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Tolerance and Resistance to Organic Nitrates in Human Blood Vessels

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Thesis submitted for the degree of

Doctor of Philosophy

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Summary

1) The major categories of hypotheses of nitrate tolerance are a) impaired nitrate bioconversion resulting in diminished nitric oxide (NO) release b) increased NO clearance mediated via increased generation of superoxide (O_2^-) and/or c) desensitization of guanylate cyclase. The supporting evidence for these studies is based largely on animal studies.

2) The primary aim of the experiments described in this thesis was to investigate the mechanism(s) of nitrate tolerance) in vessels from patients receiving a 24 hour intravenous infusion of glyceryl trinitrate (GTN) at 10 µg/min. The vessels studied were isolated segments of internal mammary artery (IMA) and saphenous vein (SV) obtained from patients undergoing coronary bypass surgery.

3) Pharmacological studies were performed in organ baths with the vessels mounted under tension (2g for IMA; 1g for SV).

4) Responses to GTN were reduced 3- to 5-fold in the vessels from the nitrate group compared with vessels from control patients, demonstrating induction of a moderate degree of tolerance with this GTN regimen.

5) Tolerance was associated with minimal cross-tolerance to sodium nitroprusside (SNP) and A23187, indicting that tolerance was nitrate-specific. Other studies confirmed that GTN and SNP vasodilator action are mediated via guanylate cyclase in human vessels. The lack of significant cross-tolerance was therefore consistent with the impaired bioconversion hypothesis

6) This finding was supported by measuring bioconversion of GTN to 1,2-glyceryl dinitrate (1,2-GDN) following brief exposure to GTN. The concentration of 1,2-GDN was lower in segments of SV from the nitrate group compared with segments from the control group, indicating impairment of the bioconversion process.

7) With regard to the O_2^- hypothesis, IMA O_2^- generation was found to be greater in segments from the nitrate group than from those in the control group. However, inhibition of superoxide dismutase in vitro, which produced a 3-fold increase in IMA O_2^- generation, did not affect responses to GTN or bioconversion of GTN. These results suggest GTN action is insensitive to acute increases in redox stress.

8) Removal of the endothelium did not affect GTN responsiveness in IMA segments from either control or tolerant patients, suggesting that tolerance induction is independent of the endothelium.

9) Diphenyleneiodonium (DPI) was found to have only a small inhibitory effect on the relaxant responses of IMA to GTN in control segments, suggesting that the cyctochrome-P450 enzyme system play a minor role in GTN-induced vasodilation in human vessels.

10) Co-infusion of intravenous N-acetylcysteine (NAC) at 10 g/24 hours with GTN had minimal effect on the vasodilator responses to GTN and bioconversion of GTN. Similarly, exposure of tolerant and non-tolerant vessels to NAC in vitro did not affect responsiveness to GTN. These results provide evidence that NAC does not prevent or reverse GTN tolerance in these human vessels.

11) Vessels obtained from patients receiving prophylactic oral nitrate therapy (60-120 mg ISMN-SR once-daily or 10-20 mg ISDN thrice-daily) exhibited cross-tolerance to GTN. A secondary finding was that tolerance to GTN was seen up to 29 hours following the last dose of ISMN-SR, whereas there was no evidence of tolerance 17 hours after the last dose of ISDN..

12) The availability of control data permitted evaluation of the determinants of de novo vasodilator responses to GTN. Hyporesponsiveness to GTN (GTN resistance) was observed in the IMA but not SV, and was associated with increasing total number of risk factors for coronary artery disease and specifically with prior hypercholesterolaemia, smoking or diabetes mellitus. In addition, a correlation was

found between IMA responses to GTN and responses to A23187, a conventional marker of endothelial function.

13) Prior hypercholesterolaemia was also associated with higher levels of O_2^- generation. These experiments demonstrate that the presence of some risk factors for coronary artery disease may impair responses of arterial smooth muscle to GTN. Furthermore, the results suggest that the mechanism underlying this “resistance” to GTN may be related to increased redox stress.

Declaration

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

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

Peter Radford Sage (January 2001)

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Abbreviations

1,2-GDN	glyceryl-1,2-dinitrate
1,3-GDN	glyceryl-1,3-dinitrate
ACE	angiotensin-converting enzyme
ADMA	asymmetric dymethyl arginine
ATP	adenosine-5-triphosphate
CABG	coronary artery bypass graft
CAD	coronary artery disease
cGMP	cyclic guanosine 3'5'-monophosphate
CHF	congestive heart failure
COX	cyclo-oxygenase
DETCA	diethyldithiocarbamate
DPI	diphenyleneiodonium
EDRF	endothelium-derived relaxing factor
eNOS	endothelial nitric oxide synthase
GMN	glycerol mononitrate
GST	glutathione-S-transferase
GTN	glyceryl trinitrate
GTP	guanosine-5-triphosphate
IMA	internal mammary artery
ISDN	isosorbide dinitrate
ISMN	isosorbide mononitrate
NAC	N-acetylcysteine
NAD(P)H	reduced nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NO^-	nitroxyl anion
NO_2^-	nitrite
O_2^-	superoxide
ODQ	1*H*-[1,2,4]oxadiazolo-[4,3,-*a*]quinoxalin-1-one
PETN	pentaerythritol tetranitrate
SNP	sodium nitroprusside
SOD	superoxide dismutase
SV	saphenous vein

Publications

1. Sage PR, de la Lande IS, Stafford I, Bennett CL, Philipov G, Stubberfield J, Horowitz JD. Nitroglycerin tolerance in human vessels. Evidence of impaired nitroglycerin bioconversion. Circulation 2000;102:2810-2815.
2. Chirkov YY, Holmes AS, Willoughby SR, Stewart S, Wuttke RD, Sage PR, Horowitz JD. Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets. J Am Coll Cardiol (under review).

Abstracts

1. Sage PR, de la Lande IS, Stafford I, Stubberfield J, Horowitz JD. Nitrate tolerance and cross-tolerance in human internal mammary artery and saphenous vein following low-dose nitroglycerine infusion. Eur Heart J 1999;20(Suppl):541.
2. Chirkov YY, Holmes AS, Wuttke RD, Willoughby SR, Stewart S, Sage PR. Determinants of platelet responsiveness to nitric oxide donors in the presence and absence of ischaemic heart disease. Eur Heart J 1999;20(Suppl):545.
3. Sage PR, de la Lande IS, Stafford IS, Bennett CL, Stubberfield J, Horowitz JD. Nitrate tolerance to low-dose nitroglycerin is associated with reduce bioconversion. Circulation 1999;100(Suppl 1):186.
4. Sage PR, de la Lande IS, Stafford I, Stubberfield J, Horowitz JD. Nitrate tolerance and cross-tolerance in human internal mammary artery and saphenous vein following low-dose nitroglycerine infusion. Aust NZ J Med 2000;30:182.
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1. Introduction

1.1 Historical Perspective

The organic nitrates are the oldest class of antianginal agents, with reports of nitrate use in the late 19th century. Brunton, in 1857, was the first to describe the relief of angina with amyl nitrite, a compound related to the organic nitrates. However, it was not until 1879 that Murrell recognized that the organic nitrate, glyceryl trinitrate (GTN; nitroglycerin[e]) was effective in the relief and prophylaxis of acute angina pectoris(Murrell 1879).

Shortly after this it became evident that some of the effects of nitrates diminished with continued exposure. Munitions workers who suffered side effects following acute exposure to nitroglycerin, noted that these effects disappeared with time(Ebright 1914). Two early clinical reports also observed attenuation of nitrate effects during chronic dosing for hypertension(Crandall, *et al.* 1931; Stewart 1888).

Despite these reports, nitrates continued to be used clinically in the management of angina pectoris. Nitrate tolerance was largely regarded as a curiosity until the late 1960s.

1.2 Chemical Structure and Available Preparations

The organic nitrates are nitric acid esters of aliphatic or aromatic alcohols. The structure of several nitrates used therapeutically, is shown in Figure 1.1. The currently available preparations are listed in Table 1.1.

GTN is available as sublingual tablets or spray for relief of acute anginal attacks, as transdermal patches or ointment for anginal prophylaxis or congestive heart failure, and as an intravenous preparation for use in unstable angina, acute myocardial infarction or acute pulmonary oedema. Intravenous infusion of GTN (and other nitrates)

necessitates use of special infusion lines made of polyethylene, to avoid significant losses through adsorption onto standard plastic lines(Roberts, *et al.* 1980).

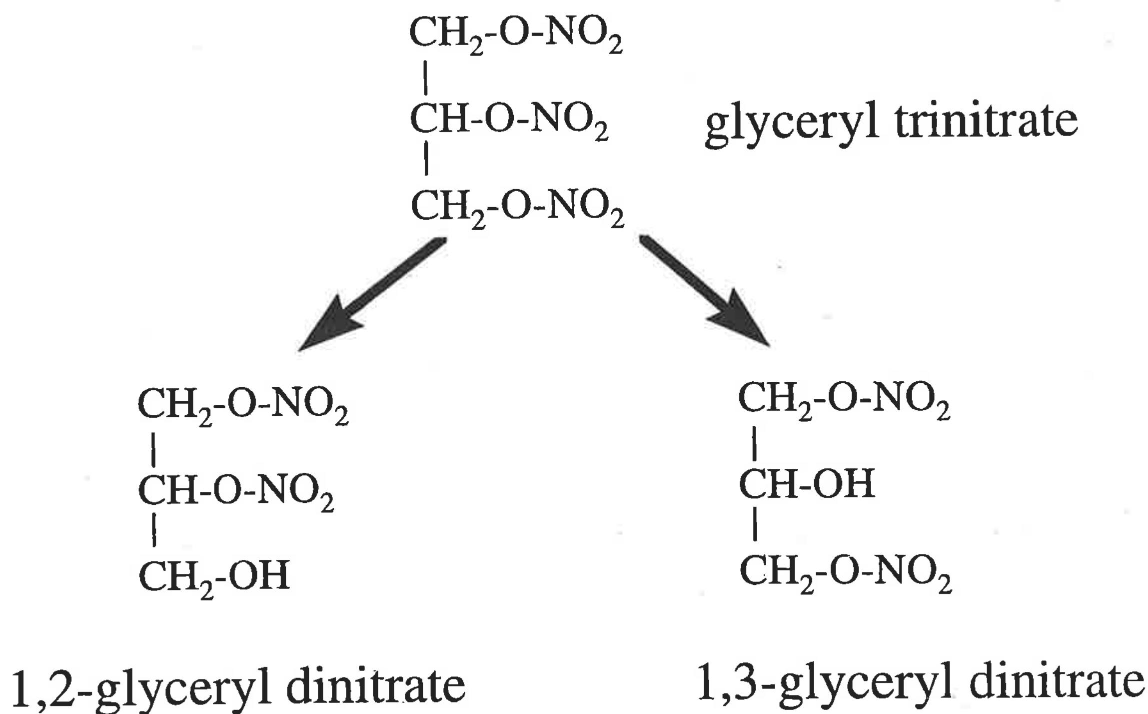
The other agents are available predominantly as oral preparations (including sustained-release preparations), for use in anginal prophylaxis and congestive heart failure. Isosorbide dinitrate is also available as sublingual tablets and intravenous injection.

A number of chimeric compounds exist possessing additional properties, such as potassium-channel opening activity (eg. nicorandil).

