance P (Paulus et al., 1995).
Similar effects have been seen in the isolated ejecting guinea pig heart. Reducing NO\textsuperscript{\textprime} bioavailability (by NOS or scavenging of NO\textsuperscript{\textprime} by free haemoglobin) significantly attenuated preload-induced increases in cardiac output (Prendergast et al., 1997), moving the LV diastolic pressure-volume relationship to the left and upwards compatible with a decrease in diastolic distensibility. The effects of L-NMMA were inhibited in the presence of L-arginine, while SNP had the opposite effect. It was also noted that the effects of L-NMMA were more pronounced at higher preloads which suggests that the effects of NO\textsuperscript{\textprime} might be greater at a higher LVEDV. This raises the issue of mechanical stimulated release of NO\textsuperscript{\textprime} in the heart having contractile implications in a similar way that shear-stress liberates NO\textsuperscript{\textprime} from the vascular endothelium. Indeed eNOS is located on the plasmalemmal caveolae an ideal site for putative modulation by mechanical stimuli. In frog myocardium, cGMP levels vary in the cardiac cycle with a rise coinciding with ventricular relaxation (Wollenberger et al., 1973). Subsequently, via use of a NO\textsuperscript{\textprime} probe, it has been shown in the beating heart that there is a brisk rise in NO\textsuperscript{\textprime} around the time of early diastolic filling (Pinsky et al., 1997). Thus bursts of NO\textsuperscript{\textprime} are released at precisely the appropriate time for modulation of myocardial relaxation and diastolic tone, as well as to maximize coronary perfusion. This would be beneficial during periods of increased LV work such as with exercise. The increase in shear-stresses would augment NO\textsuperscript{\textprime} release resulting in hastened LV relaxation, thus prolonging the diastolic interval for LV filling and coronary perfusion as well as increasing LV distensibility, thus improving LV filling and subendocardial blood flow due to reduced diastolic wall stress (Paulus and Shah, 1999).

1. 2. 4. 4. Force-Frequency Relationship
The (positive) force frequency relationship (FFR), Treppe or Bowditch effect (Bowditch, 1871; Woodworth, 1902) describes the phenomenon of enhanced contractility associated with an increase in heart rate. The FFR is an important homeostatic mechanism (ie. during exercise) that contributes to the augmentation of cardiac output. The FFR is negative in rats (Henry, 1975; Spurgeon et al., 1988), hamsters (Finkel et al., 1992) and in the human failing heart (Feldman et al., 1988a; Feldman et al., 1988b; Mulieri et al., 1992; Pieske et al., 1999), resulting in a decrease in tension generation associated with an increase in frequency of stimulation. Although abnormalities of excitation-coupling, such as altered expression of SR proteins, are generally thought to be primarily responsible for the blunted FFR (Pieske et al., 1999) it has also been suggested that NO\textsuperscript{-} effects may play a significant role.

Two in vitro studies have suggested that endogenous NO\textsuperscript{-} blunts the FFR. In the hamster papillary muscle the negative FFR was converted to a partially positive one by L-NMMA, an effect reversed by L-arginine or a NO\textsuperscript{-} donor (Finkel et al., 1995). Also in adult rat myocytes, the positive FFR was augmented by NOS or guanylyl cyclase inhibitors (Kaye et al., 1996), possibly mediated through a NO\textsuperscript{-}-dependent reduction in myofilament response to Ca\textsuperscript{2+} (Kaye et al., 1999). However, in vivo human studies showed L-NMMA had no effect on the FFR in either normal or heart failure subjects (Cotton et al., 2001). Likewise, in ventricular myocytes isolated from explanted failing human hearts, L-NMMA had no effect on the negative FFR (Harding et al., 1998).

1. 2. 4. 5. Energetics
The idea that an agent has the ability to alter oxygen ($O_2$) utilization in the myocardium is an appealing therapeutic target particularly in the treatment of CAD and its acute thrombotic complications. While some have found no effect (Sadoff et al., 1996; Saeki et al., 1996) and others even enhanced $O_2$ consumption (Sherman et al., 1997), evidence supports a load-independent reduction of myocardial $O_2$ consumption in the presence of NO$^\cdot$. The first evidence to suggest that NO$^\cdot$ influenced oxygen uptake, showed that NOS inhibition increased total body $O_2$ consumption in conscious dogs, independent of haemodynamics (Shen et al., 1994). The same group duplicated these results showing a decrease in $O_2$ consumption in skeletal muscle with exogenous NO$^\cdot$ and endogenously released NO$^\cdot$ (Shen et al., 1995). This phenomenon was also seen in cardiac muscle (Xie et al., 1996) and is augmented in the presence of angiotensin converting enzyme (ACE) inhibitors (Zhang et al., 1997). Recent evidence that bradykinin-induced reduction in in vitro myocardial $O_2$ consumption was abolished in eNOS knock-out mice further supports the previous findings (Loke et al., 1999).

Complementary to these results, it has also been reported that NO$^\cdot$ may also influence substrate utilization by the heart (Recchia et al., 1998). In conscious instrumented dogs, acute NOS inhibition switched cardiac substrate utilization from free fatty acids to glucose with a resultant increase in $O_2$ consumption. This is also seen in isolated rabbit hearts subjected to low flow ischaemia, where NOS inhibition stimulates glycolysis possibly secondary to increase glucose transport (Depre et al., 1995). These effects may be mediated through the glycolytic pathway as NO$^\cdot$ can stimulate ADP-ribosylation of glyceraldehydes-3-phosphate dehydrogenase and thereby possibly inhibit glycolysis (Zhang and Snyder, 1992). As will be discussed later, these effects may be beneficial in the reactive oxygen species (ROS) rich environment of reperfusion, by minimizing the toxic effects of peroxynitrite. An
impairment of NO\textsuperscript{\textperiodcentered} mediated effects on cardiac efficiency is also one postulated mechanism behind an increase in O\textsubscript{2} consumption seen in heart failure (Recchia et al., 1998; Xie et al., 1996).

1. 3. NITRIC OXIDE (PATHOLOGY)
A characteristic anomaly associated with atherosclerotic disease, particularly CAD and associated ACS, is a reduction in NO\(^{-}\) effect. As will be discussed further, this can be subdivided into a decrease in NO\(^{-}\) production, an increase in degradation of NO\(^{-}\) or a decreased sensitivity of soluble guanylate cyclase toward NO\(^{-}\).

As previously discussed, NO\(^{-}\) is produced by a number of tissues throughout the body and is particularly important in the cardiovascular system where it is produced by and has effects on the endothelium/vasculature, myocardium and platelets. While endothelial function/dysfunction can be defined by any of its physiological functions, it is usually expressed in vascular terms and implies an imbalance in the relative contributions of endothelial derived relaxing factors (NO\(^{-}\), prostacyclin, endothelial – derived hyperpolarizing factor) and contracting factors (endothelin, angiotensin II, TXA\(_2\)) (Verma and Anderson, 2002a). The major effect on this balance however is the bioavailability of endothelial-derived NO\(^{-}\).

Endothelial dysfunction occurs in the setting of atherosclerosis (Bossaller et al., 1987; Ludmer et al., 1986) hypertension (Panza et al., 1990), hypercholesterolaemia (Creager et al., 1990) and DM (Cohen, 1993a; Nitenberg et al., 1993) and has been associated with adverse risk and future cardiovascular events (Schachinger et al., 2000). While Table 3 summarizes a number of methods in assessing endothelial function, response to flow mediated dilatation (FMD) and subsequent analysis of the peripheral forearm arterial system is one of the common methods used.
Flow mediated dilatation is known to be dependent on an intact endothelium (Pohl et al., 1986) and this phenomenon was first described after forearm ischaemia in the

<table>
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<th><strong>Invasive coronary vascular testing</strong></th>
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<tr>
<td>Conduit vessels - ACh, L-NMMA, FMD, cold pressor test, mental stress</td>
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<tr>
<td>Resistance vessels - ACh, L-NMMA, cold pressor test, mental stress, CFR</td>
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<th><strong>Non-invasive coronary vascular testing</strong></th>
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<td>PET - CFR, cold pressor test</td>
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<td>Echocardiography - CFR</td>
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<td>Phase-contrast magnetic resonance imaging - CFR</td>
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<th><strong>Peripheral arterial assessment</strong></th>
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<tr>
<td>Conduit vessels - conduit artery FMD by non-invasive ultrasound</td>
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<tr>
<td>Resistance vessels - forearm blood flow by plethysmography (Ach, L-NMMA)</td>
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<tr>
<td>Cutaneous microcirculation - laser Doppler flowmetry</td>
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<td>Peripheral pulse waveform analysis - photoplethysmography, applanation tonometry</td>
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<th><strong>Circulating biomarkers</strong></th>
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<td>ADMA, serum nitrite</td>
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<tr>
<td>High sensitivity CRP</td>
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<tr>
<td>Endothelin-1</td>
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<tr>
<td>Haematological markers: vWF, PAI-1, tPA, thrombomodulin</td>
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<tr>
<td>Soluble cell adhesion molecules: ICAM-1, VCAM-1, E-selectin, P-selectin.</td>
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Table 3. Methods of assessing endothelial function, modified from Farouque and Meridith (2001).
brachial artery of humans in 1989 (Sinoway et al., 1989). With modification of a previously described ultrasound technique (Anderson and Mark, 1989), a technique was introduced (Celermajer et al., 1992) utilizing high resolution B-mode ultrasound to assess changes in brachial and femoral arterial diameter in response to reactive hyperaemia and sublingual nitrates. This technique in experienced hands has been found to be reproducible (Sorensen et al., 1995; Uehata et al., 1997), although not all investigators have found this to be the case (Hardie et al., 1997) and is widely used to assess endothelial function today. Applanation tonometry is also becoming accepted as a non-invasive tool for the assessment of endothelial dysfunction (Hayward et al., 2002; Wilkinson et al., 2002). With applanation tonometry, the most common parameter to assess endothelial function is the ‘augmentation index’ \( \{ \text{AI}(x) \} \). This measurement (Kelly et al., 1989) represents the quantification of the reflected arterial wave to the primary arterial wave. It is calculated as the ratio of the pulse pressure at the second systolic peak to that at the first systolic peak (Wilkinson et al., 1998a; Wilkinson et al., 2000). An increase in ‘augmentation index’, an approximation of arterial ‘stiffness’, is associated with age (O'Rourke et al., 2001), DM (Lehmann et al., 1992; Wilkinson et al., 2000) and atherosclerosis (Wada et al., 1994). As previously discussed a number of factors are responsible for the intact vasodilator function dependent on the endothelium.

Endothelial dysfunction has been classically defined as an impaired vasodilator response to agents inducing release of NO\(^\bullet\), with an intact response to exogenous sources of NO\(^\bullet\) (Ludmer et al., 1986; McLenachan et al., 1990; Reddy et al., 1994). However, a number of studies have now shown an impaired response to exogenous NO\(^\bullet\) in both the peripheral (Katz et al., 1992) and coronary (Schachinger and Zeiher, 1995) circulations with some suggesting that a more appropriate term should be ‘vasodilator dysfunction’ (Schachinger et al., 2000). Examples of this in the
vasculature include a reduction in SNP-mediated dilatation in hypercholesterolaemic human subjects (Creager et al., 1990) as well as an impaired response to authentic NO– in arterial tissue isolated from hypercholesterolaemic rabbits (Weisbrod et al., 1997). An impaired vasodilating response to nitroglycerin has also been shown in 800 patients with coronary risk factors (Adams et al., 1998). This phenomenon is not only localized to the vasculature, but has also been seen in platelets where an impaired NO– response has been seen in patients with stable angina pectoris (Chirkov et al., 1996; Chirkov et al., 1999; Chirkov et al., 1993a). Further examples of impaired platelet response to NO– are seen in patients with hypertension (Woods et al., 1993), diabetes/obesity (Anfossi et al., 1998) and ACS (Chirkov et al., 2001).

1. 3. 1. Nitric oxide bioavailability

1. 3. 1. 1. Decreased Nitric Oxide Production

L-arginine is obviously required in sufficient amounts for NO– production. There is however little evidence to support absolute dietary deficiency as a cause of vascular dysfunction in humans. In animal models, a restricted L-arginine diet is associated with a decrease in NO– production (Wu et al., 1999). Stimulation of alternative metabolism of L-arginine is also another possible mechanism for its decreased bioavailability in NO– deficient states. This is seen in patients with heart failure, where an increase in arginase activity may account for the observed low plasma levels of L-arginine (Dosiak and Kocot, 1980; Hanssen et al., 1998). Levels of L-arginine can also be depleted in cigarette smokers, through the formation of cyanomethyl-L-arginine, a substance that also inhibits eNOS activity (Wong et al., 2000). Impaired cellular uptake of L-arginine, mediated through the y+ transport system, is seen in the presence of long-standing hyperglycaemia (Sobrevia et al.,
or oxidized lipoproteins (Chen et al., 1996). L-arginine has been shown to improve endothelium-dependent vasodilation in traditional ‘NO’ deficient’ states such as hypercholesterolaemia (Clarkson et al., 1996a; Creager et al., 1992; Drexler et al., 1991), coronary artery disease (Adams et al., 1997; Dubois-Randle et al., 1992) and heart failure (Hirooka et al., 1994; Kubota et al., 1997; Rector et al., 1996). However, predominantly negative results have been seen in hypertensive patients (Panza et al., 1993) and mixed results in normal healthy subjects (Calver et al., 1991; Chin-Dusting et al., 1996; Creager et al., 1992; Imaizumi et al., 1992; Panza et al., 1993).

The first assumption of the mechanism of action of L-arginine is that it acts as a substrate for increased NO production. However, as previously discussed, with the $K_m$ for isolated purified endothelial NOS at 2.9 $\mu$M and plasma L-arginine concentration between 60-100$\mu$M at normal physiological levels, NOS should be saturated with substrate. The vascular effect seen with L-arginine therefore constitute the ‘L-arginine paradox’. A number of explanations have been proposed to explain this paradox. Possibly intracellular levels in the vicinity of eNOS may be significantly reduced when compared to those from whole-cell homogenates (Boger and Bode-Boger, 2001). L-arginine itself stimulates growth hormone, glucagon and insulin secretion all of which have vasodilating properties (Boger and Bode-Boger, 2001; Maxwell and Cooke, 2000). Levels of agmatine, which also has vasodilating properties, mediated through the $\alpha_2$-adrenergic and imidazoline receptors may also increase (Li et al., 1994). In vascular disease arginine may be depleted secondary to an increase in arginase activity (Loscalzo, 2000b). L-arginine may also directly scavenge superoxide anions, thus minimizing inactivation of NO’ (Wascher et al., 1997). The most plausible explanation however is the presence of an endogenous NOS inhibitor (Tsikas et al., 2000).
Asymmetric dimethylarginine (ADMA) is a competitive inhibitor of nitric oxide synthase (Cooke, 2000). It was first noted to antagonize endothelium-dependent vasodilation in 1992 (Vallance et al., 1992) and subsequently plasma ADMA levels have been associated with chronic renal failure (Kielstein et al., 1999; Vallance et al., 1992), peripheral vascular disease (Boger et al., 1997), hypercholesterolaemia (Bode-Boger et al., 1996; Boger et al., 1998; Yu et al., 1994), hypertension (Matsuoka et al., 1997; Surdacki et al., 1999), homocysteinaemia (Stuhlinger et al., 2001), insulin resistance (Stuhlinger et al., 2002) and DM (Abbasi et al., 2001). As L-arginine and ADMA directly compete for NOS, it is felt by some to best be represented as a L-arginine/ADMA ratio (Bode-Boger et al., 1996; Boger et al., 1998; Walker et al., 2001).

Asymmetric dimethylarginine is derived from the catabolism of proteins containing methylated arginine residues, predominantly found in the nucleus and are involved in RNA processing and transcriptional control (Najbauer et al., 2000). There are two types of enzymes that methylate arginine residues. These are protein arginine methyltransferase (PRMT) type I which forms ADMA and N-monomethylarginine (NMA) and PRMT type II which forms symmetric dimethylarginine (SDMA) (Ghosh et al., 1988; Tang et al., 2000). Methylated arginines are excreted in the urine (Kakimoto and Akazawa, 1970), hence their elevated levels in chronic renal failure. While a minor amount of ADMA and NMA can be metabolized by dimethylarginine pyruvate transferase (DPT) in the kidney and possibly also via acetylation in the liver (Cooke, 2000), the major metabolic pathway is the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (Ogawa et al., 1987). There are two isoforms of DDAH :- type I is typically found in tissues expressing nNOS, while type II
predominates in tissues expressing eNOS (Leiper et al., 1999). A summary of the metabolism of ADMA is shown in figure 3.

![Fig 3. Generation and metabolism of ADMA (Cooke., 2000).](image)

A number of other factors may disrupt the ability of NOS to produce NO•. As previously mentioned BH₄ is an essential cofactor for the proper functioning of NOS. At suboptimal concentrations of BH₄ or L-arginine (Cai and Harrison, 2000), NOS becomes 'uncoupled', acting as an NADPH oxidase in which molecular oxygen, rather than L-arginine, becomes an electron acceptor. This leads to the production of O₂•⁻ and H₂O₂ thus decreasing NO• production and increasing its inactivation (Cosentino and Katusic, 1995; Vasquez-Vivar et al., 1998; Wermer et al., 1995; Xia et al., 1998). In animal models of DM (Brunner et al., 2000; Pieper et al., 1997a) and hypertension (Cosentino et al., 1998) enhanced endothelium-dependent relaxation is seen with BH₄. In human studies improved endothelial function is also seen with
BH$_4$ in patients with known CAD (Maier et al., 2000), hypercholesterolaemia (Stroes et al., 1997), smokers (Heitzer et al., 2000a) or diabetics (Heitzer et al., 2000b).

Dysfunction of the NO$^\cdot$ pathway can occur by a decrease in the expression of eNOS. This can occur via decreased gene transcription or by decreased half-life of eNOS mRNA. One such example is a decrease eNOS expression in the endothelial cells over advanced atherosclerotic lesions (Wilcox et al., 1997). Tumour necrosis factor-alpha and glucocorticoids are known to interfere with the expression of eNOS possibly by destabilizing eNOS mRNA (Förstermann and Kleinert, 1995; Yoshizumi et al., 1993). An association has also been shown between a missense variant Glu298Asp in exon 7 of the eNOS gene with essential hypertension (Miyamoto et al., 1998), AMI (Hibi et al., 1998) and variant angina (Yoshimura et al., 1998) in three independent Japanese populations. However, others have failed to find any association with NOS polymorphisms and CVD (Bonnardeaux et al., 1995; Markus et al., 1998). Furthermore a recent association has been made between the polymorphism T$^{786}$C promoter for eNOS and endothelial dysfunction (Rossi et al., 2003a) and multivessel CAD (Rossi et al., 2003b).

**1. 3. 1. 2.Enhanced Nitric Oxide Degradation**

One of the main postulated mechanisms behind a reduction in NO$^\cdot$ effect is excessive oxidative stress. The term oxidative stress is defined as an imbalance between oxidants and antioxidants in favour of the former (Sies, 1985). The main oxidants in human biology are derived from oxygen (Maxwell and Lip, 1997). It is estimated that 5% of the oxygen consumed by normal tissues is transformed to ROS (Lefer and Granger, 2000). These ROS include superoxide anion O$_2^\cdot$ and hydroxyl radical (OH$^\cdot$) which are free radicals due to the existence of an unpaired electron.
Other ROS such as hydrogen peroxide (H₂O₂), peroxynitrite (ONOO⁻) and hydrochlorous acid (HOCL) are not free radicals but have oxidizing effects that contribute to oxidative stress. These ROS cause damage due to their high reactivity with other molecules, resulting in transfer of electrons and destabilization of cellular structure and function.

One of the main effects that ROS species have on cardiovascular homeostasis is through their interaction with NO⁻. The interaction between NO⁻ and O₂⁻ is three times faster than the reaction rate for O₂⁻ with the O₂⁻ scavenger superoxide dismutase (SOD) (Cai and Harrison, 2000). This inactivation of NO⁻ by O₂⁻ and subsequent formation of peroxynitrite (Gryglewski et al., 1986) has direct implications in cardiovascular disease and its associated risk factors. Peroxynitrite, in turn, imparts further nitrosative and oxidative injury to the endothelium (Beckman et al., 1990) and, as will be discussed, produces detrimental effects during I/R injury.

Reactive oxygen species also exert their toxicity in the vasculature by oxidation of polyunsaturated fatty acid species in the low-density lipoprotein (LDL) particle to form oxidized LDL in a process called lipid peroxidation (Forgione et al., 2000). Lipid peroxides have been shown to deplete NO⁻ levels by directly forming lipid peroxynitrates (Rubbo et al., 1994) or indirectly by attenuating NO⁻ release (Chin et al., 1992). With this in mind it is of no surprise to find impaired endothelium-dependent vascular relaxation associated with enhanced ROS generation in hypertension (Lockette et al., 1986), DM (Durante et al., 1988; Oyama et al., 1986), heart failure (Landmesser et al., 2002), cigarette smoking (Reilly et al., 1996) and hypercholesterolaemia (Davi et al., 1997). Of particular importance to this thesis is the concept of hyperglycaemia induced oxidant stress (Ceriello, 1997; Ceriello, 2000). While hyperglycaemic induced oxidative stress has long been thought as a mechanism to explain long term diabetic complications (Sheetz and King, 2002), it is
now known that hyperglycaemic associated oxidative stress is associated with acute haemodynamic effects in both normal (Marfella et al., 2001) and diabetic subjects (Marfella et al., 2000).

In mammalian cells, potential sources of ROS include NAD(P)H oxidase, xanthine oxidase, NOS, peroxidase, lipoxygenase, cyclooxygenase and cytochrome P450. The first three appear to be the main contributors in the cardiovascular system. Of these NAD(P)H oxidase is the major source of superoxide in the vascular cells and myocytes (Griendling et al., 2000). The NAD(P)H oxidases of the cardiovascular system are membrane bound enzymes that catalyze the 1 electron reduction of oxygen using NADH (preferred substrate) or NAD(P)H as the electron donor (Griendling et al., 2000). The NAD(P)H oxidases in vascular smooth muscle and endothelium are low-output, slow-release, possibly constitutive enzymes (Griendling et al., 2000) while the neutrophil form has up to three times more capacity to produce O$_2^-$ (Griendling and Ushio-Fukai, 1998). Vascular NAD(P)H oxidase activity is increased in hypertension (Fukui et al., 1997; Rajagopalan et al., 1996; Zalba et al., 2000) DM (Guzik et al., 2002; Guzik et al., 2000) and hypercholesterolaemia (Guzik et al., 2000). A major focus of recent research has been the physiological control of NAD(P)H oxidase activity. For both neutrophil and endothelial forms of NAD(P)H oxidase, angiotensin II appears to be a major physiological (and pathological) stimulus for expression of several of the component subunits of the molecule.

Xanthine oxidase is another important source of O$_2^-$. This enzyme is formed from xanthine dehydrogenase through either proteolytic cleavage or by oxidation of cysteines in the enzyme (Harrison et al., 2003). While xanthine oxidase is produced by endothelial cells, it also circulates in plasma and has been shown to reduce NO$^-$ bioactivity (Houston et al., 1999). Xanthine oxidase inhibitors improve endothelial
dysfunction in patients with hypercholesterolaemia (Cardillo et al., 1997), heavy smokers (Guthikonda et al., 2003), heart failure (Doehner et al., 2002; Farquharson et al., 2002) and diabetics with hypertension (Butler et al., 2000).

Finally as previously mentioned with a relative lack of L-arginine or BH₄, NOS favours production of ROS over NO′. This may explain a potential beneficial effect of NOS inhibitors on NO′ bioavailability in BH₄-depleted states (Kerr et al., 1999). Furthermore, the beneficial effects of folate supplementation in patients with ‘endothelial dysfunction’ have been ascribed to reversal of eNOS uncoupling.

With NO′ bioavailability heavily dependent on oxidative stress it is no surprise to find a number of small clinical studies showing an improvement in endothelium-dependent vasodilation with antioxidants in patients with CAD and associated risk factors (Levine et al., 1996; Solzbach et al., 1997; Ting et al., 1996). Further work needs to be done in this area, as large clinical trials of antioxidant therapy have not been associated with primary preventative benefits in high risk cardiovascular patients (Heart Protection Study Collaborative Group, 2002; Yusuf et al., 2000).

1. 3. 1. 3. Decreased sensitivity towards Nitric Oxide

As previously mentioned, an integral manifestation of ‘endothelial dysfunction’ is impaired vascular and platelet responses to NO′ donors. In the presence of atherosclerosis and its associated risk factors, impaired tissue response to NO′ in the vasculature (Creager et al., 1990; Katz et al., 1992; Schachinger and Zeiher, 1995; Weisbrod et al., 1997) and platelets (Anfossi et al., 1998; Chirkov et al., 1996; Chirkov et al., 1999; Chirkov et al., 2001; Chirkov et al., 1993a; Woods et al., 1993) has been shown. One possible mechanism to explain this observation is impaired
soluble guanylate cyclase activity. Oxidized LDL, hypertension, ageing and hyperglycaemia have all been shown to decrease the expression of this enzyme (Schmidt et al., 2000; Thakur et al., 2000). In spontaneously hypertensive rats the expression of both the α and β subunits of heterodimeric soluble guanylate cyclase and basal levels of cGMP were reduced in aortic rings (Ruetten et al., 1999). A reduction in the anti-aggregatory action of NO− in patients with stable angina pectoris is also in part explained by an impaired guanylate cyclase sensitivity (Chirkov et al., 1999). Interestingly a number of cardiovascular therapies may act in part by improving NO− responsiveness in certain tissues. In platelets (Failli et al., 1998) and kidney epithelial cells (Grosser and Schroeder, 2000) aspirin surprisingly increases sensitivity to NO− with associated increases in cGMP levels. In the platelets of patients with symptomatic CAD, statin therapy (Chirkov et al., 2001) or perhexiline therapy (Chirkov et al., 2001; Willoughby et al., 2002) are markers of preserved NO− responses, the latter of which is largely secondary to an increase in platelet cGMP responsiveness (Willoughby et al., 2002). It must also be added that incremental O2− generation is likely to play a part in observed impairment of tissue responsiveness to NO− in many circumstances. This may be difficult to assess utilizing exogenous NO− donors, which are relatively unaffected by moderate increases in vascular O2− generation (Sage et al., 2000). Delineation of the relative contributions of increased NO− clearance and decreased soluble guanylate cyclase responsiveness to NO− is problematic in many disease states.
1.3.2. Nitric oxide and Myocardial Pathology

1.3.2.1 Ischaemia-Reperfusion

Over recent times it has become apparent that in the treatment of ACS, it is not just the acute insult of myocardial ischaemia that is detrimental. With modern therapies, such as thrombolytics and percutaneous coronary angioplasty, the clinical consequences of reperfusion injury also can have direct effects on cardiac myocyte function and indirect effects due to changes in coronary perfusion. The modifying effect that NO\(^{-}\) has on this process remains critically important as we tailor therapies to minimize the impact of this life threatening process.

Ischaemia/reperfusion injury has broad-ranging consequences on a number of different cardiovascular structures. A number of these changes can be directly attributable to the burst of ROS that occurs on reperfusion (Shah and MacCarthy, 2000). This is seen in an I/R rat model, where reperfused myocardium was shown to produce large amounts of superoxide radicals which lead to the development of severe endothelial dysfunction (Lefer et al., 1990; Tsao and Lefer, 1990a). Endothelial dysfunction following reperfusion has been observed in the coronary (Hayward et al., 1997; Ku, 1982; Mehta et al., 1989a; Tsao et al., 1990b; Tsao and Lefer, 1990a; Van Benthuyzen et al., 1987), mesenteric (Hayward and Lefer, 1998), renal (Lieberthal et al., 1989) and cerebral (Mayhan et al., 1988) circulations. While endothelium-dependent vasodilators have little change in vascular response during ischaemia (Hayward and Lefer, 1998; Viehman et al., 1991) as early as 2.5 minutes after reperfusion there is an impaired response to the endothelium-dependent vasodilator acetylcholine in both the rat (Tsao and Lefer, 1990a) and cat (Tsao et al., 1990b) coronary circulations.
Myocardial effects are also pronounced, resulting in transient impairment of left ventricular systolic contractile function or ‘myocardial stunning’ (Braunwald and Kloner, 1982), acute diastolic dysfunction and arrhythmias (Shah and MacCarthy, 2000). A prolonged ischaemic insult obviously also results in myocardial necrosis.

1. 3. 2. 1. Ischaemia

A number of studies now confirm that in the initial stages of acute ischaemia (up to 30 mins) there is an increase in NO$^\cdot$ production. Nitric oxide synthase activity is enhanced during ischaemia in isolated rabbit hearts (Depre et al., 1997). In a number of different models, including open chested dogs (Kitakaze et al., 1996; Node et al., 1996; Node et al., 1995) and isolated rat hearts (Komarov et al., 1997), NO$^\cdot$ production increases during ischaemia and stimulates guanylate cyclase (Kitakaze et al., 1995; Park et al., 1992). An inhibition of this rise has also been shown in the presence of a NOS inhibitor (Kitakaze et al., 1995; Park et al., 1992). In this acute stage of ischaemia NO$^\cdot$ impairs contractility but improves myocardial metabolic function (Node et al., 1996), with a reversal of this negative inotropic effect in the presence of a NOS inhibitor (Depre et al., 1997). Following prolonged ischaemia however, NO$^\cdot$ production may decline. In isolated rat hearts NOS activity was significantly reduced after exposed to greater than 60 mins of ischaemia, which was attributed to loss of eNOS protein (Giraldez et al., 1997).

1. 3. 2. 1. 2. Reperfusion (influence of ROS)

Reperfusion injury is largely a result of the detremental effects of ROS (reactive oxygen species). There are three major lines of evidence that implicate ROS in the pathogenesis of myocardial reperfusion injury: 1) ROS can be detected in
postischaemic myocardium, 2) exposure of myocardium to exogenous ROS results in myocyte and myocardial tissue dysfunction that is comparable to that elicited by ischaemic reperfusion injury and 3) pre-treatment of animals with anti-oxidant enzymes (eg, superoxide dismutase) or genetic overexpression of these enzymes in experimental animals affords protection against reperfusion injury (Lefer and Granger, 2000).

The potential major sources of formation of the predominant ROS, $O_2^-$, are NAD(P)H oxidase, xanthine oxidase, cyclooxygenase, NOS and mitochondrial oxidases, but uncertainty remains as to the actual primary source. NAD(P)H oxidase, produced predominantly by neutrophils, was thought a likely candidate as a reduction in I/R-induced myocardial necrosis is seen in experimental animals that are either rendered neutropenic or that receive antibodies that neutralize adhesion molecules critical for neutrophil recruitment (Lefer and Lefer, 1996). However, the lack of benefit seen in I-R injury in NAD(P)H oxidase deficient mice suggests it is not the major producer of ROS in this setting (Hoffmeyer et al., 2000). Furthermore allopurinol, an inhibitor of xanthine oxidase, has been shown to improve functional recovery from I/R injury (Bolli, 1990) and to reduce infarct size in dogs (Downey and Yellon, 1992). There is also clinical evidence that allopurinol pre-treatment improves postoperative cardiac performance in patients undergoing CABG (Flitter, 1993). This is somewhat surprising as little or no xanthine oxidase activity is detectable in the human heart (Downey and Yellon, 1992).

Nitric oxide synthase, in the absence of L-arginine and BH$_4$ can produce superoxide (Vasquez-Vivar et al., 1998; Xia et al., 1996b; Xia et al., 1998), but this appears not to contribute to the ROS burst in I/R injury (Vergely et al., 2002). This evidence suggests that the ROS burst in reperfusion injury comes from a number of sources.
Electron paramagnetic resonance spectroscopy, widely used for the detection of ROS generation in the heart, has shown a rapid and profound increase in ROS following reperfusion of ischaemic myocardium (Garlick et al., 1987; Kramer et al., 1987; Zweier et al., 1987; Zweier et al., 1989). Reactive oxygen species have been shown to exert a direct inhibitory effect on myocardial function in vivo and in vitro. Indeed, exposure of the normal myocardium to ROS-generating systems or hydrogen peroxide alters myocardial function in a fashion that mimics reperfusion injury. This includes cellular loss of $K^+$, depletion of high-energy phosphates, elevated intracellular calcium concentration, loss of systolic force development, a progressive increase in diastolic tension, depressed metabolic function, and arrhythmias (Bolli, 1990; Tarr and Valenzeno, 1993; Weiss et al., 1993).