1.3 Cellular/biochemical mechanism of action

The diversity of structures possessing a biological action in common led to the early appreciation that organic nitrates were prodrugs, acting via a common biological intermediate. It is now accepted that they require release of nitric oxide (NO) or a closely related moiety (see below) for their pharmacological activity. The supporting evidence stemmed from findings in the mid to late 1970s that nitrates act primarily via activation of the enzyme soluble guanylate cyclase, resulting in elevation of intracellular cyclic guanosine 3',5'-monophosphate (cGMP)(Diamond, *et al.* 1976; Katsuki, *et al.* 1977; Kukovetz, *et al.* 1979). Since NO was subsequently shown to activate soluble guanylate cyclase(Murad 1986), it was postulated that nitrates act via formation of NO. Subsequently, formation of NO from GTN has been demonstrated in vascular smooth muscle and endothelial cells.

The major mechanism of NO release from GTN, is reductive denitration (largely or entirely enzymatic) at the 1 or 3 Carbons, and/or the 2 Carbon, with the co-products 1,2-glyceryl dinitrate (1,2-GDN) and/or 1,3-glyceryl dinitrate (1,3-GDN). Formation of glycerol mononitrates (GMNs) appears to occur only to a minimal extent.



However a number of aspects of the cellular mechanism of action of organic nitrates remain uncertain. These include:

1. What is the pharmacologically active moiety?
2. How is this moiety formed?
3. Which enzyme system(s) are involved in nitrate bioconversion?
4. What is the role of sulphydryl groups in nitrate action?

Studies over the last two decades have partially resolved these questions.

1.3.1 What is the pharmacologically active moiety?

The breakdown of GTN to inorganic nitrite (NO_2^-) in blood in vitro, was noted as early as 1883(Hay 1883). Ignarro et al(1981) suggested that nitrates were denitrated to nitrite, which can react with intracellular hydrogen to form nitrous acid. Under acidic conditions, nitrous acid is decomposed to form NO. However, subsequent studies cast doubt on the role of NO_2^- as an intermediate of nitrate action. Bennett and Marks(1984) questioned how GTN-derived NO_2^- could incrementally affect cell function, given that endogenous NO_2^- concentration is in the micromolar range. Other

investigators(Romanin, *et al.* 1988), demonstrated that at equipotent concentrations of GTN and sodium nitrite for stimulation of guanylate cyclase, the NO_2^- concentrations produced were different by 1000-fold. Feelisch(1987b) also dissociated formation of NO_2^-, from GTN-induced activation of guanylate cyclase. It is therefore unlikely that breakdown of GTN to NO_2^- contributes significantly to guanylate cyclase activation(Bennett, *et al.* 1994), although changes in NO_2^- generation might nevertheless modulate effectiveness of nitrates.

Several studies have supported the concept that NO is the active moiety formed from nitrates. Formation of NO from GTN has been demonstrated in porcine vascular smooth muscle and endothelial cells by a spectrophotometric technique(Feelisch, *et al.* 1991), in bovine coronary artery via chemiluminescence-headspace gas method(Chung, *et al.* 1993), in perfused rabbit heart by oxyhaemoglobin(Forster, *et al.* 1991) and in intact rabbit aorta via bioassay(Hussain, *et al.* 1994). In the latter study, release of NO was concurrent with vasorelaxation. Recent studies provide evidence for NO generation from GTN in vascular tissues using electron spin resonance spectroscopy both in vivo(Mulsch, *et al.* 1995), and in vitro(Munzel, *et al.* 2000a) and have demonstrated a close correlation between NO generation and GTN-induced haemodynamic responses(Mulsch, *et al.* 1995). Despite this, it is still possible that the active moiety may be an S-nitrosothiol (see below 1.3.4).

Additionally, it should be mentioned that the product of nitrate bioconversion may be a redox variant of NO, such as nitroxyl anion (NO^-). NO^- released in pure form from Angeli's salt has been shown to mimic most of the biological effects of NO, although potency varies(Li, *et al.* 1999). Furthermore, there is evidence of inter-conversion between NO and NO^- in vitro according to redox state: notably cysteine has been shown to inactivate NO^- selectively(Ellis, *et al.* 2000).

1.3.2 How is the active moiety formed?

The organic nitrates must undergo bioconversion releasing the active moiety (see above) to produce pharmacological effects. While it has been shown that

nonenzymatic bioconversion of nitrates can occur in vitro in the presence of large concentrations of some thiol compounds(Feelisch, *et al.* 1987a), there is strong evidence that bioconversion of nitrates in vivo is almost entirely enzymatic(Bennett, *et al.* 1994; Fung, *et al.* 1992). Studies by Bennett's group(Bennett, *et al.* 1989; Brien, *et al.* 1988; Kawamoto, *et al.* 1990) have used dinitrate levels as a surrogate for bioconversion of GTN to the active moiety in vascular smooth muscle. If bioconversion of GTN were nonenzymatic or spontaneous, the resultant 1,2-GDN/1,3,GDN ratio would be approximately 2. However, these studies have demonstrated regioselective formation of 1,2-GDN at the low concentrations of GTN sufficient for vascular relaxation(Bennett, *et al.* 1989; Brien, *et al.* 1988; Kawamoto, *et al.* 1990). In addition, selective bioconversion of GTN to 1,2-GDN, occurs concurrently with elevation of intracellular cGMP(Brien, *et al.* 1988) and precedes vasodilation(Kawamoto, *et al.* 1990). Importantly, this regioselectivity is lost if the duration of exposure to GTN is increased(Bennett, *et al.* 1992), if the incubating concentration of GTN is high(Bennett, *et al.* 1989)or in broken-cell preparations(Marks, *et al.* 1989). Overall, these studies suggest that the bioconversion pathway for 1,2-GDN formation is largely or entirely responsible for the pharmacological effects of GTN, and hence, for generation of NO. In addition, at pharmacologically effective concentrations, cellular bioconversion is largely enzymatic.

Similarly, the nitrate isoidide dinitrate exhibits significant enantioselectivity for the D-enantiomer with regard to vasodilation and cGMP accumulation in intact cell preparations; these properties are not apparent in broken-cell preparations(Bennett, *et al.* 1988).

1.3.3 Which enzyme system(s) is involved in nitrate bioconversion?

With regard to the enzyme system(s) involved in bioconversion of organic nitrates, a number of factors need to be considered. Firstly, it is evident that multiple enzyme systems are capable of bioconverting organic nitrates, but that they may differ in their ability to generate the moiety responsible for pharmacological effects. Bennett(1994) has used the terms "mechanism-based" and "clearance-based"

biotransformation to distinguish between pathways that lead to generation of mediators of biological activity (such as NO), and pathways which lead to generation of moieties with little or no biological activity (such as nitrite(Romanin, *et al.* 1988)): the generalisation inherent in this classification has not totally limited its usefulness.

Secondly, while it is now appreciated that most tissues are capable of metabolizing organic nitrates, the bioconverting enzyme systems vary from tissue to tissue. Hence mechanism-based bioconversion will also vary. Vascular smooth muscle, for example, is capable of bioconversion yielding NO(Feelisch, *et al.* 1991) and elevation of intracellular cGMP(Diamond, *et al.* 1976), resulting in vasdodilation(Brien, *et al.* 1988), whereas bioconversion in the liver is predominantly to nitrite, with apparently little resultant pharmacological effect.

Finally, multiple pathways for bioconversion may operate in each tissue. GTN concentration-response curves have been shown to be biphasic in vitro in vascular smooth muscle and platelets. Malta demonstrated a biphasic relaxant response to GTN in rat aorta(1989), suggesting that there were two distinct mechanisms of action. Feelisch(1991) expanded on this observation by demonstrating two different components of biotransformation of GTN to NO in porcine vascular smooth muscle cells, namely a high affinity, heat-sensitive component prone to rapid desensitization, and a low-affinity, unsaturable component which may be non-enzymatic.

These factors, as well as the technical difficulty in measuring NO generation in biological systems, have complicated efforts to determine both the relationship between nitrate bioconversion and pharmacological effect, and also the identity of the enzyme system(s) responsible for mechanism-based bioconversion. However, three particular categories of investigations are of importance in this respect.

Studies by Chung and Fung(1990a) showed that an enzyme system capable of generating NO from GTN in bovine coronary artery smooth muscle cells is located in the plasma membrane. This enzyme has a molecular weight of approximately 160 kilodaltons, with activity facilitated by reduced sulphydryl groups(Seth, *et al.* 1993), and is not identical to glutathione-S-transferase(Chung, *et al.* 1992a; Chung, *et al.*

1990b). Further studies by the same group suggested that other organic nitrates are also metabolized by this enzyme(Chung, *et al.* 1992b) and that this bioconversion pathway for GTN is distinct from that of sodium nitroprusside (SNP)(Kowaluk, *et al.* 1992). However, this enzyme has yet to be characterized further and its exact identity remains uncertain.

Studies investigating the role of another putative enzyme system, glutathione-S-transferase (GST), have been conflicting, particularly as regards its role in mechanism-based bioconversion. Several isoforms of GST have been identified in vascular smooth muscle(Kurz, *et al.* 1993; Nigam, *et al.* 1996), and have been shown to be capable of bioconverting GTN, in particular the μ isoform(Kenkare, *et al.* 1994; Nigam, *et al.* 1996). However, several studies in both broken- and intact-cell preparations, have demonstrated that bioconversion of GTN by GST in vascular smooth muscle results predominantly in the formation of nitrite(Hill, *et al.* 1992; Kurz, *et al.* 1993), and 1,3-GDN(Lau, *et al.* 1992b), suggesting that GST is involved in clearance-based rather than mechanism-based bioconversion. One study(Simon, *et al.* 1996) using an eletroporation technique to introduce a GST inhibitor (basilen blue) intracellularly, demonstrated reduced bioconversion of GTN to 1,2-GDN. However, the tissue examined in this study was porcine kidney epithelial cells, not vascular smooth muscle. Studies examining the effects of GST inhibitors in isolated vessels have been conflicting, with some(Nigam, *et al.* 1993; Yeates, *et al.* 1989), but not others(Chung, *et al.* 1993; Chung, *et al.* 1990b; Lau, *et al.* 1992a; Salvemini, *et al.* 1993c) reporting inhibitory effects on GTN-induced relaxation. Finally, perhaps the most compelling evidence that GST enzymes are not involved in mechanism-based nitrate bioconversion, is the observation that humans completely lacking the GST μ isoform, have normal venodilator responses to GTN(Haefeli, *et al.* 1993).

Lastly, extensive data support the role of cytochrome P450-NADPH-cytochrome P450 reductase system in the bioconversion of organic nitrates, with some evidence that this system represents an important mechanism-based pathway, although it is clearly different from the enzyme characterized by Fung and coworkers(Chung, *et al.* 1990a). Cytochrome P450-mediated nitrate bioconversion has been demonstrated in many tissues, including rat hepatic microsomes(McDonald, *et al.* 1990; Servent, *et al.*

1989), rat aortic microsomes(McDonald, *et al.* 1993; McGuire, *et al.* 1998), and rat lung fibroblasts(Schroder 1992). This bioconversion has been shown to result in significant NO release(Servent, *et al.* 1989), as well as formation of an activator of guanylate cyclase(Bennett, *et al.* 1992). However, studies using inhibitors of the cytochrome P450 system have yielded conflicting results, possibly due to a lack of specificity of the inhibitors for the relevant cytochrome P450 enzyme(s). While in vitro bioconversion of GTN by rat aortic microsomes has been shown to be inhibited by SKF525A, carbon monoxide and oxygen(McDonald, *et al.* 1993), these agents do not appear to affect GTN-induced relaxation or GTN bioconversion in intact blood vessels(Bornfeldt, *et al.* 1987; Liu, *et al.* 1993; Salvemini, *et al.* 1993c). Studies using the flavoprotein inhibitor diphenyleneiodonium (DPI), which has been shown to inhibit c-DNA-expressed rat liver cytochrome P450 reductase, and activity of aortic and hepatic microsomal NADPH-cytochrome P450 reductase(McGuire, *et al.* 1998), have demonstrated inhibition of GTN-induced vascular relaxation and cGMP accumulation in some(McGuire, *et al.* 1994; McGuire, *et al.* 1998; Ratz, *et al.* 2000) but not all(De la Lande, *et al.* 1996a) studies. DPI appears to selectively inhibit formation of 1,2-GDN(McGuire, *et al.* 1994; McGuire, *et al.* 1998) suggesting that bioconversion of GTN to 1,2-GDN is mechanism-based. DPI has also been shown to inhibit the enantioselectivity of isoidide dinitrate action, by selectively inhibiting the more potent D-enantiomer(Ratz, *et al.* 1996). Lastly, induction of cytochrome P450 enzymes in vivo with phenobarbital(McDonald, *et al.* 1993) or in vitro with 3-methylcholanthrene(Schroder 1992), has been shown to increase GTN bioconversion in rat aortic microsomes and rat lung fibroblasts respectively.