Further evidence for ROS-mediated damage in reperfusion injury comes from a number of animal experiments demonstrating a reduction in myocardial damage following antioxidant therapy. Superoxide dismutase and catalase have received the most attention in this regard. The first assessment of antioxidant enzyme therapy in myocardial reperfusion injury was with a combination of SOD and catalase (Jolly et al., 1984). This study revealed that the combination of these antioxidant enzymes significantly reduced myocardial infarct size in dogs after 90 minutes of coronary artery ischemia and 24 hours of reflow. Since this seminal report, there have been a large number of studies from different laboratories that have similarly demonstrated a beneficial effect of SOD and/or catalase in experimental models of myocardial I/R injury (Ambrosio et al., 1986; Chambers et al., 1985; Ma et al., 1992; Mehta et al., 1989b; Naslund et al., 1986; Werns et al., 1985; Werns et al., 1998). The literature, however is conflicting, with a large and nearly equal number of articles failing to show an effect of antioxidant therapy (Gallagher et al., 1986; Nejima et al., 1989; Patel et al., 1988; Richard et al., 1988; Shirato et al., 1988; Uraizee et al., 1987).
Clinical studies published in this area are also disappointing suggesting that recombinant SOD has no effect in reducing I/R injury in patients with an acute myocardial infarction treated with either thrombolysis (Murohara et al., 1991) or angioplasty (Flaherty et al., 1994).

It has been suggested that one possible reason behind the great disparity in results observed with therapy is possibly related to the dose and/or formulation of SOD (Engler and Gilpin, 1989). Conjugation of SOD with polyethylene glycol (PEG) increases both the plasma half-life and cellular uptake of the enzyme (Lefer and Granger, 2000) and has been shown to be cardioprotective against myocardial I/R injury (Tamura et al., 1988). However, once again not all studies have confirmed these findings (Ooiwa et al., 1989). It has been suggested that PEG-SOD may in fact be too large to move into the intracellular compartment. Therefore attention was redirected to developing other long-acting (Hori et al., 1988) low-molecular-weight SOD-mimetics (Kilgore et al., 1994). A low-molecular-weight SOD mimetic has yielded a positive result in an isolated rabbit heart model of I/R (Kilgore et al., 1994). However, it remains unclear whether these agents act merely to delay, rather than to prevent myocardial necrosis (Lefer and Granger, 2000). Further focus is also being directed towards intracellular location of these antioxidant enzymes. While SOD located in the mitochondria (manganese (MN) SOD) is potentially only a minor intracellular antioxidant (Halliwell and Gutteridge, 1997), in I/R, the lethal consequences of its deficiency (Lebovitz et al., 1996) and benefit of its over-expression (Jones et al., 2003) point a pivotal pathophysiological role which is not yet paralleled in therapeutics.
1. 3. 2. 1. 3. Peroxynitrite

Given the formation of NO\(^{-}\) during ischaemia and superoxide during reperfusion, it is important to understand the impact of peroxynitrite in the I/R setting, particularly as the detrimental effects of peroxynitrite include structural damage, enzyme dysfunction, ion channel and transporter malfunction and eventually cell death (Lalu et al., 2002). Examples of the cytotoxic nature of peroxynitrite include the oxidation of lipids, proteins (mitochondrial and nuclear), DNA and nitration of tyrosine residues (Beckman and Koppenol, 1996) which can lead to membrane instability following I/R injury (Lalu et al., 2002). Mitochondrial aconitase, an enzyme involved in the Krebs cycle, is irreversibly inhibited by peroxynitrite (Castro et al., 1994) which may contribute to the disrupted metabolic state of reperfused myocardium (Lopaschuk, 1998).

Recent focus has been directed on the matrix metalloproteinases (MMPs) and their role in I/R injury. This family of zinc-containing endopeptidases which when activated can cleave a variety of extracellular matrix proteins. Categorized into five major groups, (collagenases, gelatinases, stromelysins, membrane type and miscellaneous) (Woessner, 1998). Matrix metalloproteinase-2 (gelatinase A, type IV collagenase) is of particular interest as it is a constitutive enzyme which is ubiquitously expressed at a higher level than any other. It has been shown to play an important role in angiogenesis, atherosclerosis and heart failure (Yu et al., 1998) and its expression is increased in the I/R setting (Cheung et al., 2000a; Wang et al., 2002). Indeed in isolated rat hearts a peroxynitrite infusion has been shown to be associated with an increase of MMP-2 levels and a decline in cardiac mechanical function. The mechanical dysfunction associated with peroxynitrite can be ameliorated by the co-infusion of an MMP inhibitor (Wang et al., 2002). These
findings suggest that peroxynitrite induced myocardial injury is mediated, at least in part, through MMPs.

Poly (ADP-ribose) polymerase (PARP) is a chromatin bound enzyme that is found in the nuclei of a variety of cells including cardiac myocytes (Ikai and Ueda, 1983). Under normal physiological conditions it is involved in a number of processes, such as gene expression and cellular differentiation which rely on DNA replication and repair (Pieper et al., 1999). However, under conditions of oxidative stress, peroxynitrite can serve as a powerful trigger of DNA single strand breaks with subsequent activation of PARP (Szabo et al., 1995). PARP can impair mitochondrial respiration resulting in cellular dysfunction and death (Lalu et al., 2002). This has been shown in rat cardiomyocytes where peroxynitrite caused a significant activation in PARP and subsequent depression in mitochondrial respiration (Pieper et al., 1999). This deleterious effect of peroxynitrite however could be blocked by a PARP inhibitor (Zingarelli et al., 1997).

Other detrimental effects of peroxynitrite include inactivation of Mn SOD (Yamakura et al., 1998), inactivation of the sarcoplasmic Ca^{2+}-ATPase and subsequent disruption of calcium homeostasis (Klebl et al., 1998) and the inactivation of PGI_{2} synthase thus reducing the production of this cytoprotective eicosanoid (Zou et al., 1997).

An increased production of peroxynitrite has been observed in the I/R setting (Liu et al., 1997b; Szabo et al., 1995; Wang and Zweier, 1996; Yasmin et al., 1997; Zhang et al., 2001), with blocking of its production resulting in functional protection on the heart (Wang and Zweier, 1996; Yasmin et al., 1997). Evidence to support this also comes from experiments involving direct infusions of peroxynitrite. A continuous
infusion of 40µM of peroxynitrite in isolated working rat hearts causes a significant decrease in cardiac efficiency (Schulz et al., 1997). The infusion of 3-morpholinosydnonimine (SIN-1), a spontaneous donor of both NO˙ and O₂⁻ and thus considered in part a peroxynitrite donor enhances mechanical dysfunction and loss of CK during reperfusion. Interestingly with co-administration of SOD, both of these measures of I/R injury were improved (Ma et al., 1997).

Paradoxically there is also evidence to support a protective effect of peroxynitrite on I/R injury. These effects have only been observed in experiments perfusing the heart with blood, neutrophils or a crystalloid solution containing antioxidants (Cheung et al., 2000b; Lefer et al., 1997; Nakamura et al., 2000; Nossuli et al., 1998; Ronson et al., 1999). These beneficial effects are thought to be mediated through its ability to form S-nitrosothiols and thus act as a NO˙ donor (Balazy et al., 1998; Wu et al., 1994) and its ability to inhibit superoxide-generating enzymes such as xanthine oxidase (Lee et al., 2000), eNOS and iNOS (Pasquet et al., 1996).

In summary, the contribution of peroxynitrite to I/R injury remains complex. Further complicating experimental protocols is an inability to accurately measure peroxynitrite levels due to its extremely short half-life (Beckman and Koppenol, 1996), with nitrotyrosine, regarded as a ‘footprint’ of peroxynitrite formation, often being used as a substitute. Evidence supports the notion that peroxynitrite produced within the myocardium (endogenously) is probably cytotoxic and contributes to myocardial I/R injury. Conversely exogenously administered peroxynitrite may exert protective effects via its formation of S-nitrosothiols and subsequent NO˙ donating capabilities, as well as inhibiting further ROS producing enzymes.
1. 3. 2. 1. 4. Implications: NO donor pharmacotherapy

As we have reviewed thus far, NO\textsuperscript{-} donors have the potential to minimize I/R injury at a number of different levels. These mechanisms are predominantly directed towards maximizing coronary perfusion, not only through its vasodilating properties, but also via its inhibition of platelet adhesion and aggregation (Folts et al., 1991; Golino et al., 1992) and limiting the expression of adhesion molecules. This reduces the impact of the detrimental effects of cell-cell interactions (ie. leukocyte-endothelial interaction). Likewise during ischemia, reducing myocardial \( O_2 \) demand and thus improving myocardial energetics also can reduce the long-term effects I/R injury.

For over ten years, a number of studies have supported a beneficial effect of NO\textsuperscript{-} donors in I/R myocardial damage. This was first shown in a feline model where acidified NaNO\textsubscript{2} significantly attenuated reperfusion induced myocardial necrosis and cardiac infiltration of polymorphonucleocytes (PMN), as well as inhibiting platelet aggregation (Johnson et al., 1990). This was confirmed by the same group two years later using SIN-1 and C87-3754 in a feline model of I/R, which both attenuated cardiac necrosis by 65-70\% and did so without affecting haemodynamics. Improved post-ischaemic contractile function in the presence of NO\textsuperscript{-} donors has also been observed in isolated rat (du Toit et al., 1998), guinea-pig (Massoudy et al., 1995) and dog models (Lefer et al., 1993a; Pabla et al., 1995b). In the guinea-pig heart this was associated with a decreased glutathione release, suggestive that the beneficial effects of NO\textsuperscript{-} donors in this model may relate to a ROS scavenging effect. Beneficial effects have also been seen in conscious rabbits with nitroglycerin, despite the development of tolerance (Hill et al., 2001), possibly by a PKC dependent signalling mechanism (Banerjee et al., 1999). The advantageous effects of NO\textsuperscript{-} are also confirmed by a number of studies confirming the detrimental effects observed...
with NOS inhibitors (Dunker and Bache, 1994; Hasebe et al., 1993; Pabla et al., 1996).

Diastolic impairment, although transient, is also a feature of I/R injury. In adult rat cardiac myocytes, pretreatment with 8-bromo-cGMP fully prevented the impaired relaxation following I/R injury (Shah et al., 1995). Similarly, in the isolated rat heart subjected to brief hypoxia at constant coronary flow, treatment with SNP during hypoxia improved post-hypoxic LV relaxation (Draper and Shah, 1997). Nitric oxide donors also attenuated I/R induced endothelial dysfunction (Sieglfried et al., 1992). One mechanism by which NO’ donors exert their protective effects is by impairing superoxide mediated coronary vasoconstriction from PMNs (Lefer et al., 1993; Lefer et al., 1993a; Lefer et al., 1993b; Pabla et al., 1996) and the subsequent inactivation of endothelial derived NO’ (Ma et al., 1991). Nitric oxide donors have also been shown to suppress ischaemic arrhythmias in the porcine model (Wainwright and Martorana, 1993).

These findings obviously have significant clinical implications. Intravenous nitroglycerin given in low dose before, during and after coronary reperfusion to patients after anterior myocardial infarction accelerates recovery of left ventricular function (Jugdutt et al., 1997; Leesar et al., 2001; Tymachak et al., 1988). The development of certain reperfusion therapies that involve a NO’ moiety also offers new and exciting alternatives to the treatment of I/R injury. This has been shown with an S-nitrosated tissue plasminogen activator (NO’-tPA) which has been found in a feline model to attenuate myocardial I/R injury (Delyani et al., 1996). Preservation of coronary endothelial function, along with inhibiting PMN adherance to and P-selectin expression on coronary endothelial surface layer was also observed (Delyani et al., 1996).
The beneficial effects of NO\(^-\) on I/R injury are dependent however on a number of variables. This is apparent in a number of articles implying a detrimental effect of NO\(^-\) in this setting by observing an improved myocardial function in the presence of NOS inhibitors. The observed benefit is thought to be linked to an associated reduction of peroxynitrite (Depre et al., 1995; Mathies et al., 1992; Schulz and Wambolt, 1995; Wang and Zweier, 1996; Xie et al., 1998; Yasmin et al., 1997). The optimal conditions for excess peroxynitrite production is excess production of NO\(^-\), on the background of reduced antioxidant capacity. Such situations are as a result of an increase in iNOS expression. In the I/R setting, an increase in iNOS expression has been associated with adverse outcomes (El-Omar et al., 1998; Mungrue et al., 2002). This is thought to be a key mechanism behind hypotension associated with severe sepsis. With various cytokines increasing iNOS expression in sepsis, the resultant myocardial depression and inappropriate vasodilation can result in irreversible shock (Annane et al., 2000; Thiemermann, 1997). Limited studies have shown reduced hypotension in this setting with NOS inhibitors (Petros et al., 1991).

With recent studies demonstrating that both atherogenesis and precipitation of inflammatory changes (Libby, 2001; Libby et al., 2002; Ross, 1999) it is possible that this process may also be contributing to patients with severe ischaemia. We know a number of inflammatory markers such as C-reactive protein (CRP) are associated with adverse cardiovascular outcomes (Ridker, 2001). Indeed high-sensitivity CRP levels are a strong independent predictor of risk of future myocardial infarction, cerebrovascular accidents, peripheral vascular disease and vascular death among individuals with no known CVD (Kuller et al., 1996; Mendall et al., 2000; Ridker et al., 1998a; Ridker et al., 1997; Ridker et al., 1998b; Ridker et al., 2000; Tracey et al., 1997). In addition, among patients with acute myocardial ischaemia (Biasucci et al., 1999; Lindahl et al., 2000; Liuzzo et al., 1994; Morrow et al., 1998)
stable angina pectoris (Haerkate et al., 1997) and a history of myocardial infarction (Ridker et al., 1998c) levels of high-sensitivity CRP have been associated with increased vascular events. C-reactive protein may not be an innocent bystander in CAD as once thought, with possible adverse effects through its ability to both augment iNOS (Ikeda et al., 2002) and inhibit eNOS (Verma et al., 2002b). Endothelial function testing in forearm resistance vessels has shown that reductions in basal endothelial nitric oxide synthesis are correlated with increased serum CRP concentrations in healthy subjects (Cleland et al., 2000a). Similarly, in patients with CAD, forearm blood flow responses to ACh correlated inversely with elevated CRP levels, reduction of which were associated with improved vascular responses (Fichtlscherer et al., 2000). Although the vasoconstrictive nature of cardiogenic shock sets this apart from septic shock, it is not with complete surprise that preliminary animal (Lorente et al., 1996) and human (Cotter et al., 2000) data show benefit with NOS inhibitors in this condition.

1. 3. 3. **Nitric Oxide and Diabetes Mellitus**

The term diabetes mellitus describes a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both (World Health Organization, 1999). Along with DM, impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) represent disorders of impaired glucose handling, all of which can be diagnosed on a 75gm oral glucose tolerance test (OGTT) as shown in table 4.
Blood Sugar Level

<table>
<thead>
<tr>
<th>Classification</th>
<th>Fasting (mmol/L)</th>
<th>2 hrs (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt; 6.1</td>
<td>&lt; 6.9</td>
</tr>
<tr>
<td>Impaired fasting glucose</td>
<td>6.1 – 6.9</td>
<td>&lt; 7.8</td>
</tr>
<tr>
<td>Impaired glucose tolerance</td>
<td>&lt; 7.0</td>
<td>7.8 – 11.0</td>
</tr>
<tr>
<td>Diabetes</td>
<td>≥ 7.0</td>
<td>≥ 11.1</td>
</tr>
</tbody>
</table>

Table 4. Glucose handling classification based upon fasting blood sugar level (BSL) and response to oral glucose tolerance test (World Health Organization., 1999).

While the terms of IFG and IGT are relatively new terms, they refer to a metabolic state intermediate between normal glucose homeostasis and DM (World Health Organization, 1999). Impaired glucose tolerance possibly slightly more than IFG (Shaw and al., 1999; Tominaga et al., 1999; Zimmet et al., 2001), has been associated with an increased risk in progressing to DM as well as an increased risk for CVD and all cause mortality (Alberti, 1996; Fuller et al., 1980). Evidence even supports an increase in CVD in subjects with a ‘high’ normal fasting blood sugar level (BSL) (Balkau et al., 1998; Bjornholt et al., 1999; Coutinho et al., 1999).

A clustering of a number of cardiovascular risk factors with diabetes termed the ‘metabolic syndrome’ further increases cardiovascular mortality (Kaplan, 1989). Initially described in the 1960’s (Avogaro and Creapaldi, 1965) this syndrome has been known by a variety of names such as syndrome X, insulin resistance syndrome and cardiovascular metabolic syndrome (Groop and Orho-Melander, 2001). Insulin resistance is defined as a state in which a normal amount of insulin produces subnormal insulin-mediated glucose disposal, frequently resulting in a compensatory
hyperinsulinaemia and appears to be a common aetiological factor for the individual components of the metabolic syndrome (Haffner et al., 1992; Stern, 1997; Zimmet, 1992) For uniformity the World Health Organization (WHO) in 1999 defined the requirements for the metabolic syndrome as shown in figure 4.

![Figure 4. Diagnostic criteria for the Metabolic Syndrome (Zimmet et al., 2001).](image)

Diabetes is divided into two main types. Type I is an early onset form attributable to an autoimmune process with β cell destruction with sufferers prone to ketoacidosis. Type II, which is generally a later onset condition, has the associated aberration of insulin resistance (World Health Organization, 1999). While both forms are on the increase (Howard et al., 2002) Type II DM accounts for approximately 90-95% of all cases (Harris et al., 1998), with up to half of these cases undiagnosed (Australian Diabetes Obesity and Lifestyle Study, 2001; Howard et al., 2002). From the recent AUSDIAB report, of 11,247 Australians studied, the prevalence of DM, IFG and IGT was 7.5%, 5.7% and 10.6% respectively, twice what was expected and these
numbers are expected to continue to rise (Australian Diabetes Obesity and Lifestyle Study, 2001).

Although diabetes is a multi-system disease affecting eyes, kidneys and the nervous system, the leading cause of death among adult diabetics is CVD (Schernthaner, 1996). Cardiovascular disease is 2-8 times more common in the diabetic than the non-diabetic population (Howard et al., 2002; Jarrett et al., 1982; Kannel and McGee, 1979). Diabetics’ absolute rates of CVD increase with age and are higher in men than women (Howard et al., 2002). However, death from CVD in diabetics is significantly more frequent in women than men (Barrett-Connor and Wingard, 1983; Barrett-Connor et al., 1991; Heyden et al., 1980). Indeed it has been shown that DM renders an incidence of MI over a seven year period similar to that associated with a previous MI, 20% (Haffner et al., 1998). This has led the Adult Treatment Panel III of the National Cholesterol Education Program to establish diabetes as a CAD risk equivalent mandating aggressive antiatherosclerotic therapy (NECP, 2001). In patients with ACS a number of studies have shown that in hospital mortality rates are significantly higher in diabetics than non-diabetics (Granger et al., 1993; Malmberg and Ryden, 1988; Savage et al., 1988; Stone et al., 1989). In both ST segment elevation (Mak et al., 1997) and non-ST segment elevation AMI (Malmberg et al., 2000), the 30 day and 2 year mortality rates respectively are between 1.5 – 2 times higher in diabetes than non diabetic patients. Patients in cardiogenic shock also have poorer outcomes if diabetic (Shindler et al., 2000). The exact mechanism and contributing factors that result in the poor outcomes in diabetics with CVD remain uncertain.

Diabetic patients get incremental benefit from a number of therapies for both primary and secondary prevention of CVD. These therapies include aspirin (Antiplatelet
Trialists' Collaboration, 1994; Physician's Health Study, 1989), statin therapy (Heart Protection Study Collaborative Group, 2002; Pyorala et al., 1997), β-adrenoceptor antagonists (Chen et al., 1999; Gundersen and Kjekshus, 1983; Kendall et al., 1995; Kjekshus et al., 1990; Malmberg et al., 1989; Mangano et al., 1996) and ACE inhibitors (Gustafsson et al., 1999; Moye et al., 1994; Yusuf et al., 2000; Zuanetti et al., 1997). Yet in the diabetic group a number of these therapies including thrombolytic therapy in AMI are underutilized (Lim et al., 1998; Norhammar et al., 2003). In order to attenuate the increase in mortality associated with AMI, the DIGAMI study was undertaken. Randomizing patients with DM and AMI to an intensive glycaemic control (initiated with an intravenous insulin) was associated with a 52% reduction in the 1 year mortality rate (Malmberg et al., 1995), with this benefit remaining significant for up to a five year follow up (Malmberg, 1997; Malmberg et al., 1999). While insulin infusions have also been shown to be beneficial in diabetics undergoing coronary artery bypass surgery (Furnary et al., 1999; Lazar et al., 2000), the mechanistic explanation behind the benefit observed from insulin infusions and associated glycaemic control in this cohort remains uncertain. A large part of this thesis has looked at addressing these issues.

1. 3. 3. 1. Impact on Nitric Oxide Bioavailability

There is overwhelming evidence, in both animal models of DM and diabetic human studies, that there is impaired NO† bioavailability. A variety of animal models (rats, mice, rabbits, hamsters and dogs) have shown that chemically induced DM is associated with impaired endothelium-dependent vasodilation in both conduit (Abiru et al., 1990; Cameron and Cotter, 1992; Chang and Stevens, 1992; Gebremedhin et al., 1988; Hattori et al., 1991; Kamata et al., 1989; Matsunaga et al., 1996; Meraji et
al., 1987; Oyama et al., 1986; Tesfamariam et al., 1989) and resistance vessels (Diederich et al., 1994; Hill and Ege, 1994; Lash and Bohlen, 1991; Mayhan et al., 1991; Ralevic et al., 1995; Taylor et al., 1995). Further confirmation of a reduced effect of NO’ is seen with an impaired ACh-stimulated cGMP production in diabetic rat, rabbit and canine models (Abiru et al., 1990; Kamata et al., 1989; Koltai et al., 1997). Endothelium-dependent vasodilation is largely mediated by soluble guanylate cyclase activation in diabetic rat aorta, as its inhibition (by ODQ) blocks relaxation by up to 80% (Pieper and Siebeneich, 1997b). Human studies support the findings of the animal data. Endothelial dysfunction in diabetic humans was first shown in penile smooth muscle in subjects with impotence (Saenz de Tejada et al., 1989).

Subsequently a number of studies have shown impaired endothelium-dependent vasodilation in both conduit and resistance vessels of insulin dependent (Clarkson et al., 1996b; Johnstone et al., 1993; McNally et al., 1994; Nitenberg et al., 1993) and non-insulin dependent diabetics (Karasu et al., 1995; McVeigh et al., 1992; Morris et al., 1995; Nitenberg et al., 1993; Watts et al., 1996). A number of investigators have also shown attenuated responses to nitrovasodilators in both insulin dependent (Calver et al., 1992b; Clarkson et al., 1996b; Lambert et al., 1996; Zenere et al., 1995) and non-insulin dependent (Huvers et al., 1997; McVeigh et al., 1992; Morris et al., 1995; Watts et al., 1996) diabetic subjects. This ‘vasodilator dysfunction’ manifested by impaired response to exogenous vasodilators remains controversial as not all studies have shown its presence (Johnstone et al., 1993; Karasu et al., 1995; McNally et al., 1994; Nitenberg et al., 1993). As will be discussed, the pathogenesis of impaired NO’ bioavailability in DM is multifactorial.

There is evidence to support impaired NO’ synthesis as a mechanism behind a reduction in NO’ bioavailability in DM. Plasma arginine is decreased in diabetic rats (Pieper and Peltier, 1995; Pieper et al., 1996; Reyes et al., 1993) and in human
subjects (Grill et al., 1992; Hagenfeldt et al., 1989). Supplemental L-arginine improves endothelial function in both diabetic animal models (Pieper and Dondlinger, 1997c; Pieper and Peltier, 1995; Wu and Meininger, 1995) and human diabetic subjects (Chowienczyk et al., 1995), an effect that is cGMP mediated (Smulders et al., 1994). This ‘arginine paradox’ is explained in part in DM due to the high levels of ADMA. Asymmetric dimethylarginine is increased in DM (Abbasi et al., 2001; Lin et al., 2002) and in insulin resistance (Stuhlinger et al., 2002). Indeed high fat meals in diabetics are associated with an increase in ADMA and subsequent decrease in FMD (Fard et al., 2000).

One study however found ADMA was decreased in diabetics as well as being inversely correlated with HbA1c levels. This study also found that an increase in glomerular filtration rate in diabetics (often a manifestation of early diabetic kidney disease) inversely correlated with ADMA levels perhaps reflecting an increase in its renal clearance (Paiva et al., 2003).

Further evidence to support a decrease in NO\(^{-}\) synthesis comes from studies with BH\(_4\). Sepiapterin (a precursor of BH\(_4\)) (Bagi and Koller, 2003) and BH\(_4\) (Heitzer et al., 2000b; Pieper, 1997d) restores endothelium-dependent vasodilation in diabetic rats (Bagi and Koller, 2003). Improving availability of both L-arginine and BH\(_4\) have beneficial effects, switching ‘uncoupled’ NOS from superoxide to NO\(^{-}\) production. Free fatty acids (FFA), increased in diabetics, (Steinberg et al., 1996) may also contribute to a reduction in NO\(^{-}\) bioavailability. This has been shown by FFA mediated reduction in endothelium-dependent vasodilation of the femoral artery in insulin-resistant obese subjects (Steinberg et al., 1997). The mechanism underlying this effect is uncertain, but may relate to a decrease in eNOS activity (Davada et al.,
1995), offering further mechanistic support for insulin’s beneficial effect on vascular reactivity.

A number of studies now support an increase in oxidative stress as one of the main mechanisms behind the impaired NO\textsuperscript{\cdot} bioavailability observed in DM. This is confirmed by a number of studies showing an increase in nitrotyrosine in DM compared to non-diabetic patients (Ceriello, 2002a; Ceriello et al., 2001; Ceriello et al., 2002b), with further increases in diabetics following postprandial hypertriglyceridaemia (Ceriello et al., 2002c) and hyperglycaemia (Ceriello et al., 2002b; Ceriello et al., 2002c).

A number of other markers of oxidative stress are increased in DM. Concentrations of malondialdehyde, (MDA) formed when polyunsaturated fatty acyl chains are attacked by hydroxyl radicals, are increased in both Type 1 and Type 2 DM patients (Akkus et al., 1996; Laaksonen et al., 1996; Nishigaki et al., 1981; Santini et al., 1997; Skrha et al., 1996). Other derivatives of lipid peroxidation such as the non-enzymatic peroxidation product of arachidonic acid, esterified 8-epi-prostaglandin F\textsubscript{2} alpha, are elevated threefold in diabetics when compared to controls (Gopaul et al., 1995). Reactive oxygen species directed DNA damage characterized by 8-hydroxy-2’-deoxy-guanosine (8-OhdG) (Dandona et al., 1994; Leinonen et al., 1997) and protein damage as characterized by protein carbonyls (Altomare et al., 1997) are also increased in diabetic subjects. Evidence supports the origin of these ROS via \textit{O}_{2}^\cdot production predominantly from NAD(P)H oxidase (Guzik et al., 2002; Hink et al., 2001) or from ‘uncoupled’ NOS3 (Hink et al., 2001), although there is also evidence to suggest that xanthine oxidase adds to the oxidative stress (Pieper and Gross, 1988). It is suspected that ‘uncoupled’ NOS contributes to oxidative stress as shown
in STZ diabetic rats where NOS III expression is indeed increased 2-3 fold and superoxide generation is decreased by a NOS inhibitor (Hink et al., 2001).

Contributing to the increase in ROS in diabetics is a decrease in the anti-oxidant defences. Vitamin C (Akkus et al., 1996; Jennings et al., 1987), SOD (Atalay et al., 1997; Skrha et al., 1996), catalase (Wohaicb and Godin, 1987), reduced glutathione (Chari et al., 1984) and vitamin E (Karpen et al., 1985) are all decreased in diabetics. Intra-arterial infusion of vitamin C improve endothelium-dependent vasodilation in patients with type I (Timimi et al., 1998) and type II DM (Ting et al., 1996). Vitamin E therapy in type II diabetics also been found to improve endothelium-dependent vasodilation (Chowienczyk et al., 2000; Paolisso et al., 2000) although other studies have failed to find a benefit (Gazis et al., 1999). Other small clinical studies have also shown benefits with antioxidant therapy in diabetic patients on surrogate endpoints such as protein glycation, blood pressure as well as glucose and lipid metabolism (Ceriello et al., 1991; Coppola et al., 1992; Paolisso et al., 1993b; Paolisso et al., 1993a). Larger clinical studies have, however, failed to find a benefit from long term vitamin C/ vitamin E therapy in the prevention of cardiovascular events in high risk patients (Heart Protection Study Collaborative Group, 2002; Yusuf et al., 2000). Antioxidant therapy in the short-term treatment of ACS in DM patients however is yet to be evaluated.

1. 3. 3. 2. Detrimental effects of Glucose.

Hyperglycaemia has an adverse effect on probability of cardiovascular events. Diabetes per se is associated with an increase in incidence of CVD (Howard et al., 2002; Jarrett et al., 1982; Kannel and McGee, 1979) and death from ACS (Mak et al., 1997; Malmberg et al., 2000). In patients with myocardial infarction, hyperglycaemia
is an independent risk factor for an increase in mortality in both diabetic (Capes et al., 2000; Malmberg et al., 1999) and non-diabetic (Capes et al., 2000) subjects. A meta-analysis from three large population based studies has also shown that even high ‘normal’ blood glucose levels are associated with an increase in mortality and cardiovascular death (Balkau et al., 1998; Bjornholt et al., 1999; Coutinho et al., 1999).

A large body of evidence supports an important influence of hyperglycaemia on impaired bioavailability of NO\(^\dagger\). Normal rabbit aortic rings exposed to high concentrations of glucose for 3 hours results in a decrease in endothelium-dependent relaxation in response to acetylcholine and ADP (Tesfamariam et al., 1991; Tesfamariam et al., 1990), an effect which appears both concentration and time dependent. This effect is not due to the hyperosmolar effects of glucose, however, as mannitol did not cause any similar effect (Tesfamariam et al., 1991). A similar effect has also been shown in rat arterioles (Bohlen and Lash, 1993; Lash et al., 1999; Taylor and Poston, 1994). Hyperglycaemia per se also impairs eNOS protein expression (Noyman et al., 2002).

Conflicting results however have arisen in a number of in-vivo human studies assessing the effects of hyperglycaemia on NO\(^\dagger\) bioavailability in normal volunteers. Two studies have shown a reduction in FMD associated with hyperglycaemia (a 75gm OGTT) (Akbari et al., 1998; Title et al., 2000), but a number of studies however have failed to show any effect (Bagg et al., 2000; Houben et al., 1996; Williams et al., 1998). The difficulty with a number of these studies, in normal volunteers, is to separate the effects of an acute glucose load from the effects of the associated increase in insulin. As will be discussed, insulin has significant vasodilatory properties largely mediated through NO\(^\dagger\). These effects potentially
explain (in some studies) the beneficial effects on vascular stiffness (Wilkinson et al., 1998b) and FMD (Leyva et al., 2000) seen with an acute glucose load in normal subjects. Only one study to date has addressed this issue noting that endothelium-dependent vasodilation in normal subjects was not significantly impaired following glucose clamping, yet was significantly reduced with hyperglycaemia and co-administration of an octreotide infusion (to impair insulin release) (Williams et al., 1998). Impaired NO bioavailability may also contribute to the association between hyperglycaemia and the ‘no-reflow’ phenomenon following percutaneous coronary intervention (PCI) for AMI (Iwahura et al., 2003).

Similar patterns are seen in subjects with impaired glucose handling. Endothelial dysfunction is noted in patients with impaired fasting glucose (Vehkavaara et al., 1999), with both fasting plasma glucose and glycosylated haemoglobin (HBA1c) inversely correlated with endothelium-dependent vasodilation. Impaired endothelium-dependent vasodilation is seen with acute glucose loads in type 2 diabetics (Ceriello et al., 2002c; Lee et al., 2002; Shige et al., 1999) as well as correlating with chronic hyperglycaemia (measured by HBA1C) in insulin-dependent diabetics (Makimattila et al., 1996). A 75gm OGTT is also noted to impair endothelium-dependent vasodilation in both diabetics and patients with IGT (Kawano et al., 1999).