The effects of inhibitors of arachidonic acid metabolism have also been studied in isolated vessels(Bornfeldt, *et al.* 1987) and in vivo(Munzel, *et al.* 1988)and found to have no inhibitory effects on GTN action (see also section 1.3.5).

In summary, therefore, of the three "candidate" enzyme systems to account for the major component of NO generation from organic nitrates, GST (including its μ isomer) can be to all extent excluded. The 160 kilodalton SH-dependent plasma membrane enzyme studies by Chung and Fung(1990a) appears to be important but has not been characterized fully. Lastly, the main problem with cytochrome P450-

dependent bioconversion is that it is clearly distinct from the plasma membrane enzyme and its pharmacological importance remains less than clear-cut.

1.3.4 What is the role of sulphydryl groups in nitrate action?

Interest in the role of sulphydryl groups in the bioconversion of organic nitrates originated with the studies of Needleman(1973a), showing that exposure to a sulphydryl alkalating agent, ethacrynic acid, reduced GTN-induced vascular relaxation. In addition, both exposure to ethacrynic acid and induction of in vitro tolerance to GTN(Needleman, *et al.* 1973b), were associated with depletion of tissue sulphydryl content. It was concluded that nitrate action is sulphydryl-dependent, and the "sulphydryl depletion" hypothesis of nitrate tolerance was born (see nitrate tolerance section below). Interest in the role of sulphydryl groups was further stimulated by evidence that bioconversion of GTN in artery homogenates was thiol-dependent(Ignarro, *et al.* 1981), leading to the suggestion that S-nitrosothiols are active intermediates in nitrate action. A recent study supported this hypothesis, by dissociating GTN-induced vasodilation from NO generation, by means of ultraviolet irradiation(Hussain, *et al.* 1999). In addition, Kurz et al(Kurz, *et al.* 1991) demonstrated a stereo-selective effect of cysteine in potentiating GTN-induced vasodilation, suggesting the existence of a sulphydryl-cofactor in GTN enzymatic bioconversion.

One further possibility is that S-nitrosothiols may not merely be intermediates of NO formation: they may be the main source of direct stimulation of guanylate cyclase. A similar controversy surrounds the mode of soluble guanylate cyclase stimulation by EDRF(Myers, *et al.* 1990).

However, other studies argue against a pivotal role of S-nitrosothiols in GTN action. S-nitrosothiols themselves appear to require enzymatic release of NO to cause vasodilation(Kowaluk, *et al.* 1990b). Secondly, the systemic haemodynamic effects of GTN differ from those of S-nitrosothiols(Bauer, *et al.* 1991b), and coronary microvessel vasodilator potency also differs(Sellke, *et al.* 1990). One explanation for the latter observations, may be a relative deficiency of sulphydryl groups or the

enzymes responsible for bioconverting GTN in microvessels, resulting in diminished bioconversion of GTN to S-nitrosothiol in these vessels. An alternative conclusion however, could be that S-nitrosothiols are not involved in the action of GTN(Fung, *et al.* 1992).

On the other hand, evidence has accumulated supporting the concept that sulphydryl groups facilitate nitrate action, although their role in nitrate tolerance remains uncertain (see below). Further studies examining the effect of ethacrynic acid in isolated vessels demonstrated similar inhibition of GTN-induced relaxation(Kenkare, *et al.* 1993; Lau, *et al.* 1992a; Moffat, *et al.* 1985; Sellke, *et al.* 1991), although it appeared to have no effect in vivo(Moffat, *et al.* 1985). Later detailed experiments by Boesgaard(1993), showed that depletion of intracellular sulphydryls attenuated nitrate effects in vivo, but that increasing intracellular sulphydryls did not provide further increase in nitrate effects. Increasing extracellular sulphydryl availability, however, significantly potentiated nitrate effects. Supporting this study, Fung has demonstrated that intracellular GTN bioconversion in rat aorta is not increased by the sulphydryl donor, N-acetylcysteine (NAC)(Fung, *et al.* 1988), but that GTN and NAC react in plasma to form an S-nitrosothiol, and that GTN-induced relaxation is potentiated in the presence of NAC and plasma. The existence of an extracellular pathway for nitrate-sulphydryl interaction was postulated.

These two studies may help explain the conflicting results obtained from experiments examining the effect of sulphydryl-donating agents on nitrate action. On the one hand, most(Chong, *et al.* 1991; Fung, *et al.* 1988; Gruetter, *et al.* 1986; Henry, *et al.* 1989b; Lawson, *et al.* 1996), but not all(Torresi, *et al.* 1985) studies in isolated large vessels have shown no effect of sulphydryl donors on GTN-induced relaxation. In sharp contrast, the overwhelming majority of experiments in intact animals(Boesgaard, *et al.* 1993; Fung, *et al.* 1988; Munzel, *et al.* 1989; Munzel, *et al.* 1992) and human subjects(Boesgaard, *et al.* 1994b; Creager, *et al.* 1997; Horowitz, *et al.* 1983; Levy, *et al.* 1988; May, *et al.* 1987; Mehra, *et al.* 1994; Nishikawa, *et al.* 1998; Svendsen, *et al.* 1989; Vekshtein, *et al.* 1990; Winniford, *et al.* 1986) have demonstrated a potentiating effect of sulphydryl donors on nitrate action.

A second potential explanation for the contrasting in vitro and in vivo observations, may relate to nitrate-sulphydryl interaction in small resistance vessels. Several studies have shown that while sulphydryl donors have minimal effect on nitrate action in large vessels, they can potentiate nitrate relaxation in small microvessels(Kurz, *et al.* 1991; Munzel, *et al.* 1992; Sellke, *et al.* 1991; Wheatley, *et al.* 1994).

Overall, therefore, it appears that nitrate action is dependent on intracellular endogenous sulphydryl groups, and may be potentiated by supplemental sulphydryl groups, perhaps largely via interaction in the plasma and/or small resistance vessels. The critical therapeutic issue raised by these studies is whether the spectrum of nitrate-induced vasodilation is altered favourably or otherwise by variation in sulphydryl bioavailability.

1.3.5 Cellular mechanism of action after release of the active moiety.

As mentioned previously, nitrates have been shown to act primarily via activation of soluble guanylate cyclase resulting in elevation of intracellular cGMP(Diamond, *et al.* 1976; Katsuki, *et al.* 1977; Kukovetz, *et al.* 1979) and relaxation of vascular smooth muscle(Gruetter, *et al.* 1981). This occurs via binding of the active moiety (probably NO) to the haem-iron domain in the N-terminal portion of soluble guanylate cyclase(Hobbs 1997; Murad 1986; Murad 1996). This forms a pentacoodinate nitrosyl-haem complex and causes a conformational change which activates the enzyme. The activated enzyme is then capable of binding guanosine-5'-triphosphate (GTP) to the catalytic domain at the C-terminal end of the enzyme and converting it to cGMP(Hobbs 1997). Inhibition of soluble guanylate cyclase with nonspecific inhibitors, such as methylene blue(Gruetter, *et al.* 1981), or more selective inhibitors, such as 1*H*-[1,2,4]oxadiazolo-[4,3,-*a*]quinoxalin-1-one (ODQ)(Brunner, *et al.* 1996; Feelisch, *et al.* 1999; Olson, *et al.* 1997) has been shown to inhibit nitrate-induced vascular relaxation. However, there is some evidence that despite effective prevention of any increase in cGMP by ODQ, higher concentrations of NO or NO donors can still cause complete relaxation of vascular tissue(Brunner, *et al.* 1996;

Feelisch, *et al.* 1999; Weisbrod, *et al.* 1998). Hence, mechanisms independent of cGMP production, such as direct activation of K_{ca} channels(Bolotina, *et al.* 1994) may contribute to the vasodilator action of NO/NO donors at higher concentrations.

Elevated intracellular cGMP mediates vasodilation via several mechanisms which have not yet been completely elucidated. Current evidence suggests that cGMP activates cGMP-dependent protein kinase which in turn activates sarcolemmal Ca^{++}-extrusion ATPase in vascular smooth muscle cells(Popescu, *et al.* 1985), as well as sarcoplasmic reticulum Ca^{++}-pump ATPase(Cohen, *et al.* 1999; Luo, *et al.* 1993; Twort, *et al.* 1988), resulting in reduced cytosolic free calcium concentration. The lower calcium concentration may dephosphorylate myosin light chain via myosin light chain kinase(Murad 1996) resulting in reduced tension.

A number of studies(Dikalov, *et al.* 1999; Dikalov, *et al.* 1998a; Dikalov, *et al.* 1998c) indicate that some organic nitrates co-release superoxide (O_2^-) during bioconversion: the notable exception being pentaerythrytyl tetranitrate. While O_2^- may also exert important biological effects including conversion of NO to peroxynitrite, the extent to which this affects nitrate action is not certain (see also section 1.8.3.2.4).

There is also some evidence that NO stimulates COX-1 in tissue culture(Salvemini, *et al.* 1993a). However, as previously mentioned, studies have shown that COX-1 inhibitors not alter nitrate action(Munzel, *et al.* 1988). It is therefore likely that this stimulation is of little clinical importance.

1.4 Pharmacokinetics

Several important pharmacokinetic properties of the commonly used organic nitrates after intravenous administration are summarized in Table 1.2. The nonpolar nitrates, GTN and isosorbide dinitrate (ISDN) have a high apparent volume of distribution(Armstrong, *et al.* 1979; Lee, *et al.* 1990; McNiff, *et al.* 1981; Morrison, *et al.* 1982; Morrison, *et al.* 1983a; Yap, *et al.* 1978), while the more polar nitrate isosorbide mononitrate (ISMN) has an apparent volume of distribution closer to the

plasma volume(Abshagen, *et al.* 1981a; Major, *et al.* 1984). Systemic clearance of the nonpolar agents is rapid, resulting in short elimination half-lives(Armstrong, *et al.* 1979; McNiff, *et al.* 1981; Morrison, *et al.* 1982; Morrison, *et al.* 1983a; Yap, *et al.* 1978). GTN, in addition, undergoes significant arteriovenous extraction (present at steady-state) across the capillary bed(Armstrong, *et al.* 1982; Cossum, *et al.* 1986; Fung, *et al.* 1984) and its systemic clearance is related to cardiac output(Fung, *et al.* 1986a), suggesting that nitrates may be substantially removed from the systemic circulation by the vasculature itself(Fung, *et al.* 1984). An arteriovenous concentration gradient is far less evident for ISDN(Morrison, *et al.* 1983b) and has not been studied for ISMN. In comparison with GTN and ISDN, the half-life of ISMN is considerably longer(Abshagen, *et al.* 1981a; Abshagen, *et al.* 1981b; Major, *et al.* 1984). With regard to presystemic clearance, GTN and ISDN are extensively metabolized during their first passage through the liver, resulting in low oral bioavailability(Lee, *et al.* 1990). Bioavailability of sublingual GTN and ISDN is also incomplete(Morrison, *et al.* 1983a; Noonan, *et al.* 1985b), whereas ISMN undergoes little first-pass metabolism(Abshagen, *et al.* 1981a; Major, *et al.* 1984).