One of the main mechanisms that explains this hyperglycaemic-associated reduction in NO bioavailability is an increase in ROS, both in normal and diabetic subjects (Bonnefort-Rousselot, 2002; Ceriello, 1997; Ceriello, 2000; Sampson et al., 2002). Free radical production associated with hyperglycaemia may occur via at least 3 different routes: nonenzymatic glycation, auto oxidation of glucose or intracellular activation of the polyol pathway (Bonnefort-Rousselot, 2002; Ceriello, 1997;
Advanced glycation endproducts (AGE) are a known source of ROS (Singh et al., 2001), particularly by hyperglycaemia-induced overproduction of methylglyoxal (Shinohara et al., 1998). Advanced glycation endproduct (AGE) receptor (RAGE) stimulation upregulates oxidative stress response genes (Yan et al., 1994a) and releases oxygen radicals (Yan et al., 1994b). Advanced glycation endproducts have also been shown to quench both in vitro and in vivo NO˙ activity (Bucala et al., 1991). Furthermore, RAGE activation on endothelial cells results in the induction of oxidative stress and consequently of the transcription factor nuclear factor κB (NFκB) (Wautier et al., 1994; Yan et al., 1994a). Furthermore hyperglycaemia per se also stimulates NFκB (Hattori et al., 2000) an inducible transcription factor that has been strongly linked to the pathogenesis of atherosclerosis (Edgington, 1998). Finally, recent work shows the beneficial effect of a new class of AGE crosslink breakers on systemic arterial compliance, aortic impedance and carotid artery compliance in streptozotocin-induced diabetic rats (Wolffenbuttel et al., 1998), further confirming the detrimental effects of AGE on vascular function.

Intracellular glucose oxidation leads to the generation of the reduced forms of NADH and flavine adenine dinucleotide, which provide energy for ATP production through oxidative phosphorylation by the electron transport chain. Byproducts of mitochondrial oxidative phosphorylation such as O₂• are increased by high glucose levels (Nishikawa et al., 2000). Elevation of intracellular glucose levels can cause an increased flux through the enzyme aldose reductase, which is only activated when intracellular glucose concentrations rise to hyperglycaemic levels (Sheetz and King, 2002). Aldose reductase uses NAD(P)H to reduce glucose to sorbitol, which is then oxidized to fructose via sorbitol dehydrogenase. The decline in NAD(P)H caused by increases in aldose reductase flux is thought to contribute to a reduction in
endothelial NO˙ production (Tesfamariam, 1994). Aldose reductase inhibitors have been universally disappointing in prevention of long term diabetic complications (Sheetz and King, 2002) with no evidence to date showing any benefit in improving NO˙ bioavailability.

Diacylglycerol (DAG) and PKC are critical intracellular signalling molecules that can regulate many vascular functions, including permeability, vasodilator release, endothelial activation and growth signalling. Elevated glucose levels will increase glycolytic pathway flux in the diabetic state and lead to an elevation in the levels of intracellular glyceraldehyde-3-phosphate (Sheetz and King, 2002). Increased levels of this intermediate can stimulate increases in the de novo synthesis of DAG through glycerol-3-phosphate (Inoguchi et al., 1992). Through formation of DAG and AGEs, glucose can activate and upregulate PKC (Scivittaro et al., 2000). Hyperglycaemic induced stimulation of PKC is associated with an increase in ROS (Inoguchi et al., 2000). The source of the hyperglycaemic induced ROS is either NAD(P)H oxidase (Cosentino et al., 2003; Inoguchi et al., 2000; Mohanty et al., 2000) or NOS (Brodsky et al., 2002; Hink et al., 2001), with the latter theory supported by the reversal of hyperglycaemic associated impaired NO˙ bioavailability by BH₄ (Koshimura et al., 2002; Prabhakar, 2001). Hyperglycaemia increases DAG production and PKC activation leading to impaired endothelium-dependent vasodilation (Tesfamariam et al., 1991), with inhibition of PKC preventing the reduction in endothelium-dependent vasodilation induced by acute hyperglycaemia (Beckman et al., 2002a). Further evidence to support hyperglycaemic ROS as the predominant mechanism behind impaired NO˙ bioavailability in diabetics is an associated increase in nitrotyrosine confirming the notion of NO˙ inactivation (Ceriello et al., 2002b; Ceriello et al., 2002c; Zou et al., 2002).
Impairment of antioxidant defences may also contribute to the oxidative stress associated with hyperglycaemia (Price et al., 2001). As previously discussed antioxidant defence mechanisms are decreased in diabetic subjects and improvement in NO\(^\cdot\) bioavailability is associated with antioxidant therapy. This is also the case with hyperglycaemic induced reduction in NO\(^\cdot\) bioavailability. Evidence supports an increase in antioxidant defences reducing oxidatives stress and enhancing NO\(^\cdot\) availability (Cosentino et al., 2003; Title et al., 2000; Venugopal et al., 2002).

Asymmetric dimethylarginine, while increased in diabetics (Abbasi et al., 2001) and insulin resistant syndromes (Stuhlinger et al., 2002), may also play a role in hyperglycaemic induced reduction in NO\(^\cdot\) bioavailability. In diabetic rats ADMA was significantly elevated and with an associated reduction in DDAH (Lin et al., 2002). A reduction in DDAH activity is also seen in human endothelial cells exposed to high levels of glucose, which corresponds to a reduction in cGMP generation. This reduction in DDAH activity associated with hyperglycaemia was reversed in the presence of SOD confirming its redox sensitive nature (Lin et al., 2002). While chronic glycaemic control has been inversely correlated with ADMA levels (Paiva et al., 2003), a reduction in ADMA levels in type II diabetics has been associated with a chronic aggressive glycaemic control (Yasuda et al., 2001).

1. 3. 3. 3. Platelet Function.

In diabetics, there is associated significant platelet dysfunction (Mustard and Packman, 1984), which in turn is associated with adverse cardiovascular outcomes (Breddin et al., 1985). Patients with diabetes have an increased circulating platelet mass secondary to increased ploidy of megakaryocytes (Brown et al., 1997), which plays a part in a reduction of platelet survival (Schneider and Sobel, 2001). Altered
function is present in diabetics through-out all phases of platelet adhesion, activation and aggregation (Kwaan, 1992; Winocour, 1992a). Associated with an increase in platelet adherence in diabetics are elevated levels of von Willebrand factor (Van Zile et al., 1981) as well as GP Ib and GP IIb-IIIa complexes (Tschoepe et al., 1990).

Platelet hyperaggregability in diabetics has been demonstrated with a number of agonists including adrenaline, ADP and thrombin (Bastyr et al., 1987; Betteridge et al., 1982; Harrison et al., 1980; Kutti et al., 1986). Associated with increased platelet activation in diabetics is an increase in measurable contents of the α-granules such as platelet factor 4, β-thromboglobulin and P-selectin (Jilma et al., 1996; Jilma et al., 1999; Winocour et al., 1992b).

Impaired anti-aggregatory effects of NO\(^{-}\) represent one potential mechanism which can explain the above findings, yet data to support this remain scant to date.

Evidence supports impaired NOS activity in diabetics with up to a 75% reduction in associated NO\(^{-}\) production (Mazzanti and Mutus, 1997a; Rabini et al., 1998). It has been postulated that in diabetic platelets an increase in iNOS mediated O\(_2\)\(^{-}\) production on the background of reduced antioxidant defences (Muruganandam et al., 1992) contributes to hyperaggregability (Mazzanti and Mutus, 1997a) but once again little hard evidence supports this. Levels of cGMP are lower in diabetics compared to non-diabetics, indirectly implying a reduced effect of NO\(^{-}\) (Amado et al., 1993; Chirkov et al., 1990). Only one study to date has examined NO\(^{-}\) responsiveness in platelets of diabetics, showing an impaired anti-aggregatory effect of NO\(^{-}\) donors in obese diabetics rather than diabetics in general (Anfossi et al., 1998). The effect of hyperglycaemia in diabetics on platelet responsiveness to NO\(^{-}\) remains untested to date and has been a main focus of the research performed in this thesis.
Until recently little evidence supported a widely held belief that hyperglycaemia was associated with the prothrombotic state of diabetes (Bridges et al., 1965; Mustard and Packham, 1977; Mustard and Packman, 1984). Platelet dependent thrombus formation is enhanced in diabetics with long-term poor glycaemic control (Aoki et al., 1996) and in patients with stable CAD (Shechter et al., 2000). There is also a direct correlation between enhanced platelet activation and plasma glucose following an AMI (Oswald et al., 1988). An acute glucose load is also associated with an increase in platelet activation in type II diabetic patients, (Gresele et al., 2003) which may in part relate to an increased osmolarity (Keating et al., 2003). A decrease in platelet hyperaggregability is also noted in DM patients associated with improved long term glycaemic control (Davi et al., 1989; Osende et al., 2001; Turk et al., 1996), while others have found no improvement in platelet indices with intensive glycaemic control in in type I diabetic patients (Roshan et al., 2000). A favorable response is also noted with tight glycaemic control in diabetics in the coagulation cascade pathway with an increase in anti-thrombin III (Husted et al., 1989) and a decrease in factor VIII and fibrinogen (D'Elia et al., 2001).

Evidence supports oxidative stress as one of the main causes of this platelet dysfunction and thus a potential therapeutic target. In vitro experiments have shown that hyperglycaemia-enhanced platelet aggregation is associated with mitochondrial superoxide release and can be inhibited by SOD (Yamagishi et al., 2001). This has also been shown in diabetic patients in the clinical setting with improved glycaemic control and vitamin E resulting in a reduction in ROS and platelet activation (Davi et al., 1999). To date the effects of acute glucose control have not been examined at with relation to platelet function and associated platelet NO\(^\text{–}\) responsiveness, particularly in patients that potentially have the greatest to gain from such therapy, diabetics with an ACS.
A number of therapies can be used for glycaemic control in diabetics. Oral hypoglycaemic agents that improve insulin sensitivity such as metformin and thiazolidinediones have shown improvement in various parameters of NO\textsuperscript{−} responsiveness. Troglitazone upregulates NO\textsuperscript{−} synthesis in vascular smooth muscle cells (Hattori et al., 1999) as well as inhibiting platelet activity in non-diabetic patients with CAD (Sidhu et al., 2002). Metformin therapy appears to offer a reduction in cardiovascular mortality when compared to other hypoglycaemic therapies in type II diabetics (Anonymous, 1998) which may in part relate to improved NO\textsuperscript{−} bioavailability (Katakam et al., 2000; Mather et al., 2001a) or a reduction in platelet superoxide production (Gargiulo et al., 2002). The ATP-sensitive potassium channel blocking sulphonylureas however do not increase insulin sensitivity with evidence supporting a detrimental effect on endothelium-dependent relaxation in diabetic rat aorta (Pagano et al., 1998) as well as the human coronary circulation (Farouque et al., 2002). The human peripheral circulation does not show these findings (Farouque and Meredith, 2003a; Farouque and Meredith, 2003b).

Acute glycaemic control, however, is only effectively achieved with insulin, particularly when administered intravenously. Insulin enhances NO\textsuperscript{−}-dependent vasodilation particularly in skeletal muscle (Dunbar et al., 1996; Scherrer et al., 1994; Steinberg et al., 1994), an effect that is abolished by NOS inhibition (Baron et al., 1995). This obviously plays a key role in the delivery and uptake of glucose in skeletal muscle for uptake and utilization (Balon and Nadler, 1997; Young et al., 1997). Nitric oxide synthase inhibitors in humans produce a concurrent reduction in insulin sensitivity (Baron and Clark, 1997), while both eNOS and iNOS knock-out mice are insulin resistant compared to the wild type (Shanker et al., 2000). It is also well established that in endothelial cells insulin activates eNOS by a phosphorylation in serine via the phosphatidylinositol 3-kinase (PI3K)/Akt pathway of post-receptor

signalling (Zeng et al., 2000). Endothelial-dependent (Gaenzer et al., 2002; Vehkavaara et al., 2000) and endothelial independent (Vehkavaara et al., 2000) vasodilation in diabetic subjects is improved with tight glycaemic control with insulin therapy, albeit not universally so (Bagg et al., 2001). Insulin in turn also decreases release of FFA, which may also improve endothelial-dependent vasodilation (Steinberg et al., 1997).

Applanation tonometry assessment of ‘arterial stiffness’ as augmentation index also shows a reduction in this parameter following an insulin infusion in type II diabetics (Tamminen et al., 2002). The effect of insulin on augmentation index is blunted however in insulin-resistant subjects compared to normals (Westerbacka et al., 2001). A close relationship obviously exists between insulin sensitivity/resistance and NO’ sensitivity/resistance. This arterial vasodilator effect of insulin therefore resembles that of NO’, but it is not clear whether it is partially NO’-mediated.

Insulin also has significant platelet anti-aggregatory effects (Trovati and Anfossi, 2002; Trovati et al., 1988). Platelet - collagen interaction is inhibited by insulin (Westerbacka et al., 2002). Direct effects on platelet aggregation by insulin are felt to be concentration dependent. At low doses an inhibitory effect and at high doses an aggregatory effect is seen, mediated through cGMP levels (Anfossi et al., 1996). This explains evidence to support a hyperaggregatory effect noted by some of insulin on platelet function (Hu et al., 2002; Murer et al., 1994; Yngen et al., 2001). Mechanisms behind the beneficial effect of insulin on platelet function focus on enhanced NO’ bioavailability. Insulin reduces redox stress (Chen et al., 2001; Hu et al., 2002) and in vitro increases platelet concentrations of cGMP (Trovati et al., 1994). Further confirmation of insulin’s effect on the NO’ system is inhibition of its anti-aggregatory properties with either a NOS inhibitor (Trovati et al., 1996) or
methylene blue, a guanylate cyclase inhibitor (Trovati et al., 1994). In human platelets insulin increases NO’ synthesis, evaluated as tritiated citrulline generation in cells incubated with tritiated arginine (Trovati et al., 1997). Insulin’s eNOS activating effects in endothelial cells via the PI3K/Akt pathway (Zeng et al., 2000) is a posttranscriptional phenomenon, thus it is not surprising that it can occur also in the anucleated platelet. Insulin’s effects on the platelet are not only mediated by cGMP, but also cAMP (Trovati et al., 1997). Insulin’s dose dependent increase in cAMP, which was inhibited by L-NMMA (Trovati et al., 1997), has important implications on platelet function by reducing calcium fluxes (Trovati and Anfossi, 1998). As previously discussed, in the platelet this may also relate to a cGMP mediated inhibition of PDEIII with an associated increase in cAMP (Maurice and Haslam, 1990). Insulin can also increase cAMP via receptor dependent mechanisms, such as PGI₂ in the platelet, thus further explaining the mechanisms behind its anti-aggregatory properties (Trovati et al., 1988). In obese type II diabetic subjects, platelet response to NO’ donors was also impaired (Anfossi et al., 1998). The aforementioned effects of insulin on platelet function are blunted in insulin resistance states (Trovati et al., 1995), showing for the first time that insulin-resistance also occurs at the platelet level.

Confirmation of these important findings now needs to be examined in an environment where improved diabetic platelet function has potentially the greatest benefit, in ACS. This has been the main focus of the platelet studies of this thesis. The importance of aggressive glycaemic control with intravenous insulin in diabetics with ACS has the potential to have broad ranging benefits on both cardiovascular morbidity and mortality. The mechanisms are yet to be established.
1.4. AIM OF THE THESIS

The aim of the thesis is to investigate the tissue responses to nitric oxide donors in platelets and the myocardium.

1. In diabetic patients admitted with an ACS the following were assessed; the primary (null) hypothesis was that glycaemic control is not correlated with platelet reactivity to the NO\(^\cdot\) donor SNP or its major determinants (O\(_2\)\(^-\) generation or platelet cGMP response to SNP).

Secondary hypotheses were that glycaemic control is not correlated with:

- extent of platelet aggregation in response to ADP
- non esterified fatty acid levels (NEFA)
- neutrophil count, CRP and CK
- ADP enhanced O\(_2\)\(^-\) generation
- ADMA, L-arginine and L-arginine/ADMA ratio

1. In diabetic patients admitted with an ACS; the primary (null) hypothesis was that acute correction of hyperglycaemia does not modify platelet reactivity to the NO\(^\cdot\) donor SNP or its major biochemical determinants.

Secondary hypotheses were that acute correction of hyperglycaemia does not modify:

- platelet aggregation
- ADMA levels
- non esterified fatty acid levels (NEFA)
- CRP levels
3. In high risk cardiovascular patients and control subjects following a 75gm oral glucose load;
the primary hypothesis was that an acute glucose load does not modify platelet reactivity to the NO˙ donor SNP or its major biochemical determinants.
Secondary hypotheses were that an acute glucose load does not modify
- platelet aggregation or
- augmentation index

4. In the isolated rat ventricular papillary muscle, the hypothesis was tested that NO˙ released from SNP would affect contractility and the force-frequency relationship.
This hypothesis was tested:
(a) in electrically stimulated myocardium
(b) in the presence of incremental β-adrenoceptor stimulation
(c) in an ischaemia-reperfusion model
Chapter 2

METHODS
2. 1. PLATELET STUDIES

2. 1. 1. Subjects studied and blood sampling

All experiments performed on patients, or normal volunteers, were approved by the Ethics of Human Research Committee of the Queen Elizabeth Hospital and written informed consent was obtained prior to subject entry in all cases.

Blood samples were obtained from all subjects through the antecubital vein. In all cases a 21G needle was used with blood drawn into a plastic syringe utilizing minimal suction, as to prevent activation of the platelets during collection. Blood was then transferred slowly to plastic screw top tubes containing 1:10 volume of citric acid-sodium anticoagulant (two parts of 0.1M citric acid to three parts 0.1M trisodium citrate, pH 5). Acidified citrate was used to minimize the deterioration of platelet function during experiments (Kinlough-Rathbone et al., 1983). The time interval between collection of blood samples and platelet aggregation studies was less than 20 minutes.

2. 1. 2. Chemicals

Adenosine 5’-diphosphate (ADP) sodium salt, sodium nitroprusside (SNP), bis-N-methylacridinium nitrate (lucigenin) were purchased from Sigma (St.Louis, MO, USA). Physiological saline (0.9% NaCl) was purchased from Baxter Healthcare (Old Toongabbie, NSW, Australia).
2.1.3. Impedance aggregometry

Platelet aggregation in whole blood and platelet rich plasma (PRP) were examined utilizing a dual channel impedance lumi-aggregrometer (Model 560CA, Chrono-Log, Haverstown, P.A, USA). Data was collected via a Chrono-log Model 810CA Aggro/Link computer interface connected to an IBM (486DX) compatible computer utilizing Windows version 3.1 as previously described (Chirkov et al., 1999).

The principle behind impedance aggregometry is that a small electrical current is passed between two electrodes immersed in whole blood or PRP sample. During the initial contact platelets adhere to the electrodes, with the addition of known stimulants, platelets aggregate and thus increase impedance. This increase in impedance is recorded over time and is directly proportional to the mass of the platelet aggregate. Whole blood studies and platelet rich plasma studies were performed. For PRP studies, blood was centrifuged at 250g for 10 mins at room temperature. This was felt important, particularly when assessing NO\(^\cdot\) responsiveness in platelets as the neutrophil component of whole blood is felt to be the main generator of superoxide, through NAD(P)H oxidase. In this aspect whole blood is believed to represent a ‘more physiological’ system.

Blood or PRP was placed in plastic cuvettes, diluted 2 fold with 0.9% saline (final volume 1ml) and warmed in a heating block at 37\(^\circ\)C for 7 minutes. Siliconized stir bars were used to stir the blood at a rate of 900rpm. To initiate aggregation, 1\(\mu\)M ADP was added at the end of the incubation period. Aggregation was monitored continually for seven minutes and maximal responses recorded (RO-3 Rikadenki chart recorder) for electrical impedance, measured in Ohms. In whole blood each test was performed in quadruplicate and from these the mean value was calculated. Due
to time constants secondary to the potential deterioration of the sample, in PRP the test was performed twice with the average value assessed.

The inhibition of aggregation was also assessed in the presence of the NO’ donor SNP. One minute prior to the addition of 1μM ADP, 10μM of SNP was added and the inhibition of aggregation was assessed. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of SNP (figure 5). Likewise, in whole blood each test was performed in quadruplicate and in platelet rich plasma twice with the mean value calculated.

![Figure 5. Representative aggregation tracing and its inhibition by NO’Donors GTN and SNP (Chirkov et al., 1999).](image)

### 2. 1. 4. Chemiluminescence assessment of Superoxide

Detection of O$_2^-$ in whole blood was performed utilizing a chemiluminescence technique with lucigen as a probe O$_2^-$ as described previously (Gyllenhammar, 1987; Chirkov et al., 1999). High dose lucigenin has been questioned due to ‘redox-cycling’ and potential overestimation of O$_2^-$ (Liochev and Fridovich, 1997; Liochev
and Fridovich, 1998; Tarpey and Fridovich, 2001; Vasquez-Vivar et al., 1997). Low doses however <20µM are not complicated by this phenomenon (Li et al., 1998; Munzel et al., 2002) and thus 12.5µM was used in our experimental protocol.

Blood samples were diluted two-fold with 0.9% saline (final volume 1 ml) and prewarmed for 5 minutes at 37°C before the addition of lucigenin. Chemiluminescence was monitored using a photoluminometer component of the dual channel lumi-aggregometer (Model 560CA, Chrono-Log Havertown, P.A, USA). Data was collected via a Chrono-log Model 810CA Aggro/Link computer interface connected to an IBM (486DX) compatible computer utilizing Windows version 3.1. After 1 min the intensity of lucigenin derived chemiluminescence (LDCL) was measured and expressed in millivolts (mV).

Following establishment of a plateau in the luminescent signal post administration of lucigenin, aggregation-associated LDCL was assessed. In brief, 1 µM ADP was added and the associated maximal increase in the luminescent signal was measured over a 7 min period. One of the main sources of whole blood superoxide generation is from neutrophils. This methodology enabled the ability to measure platelet-neutrophil interaction, which mediated by activated platelets has been shown to liberate superoxide (Nagata et al., 1993; Ott et al., 1996).

2.1.5 Platelet cGMP assay

Intraplatelet cGMP is measured, as previously described (Chirkov et al., 1999) from platelets harvested from platelet rich plasma. In brief, platelet rich plasma, diluted twofold by 0.9% saline was prewarmed at 37°C, if the final volume was greater than 1ml subsequent concentrations of SNP were appropriately adjusted. Then 1 minute
prior to platelet harvest 10µM (or equivalent) of SNP was added. Platelet rich plasma was then filtered through GF/C Glass Microfibre Filters (Whatman, UK) held by plastic filter holders Swinnex-25 (Millpore, USA). Filters with absorbed platelets were then rinsed with 0.9% saline and placed into 0.5ml of 4mmol/L EDTA utilized to prevent cGMP decay. Tubes were then placed in a boiling water bath for 5 mins. Following this, samples were removed and centrifuged at 3000g for 10 mins. The cGMP concentration in the supernatant was then assayed using cGMP $^{125}$I radioimmunoassay kit (Amersham, UK). Results were expressed as a SNP mediated percentage increase of cGMP.

### 2. 1. 6. ADMA/SDMA/L-arginine assay

High performance liquid chromatography (HPLC) quantification of L-arginine, ADMA and SDMA in plasma were determined using precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Waters AccQ-Fluor™) based on a previously described method (Cohen and Michaud, 1993b). Plasma samples and standards were treated with 10% sulfosalicylic acid for 10 mins to precipitate protein prior to extraction on activated SCX solid-phase extraction cartridges (Varian Bondelut 100mg SCX) in an adaptation of the assay of Pettersson et al (Pettersson et al., 1997). The cartridges were washed with 0.1M phosphate buffer pH 6 (3ml) and methanol (1ml) to eliminate unwanted plasma components, before the amino acids were eluted with 2% TEA (triethylamine) in 65% MeOH/water. The eluents were evaporated in a heating block at 55°C over nitrogen and then redissolved in distilled water (0.2ml) for HPLC analysis. Arginine was quantified from 0.1ml of plasma in a separate SPE cleanup procedure to ADMA and SDMA due to arginine loss when larger volumes of plasma were used with the SCX cartridges. Due to ADMA and
SDMA’s relatively low concentrations in normal plasma, 0.5ml of plasma was used for quantification.

Reconstituted plasma extract (50µL) was added to an equivalent volume of borate buffer provided with the AccQ-Fluor™ kit and reacted with 10µL derivatization reagent. The arginine and dimethylarginines were separated on a Luna 5u C18(2) column (150x3.0mm, Phenomenex) with an acetonitrile gradient in 0.1M Na acetate buffer, pH 6 at 1.0mL/min. The HPLC system consisted of a Waters automated gradient controller with two 510 pumps connected to a 717plus autosampler. The amino acid derivatives were detected by fluorescence (Perkin Elmer LS40) at λex 250nm, λem 395nm and the data collected with LabLogic HPLC software.

Concentration of L-arginine, ADMA and SDMA in plasma was determined from standard curves of peak area ratio relative to internal standard (L-NMMA) versus standard concentration. Recovery of ADMA/SDMA spiked into plasma at 0.1 and 0.5 µg/ml (n=5) was 92-97% with a SD of 1.5-3%. Recovery of L-arginine spiked at 5 and 10 µg/ml was 122% with a SD of 2-4.3%. Arginine and the dimethylarginines can be quantitated in plasma down to 0.8 and 0.1 µM respectively.

2. 1. 7. CRP assay

The C-reactive protein assay was performed at the Frome Rd laboratories of the Institute of Medical and Veterinary Science. The high sensitivity CRP (hsCRP) values was utilized if the value was <3 mg/L otherwise the routine CRP measurements were utilized. The Beckman IMMAGE immunochemistry system was utilized for the hsCRP measurement. This system automatically dilutes the samples and delivers them to the reaction cuvette along with other reaction constituents. The system methodologies are rate turbidity and rate nephelometry.
The rate nephelometer measures the increase in the intensity of light scattered by particles suspended in a cuvette. The light source for the rate nephelometer is a 670nm laser. The detector is placed at a 90° angle from the laser beam to measure light scatter. The rate turbidimeter measures the decrease in the intensity of light as it passes through a solution of light scattering particles in a cuvette. The light source for the rate turbidimeter is an emitting diode (LED) at a wavelength of 940nm. Turbidimetric measurements are made at 0° from the incident beam.

2. 1. 8. NEFA assay

The non-esterified fatty acid (NEFA) test kit (Wako Pure Chemical Industries Ltd., Japan) utilizes an in vitro enzymatic colorimetric method for the quantitation of non-esterified (or free) fatty acids (NEFA or FFA) in serum. In brief this enzymatic method relies upon the acylation of coenzyme A (CoA) by the fatty acids in the presence of added acyl-Co-A synthase. The acyl-CoA thus produced is oxidized by added acyl-CoA oxidase (ACOD) with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase (POD) permits the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple coloured adduct which was measured colourimetrically at 550nm by a spectrophotometer (Varian Techron model 635, Australia).

2. 1. 9. Other variables assessed.

Other variables were assessed in the Queen Elizabeth Hospital and Frome Rd. Laboratories of the Institute of Medical and Veterinary Science. In brief plasma glucose and creatine kinase values are derived via spectrophotometry. Glycated haemoglobin is measured by (VARIANT™ Haemoglobin A1C Program) high performance liquid chromatography (HPLC) and the insulin levels were measured by
an immunoassay kit (AxSYM insulin, Abbott Laboratories, Il.) and expressed in μM/ml.

2. 2. MYOCARDIAL STUDIES

2. 2. 1. Papillary muscle preparation and apparatus

Male Sprague-Dawley rats (300-400gms) were used in all papillary muscle experiments. All animals were obtained from the University of Adelaide animal service. They were housed in air-conditioned rooms maintained at 22°C on a 12 hour light-12 hour dark cycle with food and tap water available ad libitum. All experimental procedures were approved by the Queen Elizabeth Hospital and University of Adelaide animal ethics committees in accordance with the guidelines set by the National Health and Medical Research Council of Australia.

The Krebs solution for this protocol contained (composition in mmol/L NaCl 98, KCl 4.7, MgSO₄·7H₂O 2.4, KH₂PO₄ 1.2, NaHCO₃ 20, CaCl₂·2H₂O 1.2, CH₃COONa 8.3, C₃H₅O₃Na 15, glucose 4.5). In all experiments except those with β-adrenergic stimulation, Atenolol at 0.02mmol/L was added to minimize unwanted ventricular irritability during the protocol. Bacterial contamination of the set-up was a problem during the early experiments that lead to a deterioration of the papillary muscle. This problem was related to the use of glass-distilled water which was either not freshly distilled or distilled into contaminated glass bottles and used in the Krebs solution. The use of Milli-Q water and meticulous washing of the apparatus after each day’s experiments overcame this problem. The Krebs solution was bubbled at 30°C with a gas mixture of 95% oxygen/5% carbon dioxide for at least one hour prior to each experiment. To cease spontaneous contraction during the set up of the papillary
muscle the same Krebs solution subjected to the same temperature and oxygenation was used with the addition of 2,3 butanedione-monoxime (BDM) at 30mmol/L.

The rats were anaesthetized with halothane and their beating hearts were quickly excised and placed in oxygenated Krebs solution. The heart in solution was allowed to continue to spontaneously contract for 30 seconds to expel all blood. The heart was then transferred to the BDM solution to arrest contraction during dissection of the papillary muscle. The left ventricle (LV) was then opened and suitable left ventricular papillary muscles were excised (<1mm in thickness). Two experimental setups (figure 6) were available enabling comparison between the effects of presence/absence of the NO˙ donor (SNP) in left ventricular papillary muscles from the same rat heart.

The tendinous end of the papillary muscle was then tied by an 8-0 silk suture (Sofsilk™, United States Surgical Corporation) to an electromagnetic length-tension transducer. A muscle pedicle from the left ventricular free wall of the non-tendinous end of the papillary muscle was then clamped in a phosphor-bronze clip. Minimal handling ensured negligible damage to the endocardial endothelium. The attached papillary muscle was then lowered into the 15ml organ bath containing the ‘BDM’ Krebs solution at 30°C bubbled with a gas mixture of 95%O₂/5%CO₂. The time from heart excision to insertion into the organ bath was less than 15 minutes. The papillary muscle was then allowed to stabilize for 1 hr. The ‘BDM’ Krebs solution was then washed-out three times by the normal Krebs solution.

The papillary muscle was then stimulated electrically at 10 or 35bpm (as documented in the initial experiments) by retangular pulses of 5ms duration and a voltage of approximately 10% above threshold via longitudinally arranged platinum electrodes.
Subsequent experiments assessing the effects of the NO donor SNP were performed at 10 bpm. The beating papillary muscle was then allowed to stabilize for a further one hour prior to data collection. A length/tension relationship was determined for each preparation, and resting tension was subsequently maintained to ensure maximum contractile force ($L_{\text{MAX}}$).

FIGURE 6. Papillary muscle setup. Selective numbering shows the phosphor bronze clip (1) in which the muscle is attached. The tendinous end tied to silk is attached to the arm of the electromagnetic length-tension transducer (2). This is then lowered into the organ bath (3), which is heated by a heating coil (4) and the muscle electrically stimulated by platinum electrodes (5).
FIGURE 7. Representative isotonic and isometric contractions.

1= peak shortening (PS), 2= time to peak shortening (tPS), 3= velocity of contraction (VC), 4= time to velocity of contraction (tVC), 5= velocity of relaxation (VR), 6= time to velocity of relaxation (tVR), 7= active tension (AT), 8= time to active tension (tAT), 9= positive δ force (PδF), 10= time to positive δ force (tP δF), 11= negative δ force (NδF), 12= time to negative δ force (tNδF), 13= time to half relaxation (tHR).
2. 2. 2. Papillary muscle measurements

Both isotonic (6 values) and isometric (7 values) were then measured as shown in the representative diagrams of figure 7. The values were assessed as a % change from baseline. The data from both papillary muscle setups were processed by a data acquisition system (DaqBook/120/CE, Iotech Inc, Cleveland Ohio) and displayed on IBM computers.

2. 2. 3. Chemicals

All salts used in the making of the Kreb’s solution were purchased from Anal R (Merck Pty.Ltd., Vic Aust) or Univar (APS Firedam, NSW Aust). Isoprenaline, sodium nitroprusside and atenolol were all purchased from Sigma (St.Louis, MO, USA). For routine experiments medical carbinox was used to oxygenate the papillary muscle 95%O₂ and 5%CO₂, and for the anoxic experiments the gas mixture used was 95%N₂ and 5%CO₂ (Linde compressed, Linde gas Pty Ltd, NSW Aust.).

2. 3. VASCULAR STUDIES

2. 3. 1. Applanation Tonometry

Applanation tonometry enables assessment of the arterial pressure waveform noninvasively. This technique is based on that pioneered for ocular tonometry for measurement of intraocular pressure (MacKay et al., 1962) and involves positioning the tonometer (a small pencil probe-like device) over the maximal arterial pulsation to minimally flatten (applanate) the arterial wall. By normalizing the circumferential
stress, the electrical resistance of a small piezoelectrical crystal within the tip of the tonometer varies directly with intra-arterial pressure, allowing accurate recording of the pressure waveform. In short, all patients were studied in a supine position in a quiet room and measurements were taken in the morning after an overnight fast. The blood pressure was recorded from the dominant arm prior to commencement of arterial pressure waveform acquisition using an automatic digital blood pressure monitor (HEM-705CP; Omron, Japan).