Despite relatively short biological half-lives, sustained venous plasma concentrations of GTN and ISDN can be maintained by manipulation of their input rates. This can be achieved by either continuous intravenous infusion, or by prolonging the absorption rate via sustained-release transdermal or oral preparations(Morrison, *et al.* 1983a; Muller, *et al.* 1982; Parker, *et al.* 1984a; Parker, *et al.* 1984b). A sustained release oral preparation of ISMN also exists(Beyerle, *et al.* 1990; Chrysant, *et al.* 1993; Glasser 1997; Wisenberg, *et al.* 1989) providing a longer elimination half-life than standard ISMN.

Several other aspects of nitrate pharmacokinetics are of relevance. Both GTN and ISDN, when metabolized to release the active moiety (see above), generate metabolites which are themselves also active (GDNs and GMNs)(Cossum, *et al.* 1985; Haefeli, *et al.* 1992). The specific metabolites formed may vary with the dose of parent drug and the route of administration(Nakashima, *et al.* 1990). While these metabolites are less potent than the parent drug, they tend to have more prolonged biological activity(Haefeli, *et al.* 1992; Lee, *et al.* 1990; Salvemini, *et al.* 1993b). Thus a

significant proportion of the parent drug's clinical efficacy may be due to generation of active metabolites.

A second interesting aspect of GTN pharmacokinetics is the high variability of its apparent steady-state concentrations in patients receiving either continuous intravenous infusion(McNiff, *et al.* 1981; Wei, *et al.* 1979) or a transdermal preparation. This observation may be related to haemodynamic variations, as well as variable nitrate bioconversion . While the hepatic extraction and metabolism of GTN and ISDN is high(Morrison, *et al.* 1983b), Fung et al(1986a) have shown that systemic venous clearance of GTN far exceeds normal hepatic blood flow and correlates with cardiac output. These findings were supported by Blei et al(1984), who showed that portal systemic shunting of rats only slightly decreased systemic clearance of GTN. As the vasculature may be the major clearing organ for systemically administered nitrates(Fung, *et al.* 1984), alterations in cardiac output may affect apparent steady-state concentrations(Armstrong, *et al.* 1980) and thus contribute to the observed variability in steady-state concentrations.

Renal disease has been shown to have little effect on nitrate pharmacokinetics(Bogaert 1983), while hepatic disease may increase the bioavailability of the oral preparations(Blei, *et al.* 1987; Bogaert 1983).

A final important aspect of nitrate pharmacokinetics is the effect of long-term administration on apparent nitrate clearance/metabolism. Noonan showed that the pharmacokinetics of GTN in humans are both dose- and time-dependent(Noonan, *et al.* 1985a), and several other studies have demonstrated a progressive increase in venous plasma concentration of nitrates with continuous administration(Bergami, *et al.* 1997; Fung, *et al.* 1981; Parker, *et al.* 1984a; Parker, *et al.* 1984b). Arterial plasma concentrations have not been measured under these circumstances. There is also evidence that end-product inhibition of nitrate effects may occur(Barba, *et al.* 1999; Chong, *et al.* 1989; Cossum, *et al.* 1985; Cossum, *et al.* 1986; Kojda, *et al.* 1998b; Sutton, *et al.* 1984). These data, therefore, suggest that long-term administration of nitrates may lead to a reduction of the body's ability to metabolize/clear these agents.

This may be related to the development of nitrate tolerance and is discussed in later sections.

1.5 Pharmacological Effects

1.5.1 Vascular effects

The organic nitrates dilate veins, arteries and (to a lesser extent) arterioles via the biochemical mechanism discussed above and it is these vasodilator actions that are responsible for their clinical efficacy. Nitrate potency varies in the different types of vessels, a factor which is also of clinical relevance. In addition, the relative importance of the site of vasodilation may vary depending on the clinical situation.

The classical view of nitrate pharmacological action is one of predominant peripheral venodilation, resulting in an increase in blood volume in the venous capacitance beds(Abrams 1996; Bassenge, *et al.* 1992). Indeed, several studies have suggested that peripheral veins are more sensitive to nitrates than arteries(Barba, *et al.* 1999; Kawamoto, *et al.* 1987; MacKenzie, *et al.* 1977; Rosen, *et al.* 1987), an observation that is possibly due to a greater capacity for nitrate bioconversion of veins(Bauer, *et al.* 1996), or a greater degree of end-product inhibition of nitrate bioconversion by endogenous NO in arteries(Kojda, *et al.* 1998b). Venodilation reduces cardiac preload and stroke volume(Burggraf, *et al.* 1974; Goldstein, *et al.* 1979; Greenberg, *et al.* 1975), thereby reducing left ventricular wall tension and myocardial oxygen demand(Greenberg, *et al.* 1975). These effects are particularly relevant in myocardial ischaemia and heart failure.

At doses perhaps slightly higher than required to elicit venodilation, nitrates cause dilation of large arteries(Bassenge, *et al.* 1992), and at higher doses still, reduce peripheral vascular resistance by dilating arterioles(Aldershvile, *et al.* 1987; Imhof, *et al.* 1980). Thus, nitrates may also have favourable effects on afterload in the setting of myocardial ischaemia and heart failure. Indeed, studies utilizing applanation tonometry have demonstrated a significant reduction in the stiffness of central arteries with similar doses of nitrates required to induce venodilation(O'Rourke 1989).

Nitrates also have effects on the coronary vessels which are clinically important. While nitrates can directly dilate epicardial coronary arteries(Feldman, *et al.* 1979), several other aspects of nitrate action are unique and closely related to their clinical efficacy in myocardial ischaemia. Firstly, nitrates appear to produce pronounced dilation of bridging coronary collaterals(Cohen, *et al.* 1973a; Feldman, *et al.* 1979). Secondly, it has been demonstrated that nitrates can cause dilation of atherosclerotic coronary stenoses(Brown, *et al.* 1981). Thirdly, there is evidence that nitrates, in contrast to other vasodilators such as sodium nitroprusside and dipyridamole(Fam, *et al.* 1968; Macho, *et al.* 1981), exert heterogeneous vasodilator effects on coronary arteries(Barba, *et al.* 1999; Berdeaux, *et al.* 1992; Cohen, *et al.* 1973b; Fam, *et al.* 1968; Feldman, *et al.* 1981; Habazettl, *et al.* 1994; Macho, *et al.* 1981; Sellke, *et al.* 1990; Winbury, *et al.* 1969) with dilation of large epicardial arteries, but little effect on smaller resistance vessels. This heterogeneity enables nitrates to avoid the detrimental phenomenon of "coronary steal", which might arise if vascular resistance beyond a severe coronary stenosis is reduced. The reduced sensitivity of smaller arteries approaching arterioles in dimension has been postulated to be due to a lack of sulphydryl groups(Kurz, *et al.* 1991), but this does not seem to be the explanation for reduced sensitivity in larger resistance vessels(De la Lande, *et al.* 1996b).

The above coronary effects would be expected to improve the supply of blood to ischaemic zones relative to non-ischaemic zones. Indeed, studies have confirmed that this redistribution of coronary blood flow occurs in both animal models of ischaemia(Blache, *et al.* 1975; Jugdutt, *et al.* 1981; Kedem, *et al.* 1985) and humans with coronary artery disease(Cohn, *et al.* 1977; Fallen, *et al.* 1995; Mehta, *et al.* 1978).

It is difficult to dissect out the relative contributions of the peripheral effects (decreased myocardial oxygen demand) versus the coronary effects (increased oxygen supply) with regard to nitrate clinical efficacy. It is likely that both actions are important in most patients. However, as mentioned above, it is possible that the peripheral effects are more important in stable angina pectoris and heart failure, whereas the coronary effects may be critical in unstable angina and acute myocardial infarction.

A final issue in relation to vascular effects, is the potentially beneficial effects of nitrates in the presence of "endothelial dysfunction", which is discussed in section 1.7.1.

1.5.2 Platelet Effects

The ability of organic nitrates to inhibit platelet function in vitro was first demonstrated in 1967(Hampton, *et al.* 1967), and these observations were subsequently supported by other investigators(Bohme, *et al.* 1978; Mehta, *et al.* 1980; Schafer, *et al.* 1980). Initially, the relevance of these studies was questioned due to the fact that suprapharmacological concentration of nitrates were required to produce the inhibitory effects. However, Loscalzo demonstrated that inhibition could be achieved at pharmacological concentrations if intracellular reduced thiols were maintained(Stamler, *et al.* 1989). Subsequently, considerable evidence has accumulated supporting the concept that nitrates have important platelet effects in vivo.

Nitrates have been shown in animal models to prolong bleeding time(Booth, *et al.* 1996), inhibit platelet deposition on the vascular wall(Hebert, *et al.* 1997; Lam, *et al.* 1988), and inhibit thrombus formation in stenosed coronary arteries(Folts, *et al.* 1991). With regard to studies in humans, nitrate inhibition of platelet aggregation has been demonstrated in both normal subjects(Andrews, *et al.* 1994; Chirkov, *et al.* 1993; Karlberg, *et al.* 1992) and patients with stable or unstable angina(Chirkov, *et al.* 1993; Diodati, *et al.* 1990; Lacoste, *et al.* 1994) using therapeutic doses of nitrates administered via intravenous(Diodati, *et al.* 1990; Karlberg, *et al.* 1992), transdermal(Andrews, *et al.* 1994; Lacoste, *et al.* 1994) and sublingual(Chirkov, *et al.* 1993) routes. Prolongation of bleeding time by nitrates has also been demonstrated(Cockroft, *et al.* 1991; DeCaterina, *et al.* 1994). In addition, there is some evidence that in vivo inhibitory effects on platelets may persist despite induction of vascular tolerance(Booth, *et al.* 1996; Hebert, *et al.* 1997), though nitrate tolerance has been shown to also occur in platelets(Chirkov, *et al.* 1997; Weber, *et al.* 1996).

The primary mechanism of nitrate anti-aggregatory effect is likely due to an increase in intraplatelet cGMP(Loscalzo 1985), following activation of soluble

guanylate cyclase by NO. Though platelets themselves are capable of bioconverting small quantities of nitrates(Weber, *et al.* 1996), most of the inhibitory effect of nitrates on platelets is due to nitrate bioconversion in plasma(Chen, *et al.* 1996).

Though it is unlikely that the abovementioned platelet effects contribute to the beneficial effects of nitrates on exercise tolerance in stable angina, it is likely that they play an important role in unstable angina and acute myocardial infarction.

1.5.3 Myocardial effects

The effects of nitrates on the vasculature produce secondary effects on myocardial contractility, which are likely to overshadow any direct effects on the myocardium. Nonetheless, there is evidence that nitrates have small direct effects on myocardial contractility. These appear to be biphasic in vitro, with positive inotropic effects at low concentrations and negative inotropic effects at higher concentrations(Mohan, *et al.* 1996). A positive inotropic effect on animal and human myocardial cells has also recently been demonstrated with other NO donors and NO itself(Sarkar, *et al.* 2000). Other investigators, using an animal model have demonstrated positive inotropic effects of GTN in vivo(Preckel, *et al.* 1997).