Radial artery waveforms were recorded using a Millar micromanometer tipped pressure transducer (Millar SPT 301B, Millar instruments) coupled to a Sphygmocor pulse wave velocity system (Model SCOR-Vx; PWV Medical) from the wrist of the dominant arm. A series of radial artery pressure measurements were recorded over a period of eight seconds. A central waveform was generated using a convolutional algorithm and generalized transfer function as previously described (Karamanoglu et al., 1993; O’Rourke et al., 2001; Wilkinson et al., 1998c). From the generated central waveform, an AI(x) corrected for a heart rate of 75bpm was calculated as the ratio of the pulse pressure at the second systolic peak to that at the first systolic peak (Hayward et al., 2002).

2.4. STATISTICAL ANALYSES

The D’Agostino test was used to examine conformation of data to normal (Gaussian) distribution. Non-Gaussian distributions underwent log transformation to normalize the data. For data from Chapter 3, acute and chronic glycaemic control were correlated to a number of variables using a simple linear regression with 95% confidence intervals. (Excel, Microsoft Office 98). We assessed the determinants of
platelet NO⁻ responsiveness and superoxide levels by a stepwise multiple regression analysis (multivariate analysis) (SYSTAT version 5.0, SYSTAT inc., 1990-1992).

To assess the effect of tight glycaemic control (chapter 4), a 2-way analysis of variance (ANOVA) with repeated measures was used. Statistical significance was assessed as p<0.05 and was documented in relation to the interaction between time and therapy unless otherwise stated. The same statistical test was used to assess the effects of frequency modulation and SNP on the contractile function of the rat papillary muscle. In Chapter 5 a one-way ANOVA was used to assess the effects of an oral glucose load on determinants of platelet function and applanation tonometry in both healthy volunteers and ‘high-risk’ cardiovascular patients (GB-Stat version 6.5, Dynamic Microsystems inc., 1999). All values are expressed as mean ± SEM, with non-parametric data expressed as a median value. A p value < 0.05 was used to indicate statistical significance.
Chapter 3

DETERMINANTS OF PLATELET RESPONSIVENESS TO NITRIC OXIDE IN DIABETIC PATIENTS WITH ACUTE CORONARY SYNDROMES: EFFECTS OF GLYCAEMIC CONTROL
3. 1. INTRODUCTION

Cardiovascular disease is 2-8 times more common in the diabetic compared to the non-diabetic population (Howard et al., 2002; Jarrett et al., 1982; Kannel and McGee, 1979) a concerning statistic as the incidence of diabetes worldwide is increasing (King et al., 1998). Both short-term (Kjaergaard et al., 1999; Svensson et al., 2003; Zuanetti et al., 1993) and long-term (Granger et al., 1993; Malmberg and Ryden, 1988; Savage et al., 1988; Stone et al., 1989) mortality rates are significantly higher in diabetic patients with ACS compared to non-diabetics. In all forms of ACS, such as cardiogenic shock (Shindler et al., 2000), ST segment elevation (Mak et al., 1997) and non-ST segment elevation (Malmberg et al., 2000) AMI, diabetics have an increase in adverse cardiovascular outcomes and mortality. The exact mechanism(s) to explain this increase in mortality remains uncertain.

Platelet dysfunction is common in atherosclerotic disease and its associated risk factors. Platelet activation and aggregation is a pivotal component in the pathology of ACS (Theroux and Fuster, 1998). Indeed DM (Bastyr et al., 1987; Kutti et al., 1986; Kwaan, 1992; Winocour, 1992a) and ACS (Chirkov et al., 2001; O'Brien et al., 1966; Wu and Hoak, 1976) both are associated with platelet hyperaggregability, the later of which is also a predictor of long-term mortality (Trip et al., 1990). Another manifestation of platelet dysfunction is an impaired response to the anti-platelet effects of NO˙. Platelet ‘NO˙ resistance’ is present in essential hypertension (Woods et al., 1993), obese diabetics (Anfossi et al., 1998), aortic stenosis (Chirkov et al., 2002), stable (Chirkov et al., 1996; Chirkov et al., 1993a) and unstable angina pectoris (Chirkov et al., 2001). While platelet ‘NO˙ resistance’ occurs in the presence of hyperaggregability, it is independent of increased platelet aggregation (Chirkov et al., 2002) implying these two entities differ mechanistically. Platelet ‘NO˙ resistance’
in stable angina pectoris is mediated via oxidative stress which increases clearance of NO’ by O$_2^-$ and inactivation of soluble guanylate cyclase (Chirkov et al., 1999).

In the vasculature ample data support a reduction in NO’ bioavailability in the diabetic subject, as reflected by both impaired endothelium dependent (Clarkson et al., 1996b; Johnstone et al., 1993; Karasu et al., 1995; McNally et al., 1994; McVeigh et al., 1992; Morris et al., 1995; Nitenberg et al., 1993; Watts et al., 1996) and endothelium-independent vasodilation (Calver et al., 1992; Clarkson et al., 1996b; Huvers et al., 1997; Lambert et al., 1996; McVeigh et al., 1992; Morris et al., 1995; Watts et al., 1996; Zenere et al., 1995). Strong evidence in the diabetic vasculature also supports an important role of hyperglycaemia in the impaired bioavailability of NO’ (Makimattila et al., 1996; Shige et al., 1999). This is of no surprise in the ACS setting as hyperglycaemia is an independent risk factor for an increase in mortality in both diabetic (Capes et al., 2001; Malmberg et al., 1999) and non-diabetic (Capes et al., 2001) subjects.

A number of mechanisms have been proposed to explain the ‘vasodilator dysfunction’ observed in the diabetic subject, particularly with hyperglycaemia. This may be mediated through increases in redox stress with associated inactivation of NO’ (Ceriello, 2002a; Ceriello et al., 2001). As discussed in the introduction, free radical production associated with hyperglycaemia may occur via at least 3 different routes: nonenzymatic glycation, auto oxidation of glucose or intracellular activation of the polyol pathway (Bonnefort-Rousselot, 2002; Ceriello, 1997; Ceriello, 2000). Hyperglycaemic induced stimulation of PKC is also associated with an increase in ROS (Inoguchi et al., 2000), possibly by increased generation of O$_2^-$ by NAD(P)H oxidase (Cosentino et al., 2003; Inoguchi et al., 2000; Mohanty et al., 2000), or uncoupled NOS (Brodsky et al., 2002; Hink et al., 2001). Recently, elevated
asymmetric dimethylarginine (ADMA) levels have also been proposed as a mechanism behind glucose mediated reduction in NO’ bioavailability in diabetics. In diabetic rats, ADMA was significantly elevated with an associated reduction in activity of the enzyme dimethylarginine dimethylaminohydrolase (DDAH) responsible for its clearance (Lin et al., 2002). A reduction in DDAH activity is also seen in human endothelial cells exposed to high levels of glucose, which corresponds to a reduction in cGMP generation. This reduction in DDAH activity associated with hyperglycaemia was reversed in the presence of SOD confirming its redox sensitive nature (Lin et al., 2002).

The issue of impaired NO’ responsiveness in diabetic platelets however has not received extensive previous evaluation. While impaired anti-aggregatory effects of NO’ have been shown in obese diabetics (Trovati et al., 1995) no study to date has looked at these effects in diabetics with myocardial ischaemia. This study was therefore designed to examine the determinants of platelet responsiveness to the NO’ donor SNP in diabetics admitted with ACS. We were particularly interested in looking at the effects of glycaemic control on these parameters. Both admission BSL (Capes et al., 2000; Malmberg et al., 1999) and HbA1C (Malmberg et al., 1999) are both independent predictors of poor long-term outcomes in diabetic patients with AMI. However, as admission BSL (Capes et al., 2000; Fava et al., 1996; Sala et al., 2002; Umpierrez et al., 2002; Yudkin and Oswald, 1987) not HbA1C (Roth et al., 1992) is a predictor of in-hospital short-term mortality, we chose just admission BSL when evaluating our secondary hypotheses. We tested the primary (null) hypothesis that glycaemic control (acute and chronic) was independent of whole blood platelet SNP responsiveness, superoxide generation and intraplatelet cGMP generation.

Secondary objectives were to assess possible associations of acute glycaemic control (admission BSL) with platelet aggregation, platelet SNP responsiveness in platelet
rich plasma (PRP), NEFA levels, neutrophil count, CRP levels, CK rise, ADP enhanced superoxide generation as well as ADMA, L-arginine levels and L-arginine/ADMA ratios. The determinants of both platelet SNP response and of O$_2^-$ levels were also assessed by a stepwise multivariate analysis. We assessed risk factors and therapies in our multivariate analysis that possibly may alter NO’ bioavailability which included age (Matz and Andriantsitohaina, 2003; Matz et al., 2000), BSL (Bohlen and Lash, 1993; Ceriello et al., 2002c; Lee et al., 2002; Shige et al., 1999; Taylor and Poston, 1994; Tesfamariam et al., 1990), sex (Celermajer et al., 1994), statin (Kinlay et al., 2001; Lefer et al., 2001), insulin (Dunbar et al., 1996; Scherrer et al., 1994; Steinberg et al., 1994) (Gaenzer et al., 2002; Vehkavaara et al., 2000) and ACE-inhibitor therapy (Fenster et al., 2003; O'Driscoll et al., 1999) and extent of CK elevation (as an approximation of extent of ischaemic/reperfusion injury) (Shah and MacCarthy, 2000).

3. 2. METHODS

Consecutive diabetic patients admitted to the Queen Elizabeth Hospital coronary care unit with non-ST segment elevation ACS were enrolled into the study. Diabetes mellitus was defined by a previous diagnosis or no previous diagnosis and an admission BSL $\geq$ 11.1mmol/L. Non-ST segment elevation ACS (ie. unstable angina pectoris or subendocardial MI) was diagnosed on the basis of greater than 20mins of ischaemic chest pain without S-T segment elevation. Patients were excluded if they were taking an ADP receptor antagonist (clopidogrel or ticlopidine) or perhexiline maleate; the later was shown to significantly improve platelet SNP responsiveness (Chirkov et al., 2001; Willoughby et al., 2002). Patients were also excluded if they had significant renal insufficiency (>0.300mmol/L) on the basis of admission data.
Blood samples were taken within 1 hour of admission and samples were processed as previously described in the methods section. Platelet SNP responsiveness studies were performed in whole blood and PRP. Statistical analysis was performed as described in Section 2.4.

3. 3. RESULTS

3. 3. 1. Patient characteristics/ diabetic control

76 consecutive diabetic patients were enrolled in the study. Patient characteristics are summarized in table 5. While all subjects were type II diabetics, approximately 14% were insulin dependent. Of patients taking oral hypoglycaemics, 56% were receiving a combination of sulphonylurea and metformin, with the remainder divided evenly with single therapy sulphonylurea or metformin. One patient was prescribed a thiazolidinedione. While 50% of patients were taking aspirin prior to arrival at hospital 78% had taken aspirin prior to blood collection. All patients were type II diabetics with a relatively high mean body mass index (BMI) (28 ± 0.6 kg/m²). Further evidence for an insulin resistant state in this cohort may be derived from the homeostatic model of assessment of insulin resistance (HOMA-IR) formula of insulin (µU/ml) X glucose (mmol/L)/22.5 (Matthews et al., 1985) which shows a good correlation with hyperinsulinaemic clamps for detection of insulin resistance (Bonora et al., 2000; Katsuki et al., 2001; Matthews et al., 1985). As the acute nature of the study did not ensure fasting status, the HOMA-IR median value of 15.6 in this cohort is another indicator of significant insulin resistance. A glycated haemoglobin of 8.4% suggests relatively poor prior glycaemic control. An AMI was defined on the basis of peak CK value at least three times the upper limit of normal; the median peak CK in this cohort was 330 U/L. Evidence of significant myocardial ischaemia
was also confirmed by 53% of subjects having significant ECG changes, defined as < 1mm ST segment depression or T wave inversion. An additional 11 patients were screened for this study but were ineligible (6 on an ADP receptor antagonist, 4 with renal insufficiency and 1 patient commenced on an intravenous insulin infusion 1 hour prior to potential blood collection). Only one patient was subsequently felt to have a ‘non-ACS’ cause of her chest pain with a subsequent ‘normal’ coronary angiography her data are nevertheless included in the subsequent analysis, on the basis of ‘intention to treat’ principles.
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<table>
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<tbody>
<tr>
<td><strong>Age</strong></td>
<td>66 ± 1.4</td>
</tr>
<tr>
<td><strong>Sex (M:F)</strong></td>
<td>49 : 27</td>
</tr>
<tr>
<td><strong>AMI (%)</strong></td>
<td>29</td>
</tr>
<tr>
<td><strong>BSL (mmol/L)</strong></td>
<td>14.3 ± 0.6 (range 5.1-31.7)</td>
</tr>
<tr>
<td><strong>HbA₁C (%)</strong></td>
<td>8.4 ± 0.2</td>
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**Risk Factors**

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<table>
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<tbody>
<tr>
<td><strong>Hypertension (%)</strong></td>
<td>54</td>
</tr>
<tr>
<td><strong>Smoking (%)</strong></td>
<td>17</td>
</tr>
<tr>
<td><strong>Cholesterol (mmol/L)</strong></td>
<td>4.6 ± 0.1</td>
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**Diabetic Medications (%)**

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<tbody>
<tr>
<td><strong>Diet only</strong></td>
<td>32</td>
</tr>
<tr>
<td><strong>Oral hypoglycaemics</strong></td>
<td>54</td>
</tr>
<tr>
<td><strong>Insulin therapy</strong></td>
<td>14</td>
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**Concomitant medications on admission (%)**

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<tr>
<td><strong>aspirin</strong></td>
<td>50</td>
</tr>
<tr>
<td><strong>ACEI</strong></td>
<td>40</td>
</tr>
<tr>
<td><strong>Statin</strong></td>
<td>36</td>
</tr>
</tbody>
</table>

Table 5. Patient characteristics.
AMI= acute myocardial infarction, BSL=blood sugar level, HbA₁C=glycated haemoglobin, ACEI=angiotensin converting enzyme inhibitor
### 3.3.2. Univariate Results

A summary of all univariate correlations are found in table 6.

<table>
<thead>
<tr>
<th>Hypothesis/Correlate(s)</th>
<th>Variable</th>
<th>Values</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Hypothesis</strong></td>
<td>Platelet SNP response</td>
<td>39 ± 2.7 %</td>
<td>(acute) r=-0.31, p&lt;0.01 (chronic) r=-0.18, p=0.13</td>
</tr>
<tr>
<td>(BSL/HbA₁C)</td>
<td>Superoxide generation *</td>
<td>Median 13mV</td>
<td>(acute) r=0.43, p&lt;0.0005 (chronic) r=0.28, p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Soluble guanylate cyclase activity</td>
<td>130 ± 3%</td>
<td>(acute) r=-0.22, p=0.18 (chronic) r=-0.04, p=0.83</td>
</tr>
<tr>
<td><strong>Secondary Hypotheses</strong></td>
<td>ADP induced aggregation</td>
<td>9.6 ± 0.6 Ohms</td>
<td>r=0.21, p=0.06</td>
</tr>
<tr>
<td>(BSL)</td>
<td>PRP SNP response</td>
<td>24.8 ± 3.9%</td>
<td>r=-0.14, p=0.4</td>
</tr>
<tr>
<td></td>
<td>NEFA levels</td>
<td>1.01 ± 0.07 mEq/L</td>
<td>r=0.21, p=0.11</td>
</tr>
<tr>
<td></td>
<td>Neutrophil count *</td>
<td>Median 6.7X10⁹/L</td>
<td>r=0.36, p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>CRP *</td>
<td>Median 6.8 mg/L</td>
<td>r=0.28, p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>CK *</td>
<td>Median 131.5 U/L</td>
<td>r=0.35, p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>ADP enhanced superoxide generation *</td>
<td>Median 115mV</td>
<td>r=0.28, p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>ADMA</td>
<td>0.107 ± 0.0003 µg/ml</td>
<td>r=-0.27, p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>L-arginine</td>
<td>10.4 ± 0.45 µg/ml</td>
<td>r=-0.36, p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>L-arginine/ADMA</td>
<td>98.5 ± 3.7</td>
<td>r=0.07, p=0.62</td>
</tr>
</tbody>
</table>

Table 6. Summary of univariate results: correlations of various parameters with acute BSL and HbA₁C. 
(* = log transformed data)
3.3.2.1 Primary Hypothesis

Pertaining to the primary hypothesis, admission plasma glucose level and HbA$_{1C}$ were used as indices of acute and chronic glycaemic control respectively. As seen by figures 8 and 9, platelet SNP responsiveness inversely correlated significantly with acute glycaemic control ($r=-0.31$, $p<0.01$) but not significantly with chronic glycaemic control as quantitated by HbA$_{1C}$ ($r=-0.18$, $p=0.13$). Mean platelet SNP responsiveness was $39 \pm 2.7\%$.