1.6 Clinical Efficacy

1.6.1 Stable angina pectoris

The organic nitrates are widely used both in the relief and prevention of episodes of stable angina. GTN, in either sublingual tablet or spray form, is currently the most commonly used drug for relief of acute episodes of angina(Parker, *et al.* 1998). Sublingual ISDN tablets are also available for relief of anginal episodes, but onset of action is slower.

Extensive data demonstrate the efficacy of nitrates in the prevention of episodes of angina. Efficacy has been demonstrated in studies following both acute and chronic administration, and using a variety of different preparations. However, investigations

examining long-term efficacy of nitrates have often been complicated by development of tolerance (see section below), and it took many years to determine the optimal regimen for some preparations.

Sublingual GTN is often overlooked, but can be beneficial if used prophylactically before exercise(Detry, *et al.* 1971; Goldstein, *et al.* 1971; Thadani, *et al.* 1978). This is particularly useful when patients have exertional angina of predictable onset. Similarly, sublingual ISDN has a role in this setting(Goldstein, *et al.* 1971).

Transdermal GTN delivery systems were initially hailed as a major breakthrough in nitrate therapy prior to the institution of controlled trials. Acute administration prolonged exercise time to angina(Georgopoulos, *et al.* 1982; Scardi, *et al.* 1985), and initial open-label studies suggested this effect could be maintained for 24 hours a day during chronic therapy(Georgopoulos, *et al.* 1982; Muiesan, *et al.* 1986; Scardi, *et al.* 1985). However, a large number of reports emerged showing rapid attenuation of effect with continuous transdermal therapy(Crean, *et al.* 1984; James, *et al.* 1985; Parker, *et al.* 1984a; Reichek, *et al.* 1984; Sullivan, *et al.* 1985; Thadani, *et al.* 1986). The results of these studies were confirmed by a large randomized controlled trial(1991), showing loss of transdermal GTN effects within 24 hours of continuous therapy, which could not be overcome by increasing the dose. Subsequently, several studies(Cowan, *et al.* 1987; de Milliano, *et al.* 1991; DeMots, *et al.* 1989; Ferratini, *et al.* 1989; Fox, *et al.* 1991; Luke, *et al.* 1987; Parker, *et al.* 1995b; Schaer, *et al.* 1988) demonstrated that intermittent transdermal therapy, with a 10-12 hour nitrate-free interval, could provide clinical benefit in the long-term, though only while the patches were applied. Indeed, there is an implication of a "rebound phenomenon" during the nitrate-free period(DeMots, *et al.* 1989; Ferratini, *et al.* 1989).

ISDN has been studied in standard and sustained-release preparations, administered both orally and transdermally. Acute studies of standard ISDN given orally have demonstrated prolonged exercise time to angina for at least three hours after the dose(Markis, *et al.* 1979; Thadani, *et al.* 1982). Chronic therapy with this preparation two or three times a day with an eccentric dosing schedule incorporating a space of 14 hours between two doses, also provides prolongation of exercise time to

angina(Bassan 1990; Parker, *et al.* 1987a), though partial tolerance is evident and the beneficial effect may only be present for a small proportion of the day(Bassan 1990). Increasing the dosage regimen to four times a day leads to even greater induction of tolerance and therefore less clinical benefit(Parker, *et al.* 1987a; Parker, *et al.* 1985; Wisenberg, *et al.* 1989).

Similarly, both oral and transdermal versions of the sustained-release preparation of ISDN have been shown to prolong exercise time to angina when administered acutely(Parker, *et al.* 1984b; Silber, *et al.* 1987). When given orally once a day, this effect can be sustained with chronic therapy, though for only 12 hours of the day(Silber, *et al.* 1987). However, in the setting of twice daily oral dosing(Silber, *et al.* 1987) or continuous transdermal therapy(Parker, *et al.* 1984b) complete tolerance rapidly develops.

ISMN is also available in standard and sustained-release preparations, both of which have been studied extensively. Acute studies examining the effects of a single dose of standard ISMN have shown prolongation of exercise time to angina for up to 6 hours(Kohli, *et al.* 1986; Thadani, *et al.* 1987b; Thadani, *et al.* 1987c), but induction of tolerance with loss of clinical efficacy, if given twice daily 12 hours apart(Kohli, *et al.* 1986; Thadani, *et al.* 1987b). However, chronic studies using an eccentric dosing regimen (twice daily with 7 hours between doses) demonstrated antianginal efficacy for up to 12 hours(Parker 1993; Thadani, *et al.* 1994).

Acute studies of the sustained-release preparation of ISMN have also shown significant antianginal efficacy for up to 12 hours(Beyerle, *et al.* 1990; Thadani, *et al.* 1987a; Wisenberg, *et al.* 1989) and these effects can be sustained during chronic therapy(Beyerle, *et al.* 1990; Chrysant, *et al.* 1993; Glasser 1997; Wisenberg, *et al.* 1989), though higher doses are required, indicating development of partial tolerance(Chrysant, *et al.* 1993).

The major concern regarding all of the above agents and their dosing regimens is the development of tolerance, which limits restricts clinical benefit to no more than 12 hours of each day. A second concern relates to the potential for these agents to

induce rebound during the nitrate-free period(DeMots, *et al.* 1989), an issue which will be discussed in the section on pseudotolerance.

1.6.2 Unstable angina pectoris

The organic nitrates are also widely used in the management of unstable angina(Horowitz 1992), supported largely by their known vascular and platelet effects, described above. However, there is a surprising lack of controlled data supporting this role. Intravenous GTN has been shown to be effective in relieving ischaemic symptoms in unstable angina only in small uncontrolled studies(DePace, *et al.* 1982; Roubin, *et al.* 1982). The only previous randomized study in unstable angina compared intravenous GTN as monotherapy with intravenous diltiazem(Gobel, *et al.* 1995; Gobel, *et al.* 1998), and found GTN to be less efficacious with regard to ischaemic endpoints. However, interpretation of this study is limited by the high doses of GTN used, which may have induced significant nitrate tolerance. To date, no randomized placebo-controlled trials have been performed in this setting.

1.6.3 Acute myocardial infarction

Considerable evidence has accumulated suggesting that nitrates may have a beneficial effect on prognosis as well as symptoms in the setting of acute myocardial infarction(Flaherty 1992). However, a large proportion of this data was obtained in the pre-thrombolytic era, and 2 large mortality trials have recently cast doubt on this issue (see below). Studies in animal models have generally been conducted with occluded infarct-related arteries, and have demonstrated that nitrates reduce infarct size(Jugdutt 1983; Jugdutt, *et al.* 1981; Jugdutt, *et al.* 1995b), reduce infarct-zone thinning(Jugdutt, *et al.* 1994), limit left ventricular dilatation(Jugdutt, *et al.* 1994; Jugdutt, *et al.* 1995a; Jugdutt, *et al.* 1995b), and improve left ventricular ejection fraction(Jugdutt, *et al.* 1994). Early studies in human subjects also showed evidence of beneficial effects of nitrates used early in acute myocardial infarction, in terms of myocardial ischaemia(Borer, *et al.* 1975; Flaherty, *et al.* 1983; Flaherty, *et al.* 1975), infarct size(Bussmann, *et al.* 1981; Flaherty, *et al.* 1983; Jaffe, *et al.* 1983; Jugdutt 1991; Jugdutt, *et al.* 1988), infarct expansion(Jugdutt 1991; Jugdutt, *et al.* 1988) and left

ventricular asynergy(Jugdutt, *et al.* 1983). Several early randomized mortality trials of intravenous GTN in acute myocardial infarction were also performed; individually, they were inconclusive, but a meta-analysis suggested nitrates might reduce early (7-10 days) mortality by as much as 35%(Yusuf, *et al.* 1988). However, as mentioned above, these data were obtained in the pre-thrombolytic era.

Two large multicenter trials assessing the effect of nitrates on early mortality have been performed in the post-thrombolytic era. These trials both assessed whether adding nitrates to conventional therapy for 5-6 weeks post-infarction reduced mortality. In the GISSI-3 trial(1994), intravenous GTN was used for 24 hours, followed by intermittent transdermal GTN for 6 weeks. In the ISIS-4 trial(1995), 60mg of oral sustained-release ISMN was given acutely, and for 5 weeks. The mortality reduction per 1000 nitrate-treated patients was 2.1 in ISIS-4 and 3.9 in GISSI-3, both of which were non-significant. However, a number of aspects of these studies warrant discussion. Of major concern is the fact that nearly 60% of patients in both trials used open-label nitrates. Secondly, mortality in the nitrate groups over the first 2 days was markedly reduced in ISIS-4. In addition, the combination of nitrate and an angiotensin converting enzyme (ACE) inhibitor, lisinopril in GISSI-3, produced a greater reduction in mortality than lisinopril alone, raising the possibility of a nitrate-ACE-inhibitor interaction. The nitrate group in GISSI-3 also had less post-infarction angina. These observations suggest that nitrates did have some beneficial effects. Lastly, as discussed in the section on stable angina, the dosage regimens studied would have provided beneficial effects for less than 12 hours per day, and induced partial tolerance, particularly in the case of 60mg of oral ISMN. Indeed, 60 mg/day ISMN may be inadequate for sustained anti-anginal effects during long-term therapy(Chrysant, *et al.* 1993). There is clearly the potential for more definite beneficial effects if tolerance can be circumvented.

While the above trials investigated the role of nitrates in the short-term post-infarction, there is relatively little data on their role in the long-term. As mentioned above, animal studies have suggested that nitrates can unload the left ventricle post-infarction and improve ventricular remodelling(Jugdutt, *et al.* 1994; Jugdutt, *et al.* 1995b). These studies have been supported by a recent randomized study showing a beneficial effect of 6 months transdermal GTN on left ventricular function post-

infarction(Mahmarian, *et al.* 1998). Whether this translates into a clinical improvement or survival advantage, has yet to be determined.

1.6.4 Congestive Heart Failure

The organic nitrates are used in the treatment of both acute and chronic congesive heart failure (CHF). The mechanisms underlying their beneficial effects probably relate to effects on the peripheral vasculature(Elkayam 1996) and are discussed above in section 1.5.1. A variety of different nitrate preparations have been shown to acutely reduce ventricular filling pressures, reduce pulmonary and systemic vascular resistance, and improve cardiac output in patients with CHF(Dupuis, *et al.* 1990b; Elkayam, *et al.* 1987; Elkayam, *et al.* 1991; Franciosa, *et al.* 1980; Jordan, *et al.* 1986; Leier, *et al.* 1983; Mehra, *et al.* 1994; Olivari, *et al.* 1983; Packer, *et al.* 1987; Rajfer, *et al.* 1984; Roth, *et al.* 1993; Sharpe, *et al.* 1987). These acute haemodynamic effects make nitrates suitable for the treatment of acute heart failure. There is evidence that nitrate-based therapy may provide better short-term efficacy than that of diuretics in patients with acute pulmonary oedema(Beltrame, *et al.* 1998). However, the majority of studies of nitrate therapy have involved patients with chronic heart failure. Beyond 24 hours however, tolerance again becomes an issue(Dupuis, *et al.* 1990b; Elkayam, *et al.* 1987; Elkayam, *et al.* 1991; Jordan, *et al.* 1986; Leier, *et al.* 1983; Olivari, *et al.* 1983; Packer, *et al.* 1987; Packer, *et al.* 1986), which can only be partly avoided by adjusting the nitrate regimen(Elkayam, *et al.* 1991; Sharpe, *et al.* 1987). A second problem is variability in nitrate response, with some patients displaying de novo resistance to nitrate effects(Abrams 1991; Elkayam, *et al.* 1992; Elkayam, *et al.* 1991; Packer, *et al.* 1986; Sharpe, *et al.* 1987); this will be discussed further in section 1.9.

Nonetheless, there is evidence that chronic nitrate therapy, either alone(Elkayam, *et al.* 1999; Leier, *et al.* 1983), or in combination with hydralazine(Cohn, *et al.* 1991) can improve exercise capacity in patients with CHF. Importantly, this improvement is still seen in the presence of ACE inhibition(Elkayam, *et al.* 1999).

However, the effect of nitrate therapy alone on survival in patients with CHF remains unknown, as this issue has not been studied specifically. Two studies evaluated the effects of long-term therapy with nitrate-hydralazine combination on outcome in chronic CHF(Cohn, *et al.* 1986; Cohn, *et al.* 1991). The first(Cohn, *et al.* 1986), demonstrated a reduction in mortality with nitrate-hydralazine of borderline significance. The second(Cohn, *et al.* 1991), found enalapril to be superior than nitrate-hydralazine in lowering mortality. However, neither study can address the issue of whether nitrate therapy alone, or in addition to ACE-inhibition, can improve outcome in CHF.

1.6.5 Other

There are some data supporting the use of nitrates in other clinical settings. Recently, routine intravenous GTN following coronary stenting, was demonstrated to reduce the occurrence of minor myocardial necrosis(Kurz, *et al.* 2000). In addition, another recent randomized study found intravenous nitroglycerin to be more effective than heparin in preventing ischaemic symptoms in patients with unstable angina secondary to restenosis following coronary angioplasty(Doucet, *et al.* 2000); this finding is likely to reflect the lack of a pivotal role of thrombosis in such patients.

1.7 Relationship with the endothelium and EDRF

Since the mechanism of action of the organic nitrates involves NO release, they are often referred to as exogenous NO donors. It is therefore important to have an understanding of the role of endogenous NO and its primary source, the endothelium, particularly with regard to potential interactions with nitrate efficacy.

1.7.1 EDRF and the endothelium

In 1980, Furchgott and Zawadzki(1980) demonstrated that the vasodilator effect of acetylcholine was dependent on the presence of an intact endothelium. The mediator responsible for relaxation was later termed endothelium-derived relaxing factor

(EDRF). Further studies showed that it is released in all blood vessels under basal conditions(Ignarro, *et al.* 1987a), and in response to a variety of stimuli such as acetylcholine, bradykinin, and substance P, as well as shear stress(Ignarro 1989). EDRF was found to act via activation of soluble guanylate cyclase, leading to an increase in intracellular cGMP(Rapoport, *et al.* 1983a; Rapoport, *et al.* 1983b), a mechanism which the organic nitrates shared(Katsuki, *et al.* 1977). Subsequently, several studies demonstrated that EDRF is nitric oxide (NO)(Ignarro, *et al.* 1987a; Ignarro, *et al.* 1987b; Palmer, *et al.* 1987), though some investigators suggested that it more closely resembles a NO-containing compound(Myers, *et al.* 1990). L-arginine was shown to be the precursor for the formation of NO in the endothelium by endothelial NO synthase (eNOS)(Moncada, *et al.* 1993; Palmer, *et al.* 1988a; Palmer, *et al.* 1988b).

EDRF exerts powerful vasorelaxing and platelet-inhibitory effects(Ignarro 1989), and over the last two decades a large body of evidence supporting a role of the endothelium and EDRF in the regulation of vascular tone, as well as platelet and monocyte function(for reviews see Celermajer 1997; Rubanyi 1993) has accumulated.

However, it became evident that atherosclerosis in human coronary arteries is associated with "endothelial dysfunction"(Ludmer, *et al.* 1986), manifested by a vasoconstrictor response to acetylcholine. Several other studies supported this finding in animal(Jayakody, *et al.* 1987) and human vessels(Cox, *et al.* 1989; Forstermann, *et al.* 1988; McLenachan, *et al.* 1990; Nabel, *et al.* 1990; Zeiher, *et al.* 1993) with varying degrees of atherosclerosis. Subsequently, peripheral artery endothelial dysfunction was also shown to be associated with coronary artery disease(Celermajer, *et al.* 1992) and risk factors for coronary artery disease, including hypertension(Panza, *et al.* 1990), hypercholesterolaemia(Celermajer, *et al.* 1992; Creager, *et al.* 1990; Sorensen, *et al.* 1994; Verbeuren, *et al.* 1986; Zeiher, *et al.* 1993), diabetes mellitus(Clarkson, *et al.* 1996; McVeigh, *et al.* 1992; Watts, *et al.* 1996) and cigarette smoking(Celermajer, *et al.* 1994; Celermajer, *et al.* 1992; Zeiher, *et al.* 1995). These studies led to the hypothesis that endothelial dysfunction is a precursor to atherosclerosis, and potentially is a marker for increased future atherosclerosis and cardiovascular risk.

This has stimulated efforts to try to reverse endothelial dysfunction with a variety of different agents. Postulated theories underlying the development of endothelial dysfunction include reduced NO formation from L-arginine, and increased inactivation of endothelial-derived NO by elevated O_2^-(Celermajer 1997). Certainly, NO from the endothelium is sensitive to varying levels of O_2^-(Kasten, *et al.* 1995; Langenstroer, *et al.* 1992; Moncada, *et al.* 1986; Wolin, *et al.* 1990), and there is evidence supporting the existence of a "dysfunctional" form of eNOS, which produces O_2^- rather that NO(Heitzer, *et al.* 2000). Other potential sources of vascular O_2^- generation include xanthine oxidase(Berry, *et al.* 2000) and NAD(P)H-oxidase(Berry, *et al.* 2000; Gorlach, *et al.* 2000). Alternatively, a number of studies(Boger, *et al.* 2000a; Boger, *et al.* 1998; Boger, *et al.* 2000b; Fard, *et al.* 2000; Ito, *et al.* 1999; Jang, *et al.* 2000; Surdacki, *et al.* 1999) have recently demonstrated elevated levels of asymmetric dimethylarginine (ADMA), an endogenous and competitive inhibitor of eNOS in association with atherosclerosis, risk factors for atherosclerosis and the presence of impaired endothelial function, suggesting that ADMA may be pivotal in "endothelial dysfunction".

Most studies attempting to improve endothelial dysfunction, have involved either anti-oxidants or L-arginine supplements. Indeed, these agents have been shown to improve endothelial function in a large number of studies(Cooke, *et al.* 1991; Drexler, *et al.* 1991; Heitzer, *et al.* 2000; Heitzer, *et al.* 1996; Hornig, *et al.* 1998; Keaney, *et al.* 1995; Lerman, *et al.* 1998; Levine, *et al.* 1996; Taddei, *et al.* 1998; Timimi, *et al.* 1998; Ting, *et al.* 1996; Ting, *et al.* 1997) in a variety of conditions associated with impaired endothelial function.

However, a number of questions remain unanswered with regard to endothelial dysfunction. The first issue relates to its utilization as a surrogate for future cardiovascular risk(Cannon 2000). While antioxidants, L-arginine, and oestrogen therapy have been shown to in many studies to improve endothelial function(Cannon 2000), they do not appear to reduce myocardial ischaemia and/or cardiovascular risk(Hulley, *et al.* 1998; Quyyumi 1998; Stephens, *et al.* 1996; Yusuf, *et al.* 2000a). Conversely, while lipid-lowering with HMG-CoA reductase inhibitors have been shown in large clinical trials to reduce cardiovascular morbidity and mortality in

patients with coronary artery disease(Pitt, *et al.* 1999; Sacks, *et al.* 1996), their effect on endothelial dysfunction is not consistent(Vita, *et al.* 2000). The ACE-inhibitors are currently the only agents which have been shown to have consistent beneficial effects on both endothelial dysfunction(Cashin-Hemphill, *et al.* 1999; Schlaifer, *et al.* 1999), possibly via inhibition of NAD(P)H oxidase(Zhang, *et al.* 1999), as well as cardiovascular morbidity and mortality(Latini, *et al.* 2000; Yusuf, *et al.* 2000b). These observations suggest that endothelial dysfunction, as currently assessed, may have limitations as a surrogate for predicting cardiovascular risk and/or risk reduction.

A second critical question is to what degree the "dysfunction" is limited to the endothelium. This question is raised by a number of studies also showing "dysfunction" of vascular smooth muscle in response to endothelium-independent vasodilators; this issue is discussed in detail in the section on nitrate resistance.

1.7.2 Nitrate-endothelium interactions

As mentioned above, nitrates represent exogenous sources of NO. Hence, they might be expected to be particularly beneficial in situations of diminished endogenous NO bioavailability. Recent studies have even suggested the organic nitrate pentaerythrityl-tetranitrate (PETN), may attenuate development of endothelial dysfunction and atherosclerosis in hypercholesterolaemic animals(Kojda, *et al.* 1995) and also reduce structural changes in arteries produced by long-term inhibition of eNOS(Kristek 2000). These observations may partly relate to an ability to suppress proliferation of vascular smooth muscle(Yu, *et al.* 1997).

There is also evidence that vascular responsiveness to organic nitrates is affected by the extent of endogenous NO release. Indeed, nitrates have been demonstrated to be more effective at dilating isolated vessels in vitro, when the endothelium is denuded, or in the presence of inhibition of NOS(Moncada, *et al.* 1991; Pohl, *et al.* 1987). This observation may conceivably be partly related to the issue of end-product inhibition, and has been suggested to underlie the differing sensitivities of arteries and veins to nitrates(Kojda, *et al.* 1998b). Alternatively, it may reflect the presence/absence of partial soluble guanylate cyclase activation by endogenous NO. A

recent study however, failed to observe an increase in sensitivity to GTN in the presence of endothelial dysfunction in humans(Anderson, *et al.* 1996).

A final potential nitrate-endothelium interaction is the role of the endothelium in the development of nitrate tolerance. This is discussed in section 1.8.2.3.4.

1.8 Factors limiting nitrate efficacy - Nitrate tolerance

It is evident from clinical studies that a number of factors may potentially limit nitrate efficacy. These factors can be categorized as (1) nitrate tolerance, which refers to attenuated nitrate efficacy following continuous exposure to nitrate, and (2) nitrate resistance, which is de novo impaired efficacy. It is also useful to sub-categorize nitrate tolerance as "true" or cellular tolerance, which refers to decreased activity of the nitrate-NO-cGMP cascade, and "pseudo-tolerance" which refers to activation of mechanisms which oppose the effects of nitrates.

1.8.1 Historical notes

As mentioned previously, attenuation of nitrate effects on workers in the munitions industry during chronic exposure was first reported over 100 years ago(Ebright 1914; Laws 1898). About the same time, there were also reports of tolerance to the antihypertensive effects of nitroglycerin in clinical practice(Crandall, *et al.* 1931; Stewart 1888). However, for many years nitrate tolerance was ignored.

In the late 1960s and 1970s, sporadic investigations of nitrate tolerance emerged. Schelling and Lasagna(1967) noted that the hypertensive effects of GTN diminished during continuous treatment with PETN. Bogaert noted similar observations with GTN in dogs(1968) and rabbits(1968). Needleman(1970) went a step further, and demonstrated that tolerance to the antihypertensive effects of GTN in rats was associated with reduced relaxation of aortae removed from the tolerant animals. He also showed that a similar state of reduced responsiveness could be induced in vitro, via incubation with nitrate(Needleman, *et al.* 1973b). A decade later, in vivo tolerance in rats was correlated with reduced cGMP response(Axelsson, *et al.* 1983).