Figure 8. Correlation between admission blood sugar level (BSL) and platelet SNP responsiveness. ($r=-0.31$, $p<0.01$)
Figure 9. Correlation between admission HBA1C and platelet SNP responsiveness. (r=-0.18, p=0.13)

Figure 10 illustrates the non-Gaussian distribution of the LDCL data and subsequent normalization with log transformation.
Figure 11 and 12 show a significant correlation between superoxide generation, assessed by LDCL (n=64) with both admission BSL (r=0.43, p<0.0005) and HBA1c respectively (r=0.28, p=0.02). Median LDCL was 13mV.
Cyclic GMP generation (n=36) mean response was 130 ± 3%. There was no significant correlation with either acute (r=-0.22, p=0.18) or chronic (r=-0.04, p=0.83) glycaemic control. (figures 13 and 14)
Figure 13. Correlation between BSL and cGMP response to SNP.
\((r=-0.22, \ p=0.18)\)

Figure 14. Correlation between HbA\(_{1C}\) and cGMP response to SNP.
\((r=-0.04, \ p=0.83)\)
As can be seen, the impaired SNP responsiveness in platelets correlated with acute rather than chronic glycaemic control. This also appeared (from the LDCL data) to be related to instantaneous oxidative stress and an increase in whole blood superoxide, rather than impaired soluble guanylate cyclase activity.

### 3.3.2.2 Secondary Hypotheses

In relation to the secondary hypotheses, a number of variables with non-Gaussian distribution underwent log transformation to normalize the data (Figures 15-18).

![Figure 15. Non-Gaussian distribution of neutrophil count data, normalized with log transformation.](image-url)
Figure 16. Non-Gaussian distribution of CRP values, normalized with log transformation.

Figure 17. Non-Gaussian distribution of peak CK values, normalized with log transformation.
The acute admission BSL was not significantly correlated with whole blood ADP induced platelet aggregation \((r=0.21, p=0.06)\) with a mean aggregation response of 9.6±0.6 Ohms (fig 19) or with NEFA levels \((n=61)\) \((r=0.21, p=0.11)\) with a mean value of 1.01±0.07 mEq/L (fig 20). No significant correlation was seen between SNP response in PRP and admission BSL \((n=36)\) \((r=-0.14, p=0.4)\) (fig 21). When compared to our whole blood results, this raises the possibility of NO\(^{\cdot}\) inactivation mediated through the non-platelet component of whole blood.

There was however a significant correlation between admission BSL and both the neutrophil count \((R=0.36, p=0.001\) for log transformed data) with a median count of 6.7 X 10\(^9\)/L (fig 22) and CRP values \((n=61)\) \((r=0.28, p=0.03\) for log transformed data) with a median value of 6.8mg/L (fig 23). The peak CK value (fig 24) was also correlated with admission BSL \((n=76)\) \((r=0.37, p=0.001)\) as was the ADP enhanced
LDCL (n=63) (r=0.28, p=0.02 for log transformed data) (fig 25) with a median ADP enhanced LDCL of 115mV.

Plasma asymmetric dimethylarginine (fig 26) was inversely correlated with admission BSL (n=53) (r=-0.27, p=0.04) with a mean value of 0.107±0.003 µg/ml, as was L-arginine (fig 27) (n=45)(r=-0.36,p=0.01) with a mean value of 10.4±0.45 µg/ml. No significant correlation existed between BSL and L-arginine/ADMA ratio (fig 28) (n=45) (r=0.07, p=0.62) with a mean value of 98.5±3.7.

Figure 19. Correlation between admission BSL and ADP induced aggregation. (r=0.21, p=0.06)
Figure 20. Correlation between admission BSL and NEFA levels. 
\( r=0.21, \ p=0.11 \)

Figure 21. Correlation between admission BSL and PRP platelet SNP response. 
\( r=0.14, \ p=0.4 \)
Figure 22. Correlation between admission BSL with neutrophil count (log transformed). \( r=0.36, p<0.01 \)

Figure 23. Correlation between admission BSL with CRP values (log transformed). \( r=0.28, p<0.05 \)
Figure 24. Correlation between admission BSL with peak CK values (log transformed). (r=0.35, p<0.001)

Figure 25. Correlation between admission BSL with ADP enhanced LDCL (log transformed). (r=0.28, p<0.05)
Figure 26. Correlation between admission BSL with ADMA levels. 
\( r=-0.27, \ p<0.05 \).

Figure 27. Correlation between admission BSL with L-arginine levels. 
\( r=-0.36, \ p<0.01 \)
Figure 28. Correlation between admission BSL with L-arginine/ADMA ratio.
(r=0.07, p=0.62)
3. 3. 3. Multivariate Analysis

We also assessed the determinants of (a) platelet SNP response and (b) $O_2^-$ levels by a stepwise multivariate analysis. Parameters included in the initial model were age, sex, BSL, statin, insulin and ACE-inhibitor therapy and extent of CK elevation. The results are summarized for platelet SNP response in Table 7 and for $O_2^-$ levels in Table 8. Thus, increased BSL was a significant correlate both of impaired SNP response and of increased $O_2^-$ generation: increasing age was predictive of diminished $O_2^-$ generation.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSL</td>
<td>0.28</td>
<td>-2.3,-0.2</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 7. Multivariate Analysis: Significant correlates of platelet SNP response

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSL</td>
<td>7.4</td>
<td>0.62,3.4</td>
<td>0.005</td>
</tr>
<tr>
<td>Age</td>
<td>0.5</td>
<td>-1.2,-0.6</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Table 8. Multivariate Analysis: Significant correlates of $O_2^-$ levels.

3. 4. DISCUSSION

This study is the first to examine SNP responsiveness at the platelet level in diabetics presenting with an ACS. These results show that acute BSL control in this cohort is strongly correlated with platelet SNP responsiveness as well as increased $O_2^-$ generation on both univariate and stepwise multivariate analysis.
3. 4. 1. Primary hypothesis

3. 4. 1. 1. Hyperglycaemic mediated oxidative stress

Post-prandial hyperglycaemia in diabetics is associated with both an increase in nitrotyrosine generation and a decrease in endothelial function (Ceriello et al., 2002c). These and other data suggest that hyperglycaemia-associated oxidative stress may lead to reduction in NO’ bioavailability in the vasculature, yet to date this phenomenon has not been studied in platelets. Proposed mechanisms for hyperglycaemic induced increases in redox stress are numerous and can occur in both normal and diabetic subjects (Bonnefort-Rousselot, 2002; Ceriello, 1997; Ceriello, 2000; Sampson et al., 2002). Increases in hyperglycaemic mediated ROS generation may occur via at least 3 different mechanisms: nonenzymatic glycation, auto oxidation of glucose or intracellular activation of the polyol pathway (Bonnefort-Rousselot, 2002; Ceriello, 1997; Ceriello, 2000). Hyperglycaemia-induced overproduction of methylglyoxal (Shinohara et al., 1998) is one example of AGE-mediated increases in ROS (Singh et al., 2001), which is also associated with up-regulation of oxidative stress response genes (Yan et al., 1994a). Intracellular glucose oxidation leads to the generation of the reduced forms of NADH and FAD which contributes to redox stress. Elevation of intracellular glucose levels can cause an increased flux through the enzyme aldose reductase, which is only activated when intracellular glucose concentrations rise to hyperglycaemic levels (Sheetz and King, 2002). Aldose reductase depletes NADPH and its subsequent decline contributes to a reduction in endothelial NO’ production (Tesfamariam, 1994). Hyperglycaemic mediated upregulation of PKC (Scivittaro et al., 2000), also contributes to an increase in ROS (Inoguchi et al., 2000).
High baseline $O_2^-$ levels in our study were directly correlated with both elevation of admission BSL and HbA$_{1c}$, while only poor acute glycaemic control correlated with ADP enhanced superoxide generation. The source of this $O_2^-$ is likely to be primarily neutrophil NAD(P)H oxidase, as hyperglycaemia was inversely correlated with SNP responsiveness in whole blood but not with platelet rich plasma.

3. 4. 1. 2. Mechanism and source of superoxide generation

Hyperglycaemic associated increase in NAD(P)H oxidase activity has been shown in both endothelial cells and neutrophils and may be mediated through a PKC derived mechanism (Cosentino et al., 2003; Inoguchi et al., 2000; Mohanty et al., 2000). While we have observed that increased $O_2^-$ generation is a significant correlate of impaired platelet SNP responsiveness, we found no alteration in platelet cGMP responses. While $O_2^-$ generation may impair platelet soluble guanylate activity in stable angina pectoris (Chirkov et al., 1999), our results do not support this as a major influence in diabetics with ACS. As soluble guanylate cyclase activity is potentially impaired in association with incremental redox stress, these results must, however, be taken into context: it is not implied that soluble guanylate cyclase activity in diabetes is impervious to $O_2^-$, but rather that any change induced is small in this circumstance.

3. 4. 2. Secondary hypotheses

3. 4. 2. 1. ADP enhanced aggregation, platelet rich plasma studies, NEFA levels

It has been previously shown that platelet dependent thrombus formation is enhanced in diabetics with long-term poor glycaemic control (Aoki et al., 1996) and in patients
with stable CAD (Shechter et al., 2000). There is also a direct correlation between enhanced platelet activation and plasma glucose following an AMI (Oswald et al., 1988). However, our results showed a strong trend (p=0.06) but no significant correlation between ADP enhanced aggregation and admission BSL. The absence of any significant correlation between PRP SNP response and admission BSL differs from the results found in whole blood. This raises the possibility of NO• inactivation mediated through the non-platelet component of whole blood, most likely neutrophil NAD(P)H oxidase. Free fatty acids (FFA), increased in diabetics, (Steinberg et al., 1996) may also contribute to a reduction in NO• bioavailability. This has been shown by FFA mediated reduction in endothelium-dependent vasodilation of the femoral artery in insulin-resistant obese subjects (Steinberg et al., 1997). The mechanism underlying this effect is uncertain, but may relate to a decrease in eNOS activity (Davada et al., 1995). As both elevated fatty acid levels and hyperglycaemia reflect insulin resistance in diabetics it is possible that both may impact on NO• bioavailability. No correlation existed between NEFA levels and admission BSL thus minimizing, in the presence of a high admission BSL any incremental effects that NEFA levels may have on O2•− generation (Inoguchi et al., 2000).

3. 4. 2. 2. Neutrophil count, CRP, CK, ADP enhanced superoxide generation

The initial findings of our secondary hypotheses were a ‘self-fulfilling prophecy’. As has been discussed, BSL is an independent predictor of mortality in the diabetic (Fava et al., 1996; Malmberg et al., 1999) and non-diabetic (Foo et al., 2003; Wahab et al., 2002) patients presenting with an AMI. As CRP (Biasucci et al., 1999; Lindahl et al., 2000; Liuzzo et al., 1994; Morrow et al., 1998) and neutrophils (Modan et al., 1975; Yen et al., 2001) are also associated with an increase in mortality in ACS, it is not surprising that these inflammatory markers correlated with admission BSL in our
diabetic group. The neutrophils however may not just be an ‘innocent bystander’, as neutrophil derived superoxide release has been shown to be increased in diabetics compared to controls which correlated with HbA1c levels (Shurtz-Swirski et al., 2001).

In diabetic patients with a myocardial infarction, hyperglycaemia is an independent risk factor for an increase in mortality (Capes et al., 2000; Malmberg et al., 1999). It is therefore of no surprise that we should find a significant correlation with admission BSL and CK rise in our cohort. This would support the findings that hyperglycaemic-mediated \( \mathrm{O}_2^- \) generation results in impaired platelet NO’ responsiveness leading to larger myocardial necrosis.

On activation, leukocytes respond with degranulation, increased superoxide generation, chemotaxis, and phagocytosis. All of these processes may be influenced by platelets (Bazzoni et al., 1991; Li et al., 2000). This platelet-leukocyte interaction is enhanced in patients with an AMI (Neumann et al., 1997). The positive correlation between admission BSL and ADP enhanced superoxide generation supports a possible hyperglycaemic-mediated increase in platelet-leukocyte interaction, as the most likely source of enhanced superoxide generation in whole blood is neutrophil derived NAD(P)H oxidase.

3. 4. 2. 3. ADMA and L-arginine levels.

Mean ADMA level was 0.107 µg/ml which is approximately half that in some previous diabetic studies (Abbasi et al., 2001; Asagami et al., 2002). It is likely that this difference may reflect less specific assay methodology by previous studies (see separation techniques in methods 2.1.6). There is no likelihood of significant loss of
ADMA in the current study. Asymmetric dimethylarginine is metabolized by the enzyme DDAH which is redox sensitive. In vitro studies have suggested that DDAH activity is significantly impaired by redox stress associated with high glucose levels, with resultant increases in ADMA levels (Lin et al., 2002).

Surprisingly we found ADMA levels were inversely correlated with admission BSL. While the association was not particularly strong (p=0.04) we are not the first to find such a correlation. Paiva et al. found that ADMA levels were significantly lower in their diabetic group compared to controls and were inversely correlated with HbA1c (Paiva et al., 2003). While our data showed elevated values in diabetics compared to controls (data not shown), and therefore more in keeping with diabetes-associated increases in ADMA (Abassi et al., 2001), the inverse correlation with BSL may relate to enhanced renal excretion. Glomerular filtration rate (GFR) is increased in early diabetic kidney disease and inversely correlates with ADMA levels (Paiva et al., 2003). If an increased GFR is associated with possible urinary protein loss (ie. microalbuminuria) low levels of ADMA might be seen. This would preceed diabetic end stage renal disease where ADMA levels are increased (Kielstein et al., 1999; Vallance et al., 1992).

The l-arginine results also show that there is an inverse correlation with BSL levels. This may be explained by a free radical scavenging effect of L-arginine, as O\(^2\) levels correlate with BSL in our cohort. The superoxide scavenging effect of l-arginine has been shown previously in porcine endothelial cells (Wascher et al., 1997) and a rat mesenteric ischaemic reperfusion model (Haklar et al., 1998) and offers a plausible explanation to the ‘L-arginine paradox’.
3.4.3. Study Limitations

These results support a glucose mediated increase in $O_2^-$ generation and associated impaired platelet SNP response in diabetics admitted with an ACS. There are however a number of limitations to our study. Further support to these conclusions might have been gained by nitrotyrosine measurements as a surrogate for peroxynitrite formation. A conclusion that neutrophil NAD(P)H oxidase is the major source of the $O_2^-$ generation would be premature. A number of findings support this such as BSL associated high neutrophil count and absence of a significant correlation between BSL and platelet rich plasma SNP responsiveness in the presence of a significant inverse correlation in whole blood studies. Further studies to assess an increase in NAD(P)H oxidase subunits and activity would be required before this suspicion could be confirmed. It is also possible that higher admission BSL may have resulted in greater myocardial ischaemia and thus stimulating $O_2^-$ generation indirectly rather than directly. While this study did not correlate for intensity of ischaemia, it is reassuring to note that this is likely to only play a minor part as admission BSL but not peak CK rise (a guide to ischaemic burden) significantly correlated with $O_2^-$ generation by stepwise multivariate analysis. Finally, the study design could not evaluate the possibility that hormonal perturbations associated with hyperglycaemia (such as decreased tissue responsiveness to insulin, or increased plasma catecholamine concentration) might have played a role in mediating changes in platelet reactivity to NO$^-$ donors.
3. 5. CONCLUSIONS

Hyperglycaemia is independently associated with an increase in mortality in diabetics admitted with an AMI. This study endeavors to find a mechanistic explanation to this observation. Hyperglycaemia is associated with an increase in $O_2^-$ generation and impaired platelet SNP responsiveness. This phenomenon is present in the high risk diabetic patient with associated high inflammatory markers and CK rises and may contribute to the poor prognosis in this cohort.

The next challenge is to find whether these observations can be reversed with improved glycaemic control.