In the mid-late 1980s, a large increase in research in nitrate tolerance occurred. Ironically, it was controversy over a new transdermal GTN system, initially hailed as a major breakthrough in nitrate delivery, that helped stimulate this research(Abrams 1984).

1.8.2 Clinical evidence for nitrate tolerance

Overwhelming evidence has accumulated from clinical studies that tolerance occurs with most, if not all, organic nitrate preparations(Elkayam 1991). These studies have largely examined various different aspects of nitrate effects, including anti-anginal, haemodynamic, direct vasodilator and platelet effects.

Most studies assessing the anti-anginal effects of nitrates have utilized exercise testing in patients with chronic stable angina. In this manner, tolerance, corresponding to attenuation of the prolongation of exercise time to angina/ischaemia, has been demonstrated to occur following 24 hour infusion of intravenous GTN(Zimrin, *et al.* 1988), and following continuous transdermal GTN for 24 hours(James, *et al.* 1985; Parker, *et al.* 1984a; Reichek, *et al.* 1984; Sullivan, *et al.* 1985; Thadani, *et al.* 1986) or longer(1991; Cowan, *et al.* 1987; Crean, *et al.* 1984; de Milliano, *et al.* 1991; Fox, *et al.* 1991; Hogan, *et al.* 1990; Luke, *et al.* 1987). Similar attenuation of efficacy has been demonstrated with standard-preparation ISDN administered 4 or more times a day(Boesgaard, *et al.* 1991a; Boesgaard, *et al.* 1992; Parker, *et al.* 1987a; Parker, *et al.* 1987b; Parker, *et al.* 1985), sustained-release ISDN administered 12-hourly(Silber, *et al.* 1987), standard-preparation ISMN given 12-hourly(Kohli, *et al.* 1986; Thadani, *et al.* 1987b), and sutained-release ISMN given 12-hourly. These studies led to the incorporation of a nitrate-free period into all nitrate regimens for stable angina. However, it should be noted that even the regimens currently utilized in clinical practice can induce a significant degree of partial tolerance(Chrysant, *et al.* 1993; Wagner, *et al.* 1991).

Tolerance to the haemodynamic effects of nitrates has generally been assessed by monitoring blood pressure, pulmonary artery or capillary wedge pressure, and has

been demonstrated to occur in both normal subjects, and patients with congestive heart failure. Again, all preparations appear to induce haemodynamic tolerance, including intravenously infused GTN(Elkayam, *et al.* 1987; Packer, *et al.* 1987; Pizzulli, *et al.* 1997), where attenuation of effects may be seen within 12 hours(Elkayam, *et al.* 1987), continuous transdermal GTN(Bassenge, *et al.* 1998; Hogan, *et al.* 1989; Jordan, *et al.* 1986; Milone, *et al.* 1999b; Packer, *et al.* 1986; Sharpe, *et al.* 1987; Torfgard, *et al.* 1993), where loss of efficacy may be demonstrated after 18 hours(Jordan, *et al.* 1986; Packer, *et al.* 1986), oral ISDN administered 4-6 hourly(Elkayam, *et al.* 1991; Parker, *et al.* 1983; Thadani, *et al.* 1980), oral sustained-release ISMN administered 8-hourly(Jahnchen 1992), and intravenously infused ISDN(Cotter, *et al.* 1998) and ISMN(Lehmann, *et al.* 1998). As with the anti-anginal effects, partial haemodynamic tolerance is still evident when regimens incorporating a nitrate-free period are used(Mehra, *et al.* 1995).

Attenuation of the direct vasodilator effects of nitrates have also been extensively studied in humans, utilizing techniques such as forearm venous plethysmography, vascular ultrasound, and quantitative coronary angiography. The acute effects of intravenous GTN(Boesgaard, *et al.* 1994b; Ghio, *et al.* 1992), transdermal GTN(Katz, *et al.* 1991; Levy, *et al.* 1991; Milone, *et al.* 1999a; Watanabe, *et al.* 1998a) or oral ISDN(Vincent, *et al.* 1992; Zelis, *et al.* 1975) on venodilation, are attenuated within 24 hours. Demonstration of reduction of vasodilator effects on large arteries has been less consistent. For example, radial artery diameter remains unchanged following a 48 hour infusion of GTN(Jeserich, *et al.* 1995). However, longer nitrate administration (5 days) appears to produce tolerance in epicardial coronary arteries(Kai, *et al.* 1994). In addition, a 24 hour GTN infusion has been shown to attenuate the increase in coronary blood flow produced by an intracoronary bolus of GTN in humans(May, *et al.* 1987).

As discussed above in section 1.5.2, nitrates also have inhibitory effects on platelet aggregation. While initial studies(Booth, *et al.* 1996; Hebert, *et al.* 1997) suggested that these effects were sustained despite haemodynamic tolerance, a recent study(Chirkov, *et al.* 1997) has demonstrated that the anti-aggregatory effects of GTN are reduced after a single 300 microgram dose of sublingual GTN.

In summary, therefore, there is now a large body of evidence from clinical studies proving that nitrate efficacy is attenuated with continuous nitrate exposure. It is important to appreciate, however, that it is not possible in these studies, to determine the relative contributions of true/cellular tolerance and pseudotolerance, as defined above, to this loss of efficacy.

1.8.3 True/cellular nitrate tolerance

1.8.3.1 Previous studies

The development of nitrate tolerance may be multifactorial, but there is little doubt that a large component is due to decreased activity of the nitrate-NO-cGMP cascade at the target site. Evidence supporting this concept is provided by a large number of in vitro and ex vivo studies showing reduced relaxant response of vascular smooth muscle to nitrate, following induction of nitrate tolerance. The first of these were performed by Needleman(1970; 1973b), as discussed above.

In vitro studies, on the whole, incubate tissue with relatively high (10^{-4} to 10^{-5} M) concentrations of nitrates for short periods of time (30-60 minutes). Using this method, reduced relaxation to nitrates has been demonstrated in a variety of isolated vessels, including rat aortae(Abou-Mohamed, *et al.* 2000; Bennett, *et al.* 1988; Hasegawa, *et al.* 1999; Keith, *et al.* 1982; Kowaluk, *et al.* 1990a; Kowaluk, *et al.* 1987; Lawson, *et al.* 1996; Lawson, *et al.* 1991; Mulsch, *et al.* 1988; Rapoport, *et al.* 1987), rabbit aortae(Slack, *et al.* 1989; Slack, *et al.* 1988; Smith, *et al.* 1994), bovine coronary artery(De la Lande, *et al.* 1999a; Gruetter, *et al.* 1985; Henry, *et al.* 1989a; Henry, *et al.* 1989b; Henry, *et al.* 1990; Torresi, *et al.* 1985), and canine vessels(Abdollah, *et al.* 1987; Berkenboom, *et al.* 1988), as well as in human internal mammary artery(Arnet, *et al.* 1995), saphenous vein(Ahlner, *et al.* 1986; Bohyn, *et al.* 1991) and coronary artery(Berkenboom, *et al.* 1988; Kuhn, *et al.* 1989). Other studies utilizing intact vessels(Axelsson, *et al.* 1982; Axelsson, *et al.* 1984), vessel homogenates(Mulsch, *et al.* 1989a; Romanin, *et al.* 1989) or cultures of vascular smooth muscle or endothelial cells(Mulsch, *et al.* 1989b; Salvemini, *et al.* 1993c) have shown that in vitro nitrate

tolerance is associated with diminished nitrate-induced formation of cGMP. Similar changes in cGMP formation have also been seen in other cell cultures, including rat lung fibroblasts(Schroder, *et al.* 1988) and porcine kidney epithelial cells(Grosser, *et al.* 2000; Hinz, *et al.* 1998). Lastly, in vitro tolerance in isolated bovine coronary artery(Chung, *et al.* 1993) and perfused rabbit hearts(Forster, *et al.* 1991) has been demonstrated to be associated with reduced NO levels.

Many animal studies have also demonstrated "true" nitrate tolerance using ex vivo methodology. The majority have used high doses of GTN administered over several days and then examined vascular reactivity in vitro. Using this method, reduced relaxant responses to GTN have been seen in aortae from rats(De la Lande, *et al.* 1999b; Du, *et al.* 1991; Fung, *et al.* 1986b; Laursen, *et al.* 1996b; Molina, *et al.* 1987; Ratz, *et al.* 2000; Torfgard, *et al.* 1991; Van de Voorde, *et al.* 1987) and rabbits(MacAllister, *et al.* 1995; Munzel, *et al.* 1996d; Munzel, *et al.* 1995b). Consistent with the in vitro studies, in vivo tolerance is associated with reduced vascular cGMP formation(Haj-Yehia, *et al.* 1995; Molina, *et al.* 1987), reduced vascular production of 1,2-GDN(Fung, *et al.* 1986b), and reduced NO bioavailability in some(Agvald, *et al.* 1999; Husain, *et al.* 1994), but not all(Laursen, *et al.* 1996b) studies. With regard to investigations in humans, two studies have demonstrated reduced GTN-induced relaxant responses in isolated segments of human internal mammary artery(Du, *et al.* 1992) and saphenous vein(Torfgard, *et al.* 1995) obtained from patients on long-term GTN therapy.

Overall, therefore, there is overwhelming evidence that a large component of nitrate tolerance is due to cellular tolerance of the target organ (vascular smooth muscle). Several other studies have also demonstrated tolerance to the haemodynamic(Bauer, *et al.* 1990; Bauer, *et al.* 1991a; Bauer, *et al.* 1991b; Boesgaard, *et al.* 1994a; Boesgaard, *et al.* 1991b; Laursen, *et al.* 1996a; Stewart, *et al.* 1986) or direct vasodilator effects(Munzel, *et al.* 1989; Stewart, *et al.* 1987) of nitrates in animal models, but as with the human studies, these studies cannot assess the relative contribution of true/cellular tolerance and activation of mechanisms opposing nitrate effects (pseudotolerance).

1.8.3.2 Postulated mechanisms of true/cellular tolerance

The mechanism(s) underlying the development of true/cellular tolerance is currently an area of considerable controversy. The subject is discussed in terms of the major postulated mechanisms.

1.8.3.2.1 The "Sulphydryl depletion" hypothesis

In their original paper on in vitro tolerance(1973b), Needleman et al observed that GTN tolerance was associated with decreased total sulphydryl levels, and that tolerance could be reversed with the disulfide reducing agent, dithiothreitol. They proposed firstly, that nitrate action was sulphydryl-dependent, and secondly, that nitrates oxidized critical sulphydryl groups, depleting intracellular stores of reduced sulphydryls, leading to loss of pharmacological action. This theory gained widespread acceptance, largely due to further experiments manipulating sulphydryl availability and to work on GTN metabolism by Fung and coworkers(1988). Sulphydryl-alkalating agents, such as ethacrynic acid, reduced nitrate sensitivity in vitro(Kenkare, *et al.* 1993; Lau, *et al.* 1992a; Moffat, *et al.* 1985), whereas addition of sulphydryls(Axelsson, *et al.* 1982; Ignarro, *et al.* 1981; Torresi, *et al.* 1985) enhanced nitrate action in vitro. A number of clinical studies also emerged suggesting some sulphydryl agents could potentiate(Chirkov, *et al.* 1996; Horowitz, *et al.* 1983; Svendsen, *et al.* 1989; Winniford, *et al.* 1986) nitrate effects or partially reverse(May, *et al.* 1987; Packer, *et al.* 1987; Vincent, *et al.* 1992) or prevent(Boesgaard, *et al.* 1991a; Boesgaard, *et al.* 1992) nitrate tolerance.

However, more recent data have cast considerable doubt on the validity of this theory. Firstly, in contrast to the studies of in vitro tolerance, several groups have demonstrated that in vivo nitrate tolerance is not associated with depleted arterial or venous sulphydryl levels(Boesgaard, *et al.* 1994a; Haj-Yehia, *et al.* 1996; Sakanashi, *et al.* 1991). Secondly, there is controversy regarding the exact nature of the interaction between nitrates and sulphydryl agents in vivo. This controversy is not helped by the fact that all sulphydryls are also anti-oxidants(Aruoma, *et al.* 1989), making them non-specific as regards the mechanism of tolerance limitation. In addition, there is evidence

that not all sulphydryl agents exhibit equally potent GTN interactions(Chong, *et al.* 1991).

As discussed in section 1.3.4, there is considerable evidence from both animal studies(Boesgaard, *et al.* 1993; Fung, *et al.* 1988; Hutter, *et al.* 1988; Munzel, *et al.* 1989; Munzel, *et al.* 1992) and human studies(Boesgaard, *et al.* 1994b; Creager, *et al.* 1997; Horowitz, *et al.* 1983; Levy, *et al.* 1988; Mehra, *et al.* 1994; Nishikawa, *et al.* 1998; Pizzulli, *et al.* 1997; Svendsen, *et al.* 1989; Vekshtein, *et al.* 1990; Winniford, *et al.* 1986) that sulphydryl agents potentiate nitrate effects in vivo. However, this interaction appears to occur extracellularly(Fung, *et al.* 1988), and/or at the level of the micro-vessel(Kurz, *et al.* 1991; Munzel, *et al.* 1992; Sellke, *et al.* 1991; Wheatley, *et al.* 1994), rather than at an intracellular level(Boesgaard, *et al.* 1993).

Studies showing that sulphydryl agents "reverse" nitrate tolerance in vivo(Boesgaard, *et al.* 1991b; Ghio, *et al.* 1992; Levy, *et al.* 1991; May, *et al.* 1987; Packer, *et al.* 1987; Vincent, *et al.* 1992), cannot, on the basis of their design, distinguish between actual reversal of tolerance on the one hand, and tolerance-independent potentiation of effects, on the other. In vitro studies attempting to reverse true tolerance in isolated large vessels have been largely negative(Abdollah, *et al.* 1987; Chong, *et al.* 1991; Fung, *et al.* 1988; Gruetter, *et al.* 1986; Henry, *et al.* 1989a; Lawson, *et al.* 1996).

Clinical and experimental studies attempting to prevent nitrate tolerance by co-administration of sulphydryl donors, have been conflicting. While some have shown partial prevention of tolerance(Boesgaard, *et al.* 1991a; Boesgaard, *et al.* 1992; Tsuneyoshi, *et al.* 1989), other studies(Boesgaard, *et al.* 1994b; Dupuis, *et al.* 1990b) have shown that tolerance is unaffected. It is therefore unclear whether sulphydryl agents can exert a true tolerance protective effect.

In summary, there is ample evidence that sulphydryl agents potentiate haemodynamic and platelet responses to nitrates in vivo, although probably exerting little effect on responses in large arteries. However, there are substantial defects in the "sulphydryl hypothesis" of tolerance:-

1) Sulphydryl levels may not be depleted in tolerance
2) Effects of sulphydryls in models of tolerance are similar to those in non-tolerant vessels.
3) Studies comparing sulphydryl-containing vs other anti-oxidants are lacking
4) While redox stress inevitably leads to sulphydryl depletion, this phenomenon is not well characterized in studies of nitrate tolerance.

Lastly, the clinical implications of potential nitrate-sulphydryl donor interactions also remain unclear, though there are some data suggesting they may be beneficial. Despite concern about coronary steal phenomenon(Kurz, *et al.* 1991), addition of the sulphydryl, N-acetylcysteine to nitrate therapy has been shown to limit tolerance development in patients with stable(Boesgaard, *et al.* 1992) and unstable(Ardissino, *et al.* 1997) angina pectoris, lower the incidence of acute myocardial infarction in patients with unstable angina pectoris(Horowitz, *et al.* 1988a; Horowitz, *et al.* 1988b), and reduce oxidative stress and preserve left ventricular function in patients with acute myocardial infarction(Arstall, *et al.* 1995).

1.8.3.2.2 Desensitization of guanylate cyclase

In a series of experiments, Axelsson et al showed that nitrate tolerance induced in vitro(Axelsson, *et al.* 1984), or in vivo(Axelsson, *et al.* 1983) was associated with reduced cGMP elevation in response to GTN. They observed that the cGMP response to sodium nitroprusside was also reduced, though not to such a large extent, and proposed that reduced activity of guanylate cyclase may be the cause of nitrate tolerance. Similar results were seen by other groups(Romanin, *et al.* 1989; Waldman, *et al.* 1986), in broken-cell preparations of vascular smooth muscle made tolerant to GTN.

However, studies showing no cross-tolerance to the relaxant response to non-nitrate sources of NO(Kowaluk, *et al.* 1990a; Mulsch, *et al.* 1988; Mulsch, *et al.* 1989b), soon began to emerge. The investigators suggested several explanations for the conflicting results, including the possibility that total guanylate cyclase activity may be a poor index of relaxant responses due to its abundant activity(Kowaluk, *et al.* 1990a),

or that the results reflected differences in methodology (broken-cell vs intact-cell)(Mulsch, *et al.* 1988).

These studies were the first in a large body of data examining the critical issue of whether there is cross-tolerance to other sources of NO in nitrate tolerance. This issue is discussed in detail in section 1.8.3.2.3.

Lastly, a recent study(De la Lande, *et al.* 1999a) noted that the development of in vitro nitrate tolerance in isolated bovine coronary artery was unaffected by co-incubation with an inhibitor of soluble guanulate cyclase, providing further evidence that tolerance is induced by mechanisms independent of guanylate cyclase activation.

1.8.3.2.3 Impaired nitrate bioconversion

As discussed in section 1.3, the organic nitrates are prodrugs, which must undergo bioconversion before releasing the pharmacologically active moiety, NO. In 1986, Fung et al(1986b) showed that in vivo GTN tolerance in the rat is associated with reduced vascular formation of denitrated metabolites, specifically 1,2-GDN, suggesting that bioconversion of GTN to 1,2-GDN was impaired in vessels from tolerance rats. This raised the possibility that tolerance was caused by impairment of the nitrate-NO bioconverting pathway.

Subsequently, the role of impaired bioconversion in tolerance induction has been investigated extensively, largely by two methods:- by the extent of cross-tolerance to other NO-mediated vasodilators which do not utilize the nitrate bioconversion pathway(Henry, *et al.* 1989c) and by assessment of nitrate bioconversion in tolerant tissues.

While there is strong evidence from both clinical(Manyari, *et al.* 1985; Schelling, *et al.* 1967; Thadani, *et al.* 1980; Zelis, *et al.* 1975) and experimental(Agvald, *et al.* 1999; Grosser, *et al.* 2000; Slack, *et al.* 1989; Slack, *et al.* 1988) studies, that nitrate tolerance produces cross-tolerance to other nitrates, the issue of associated cross-tolerance to <u>non-nitrate</u> sources of NO remains highly controversial.

Cross-tolerance to non-nitrate sources of NO has been sought in many in vitro tolerance induction studies in isolated animal vessels(Berkenboom, *et al.* 1988; Hasegawa, *et al.* 1999; Henry, *et al.* 1989a; Hinz, *et al.* 1998; Kowaluk, *et al.* 1990a; Kowaluk, *et al.* 1987; Miller, *et al.* 2000; Mulsch, *et al.* 1988; Mulsch, *et al.* 1989a; Mulsch, *et al.* 1989b; Rapoport, *et al.* 1987; Slack, *et al.* 1988; Van de Voorde, *et al.* 1987) and similarly in in vivo animal models of tolerance induction(Bauer, *et al.* 1991b; Berkenboom, *et al.* 1988; De la Lande, *et al.* 1999b; Du, *et al.* 1991; Laursen, *et al.* 1996a; Molina, *et al.* 1987; Munzel, *et al.* 1996d; Munzel, *et al.* 1995b; Shaffer, *et al.* 1992; Stewart, *et al.* 1987). The overwhelming majority of in vitro pharmacological studies(Berkenboom, *et al.* 1988; Hasegawa, *et al.* 1999; Henry, *et al.* 1989a; Hinz, *et al.* 1998; Kowaluk, *et al.* 1990a; Kowaluk, *et al.* 1987; Miller, *et al.* 2000; Mulsch, *et al.* 1988; Mulsch, *et al.* 1989a; Mulsch, *et al.* 1989b; Slack, *et al.* 1988; Van de Voorde, *et al.* 1987) on isolated vessels have shown minimal cross-tolerance to non-nitrate NO sources. However, the relevance of in vitro studies has been questioned, as they exclude some factors that may be of importance to tolerance induction in vivo(Harrison, *et al.* 1993; Munzel, *et al.* 1999).

The data from in vivo animal studies are somewhat more conflicting. Several studies examining isolated vessels(Berkenboom, *et al.* 1988; De la Lande, *et al.* 1999b; Du, *et al.* 1991), in situ coronary artery dilation(Stewart, *et al.* 1987) or hemodynamic indices(Bauer, *et al.* 1991b; Shaffer, *et al.* 1992) have demonstrated that tolerance induction is associated with no or minimal cross-tolerance to non-nitrate sources of NO. However, others(Laursen, *et al.* 1996a; Molina, *et al.* 1987; Munzel, *et al.* 1996d; Munzel, *et al.* 1995b) have reported varying degrees of cross-tolerance, including cross-tolerance to authentic NO.

By comparison, cross-tolerance data in humans are limited. Studies inducing tolerance in isolated vessels in vitro(Berkenboom, *et al.* 1988; Kuhn, *et al.* 1989), have found no evidence of cross-tolerance. Studies examining cross-tolerance utilizing in vivo tolerance induction, cannot assess the relative contributions of true tolerance and pseudotolerance(Sabouni, *et al.* 1990; Sutsch, *et al.* 1997). The only previous ex vivo study on isolated vessels from tolerant humans, was performed by Du et al(1992), and

found no cross-tolerance to SNP or acetylcholine in segments of internal mammary artery. Similarly, ex vivo studies of GTN tolerance induction at the level of platelet aggregation in humans showed no cross-tolerance between GTN and SNP(Chirkov, *et al.* 1997).

As discussed in section 1.3, GTN is a pro-drug which undergoes largely enzymatic bioconversion to yield dinitrates (1,2-GDN or 1,3-GDN) and NO, which is responsible for its pharmacological effects. Evidence from animal studies(Bennett, *et al.* 1989; McGuire, *et al.* 1998) suggests that conversion of GTN to 1,2-GDN and NO predominates in vascular tissue, and represents the mechanism-based pathway responsible for generation of NO. Hence, the role of impaired bioconversion in nitrate tolerance can also be assessed by measuring dinitrate (particularly 1,2-GDN), or NO release.

In addition to Fung's paper in 1986, several studies have demonstrated that GTN to 1,2-GDN bioconversion is impaired in association with tolerance induction in vitro(Bennett, *et al.* 1989; Slack, *et al.* 1989) or in vivo(Torfgard, *et al.* 1992). Other studies(Agvald, *et al.* 1999; Forster, *et al.* 1991; Husain, *et al.* 1994) have demonstrated reduced NO release from nitrates, but not other NO sources, in the presence of in vivo nitrate tolerance, though one study could find no evidence of reduced NO release(Laursen, *et al.* 1996b). Importantly, it is not known whether there is impaired bioconversion in vessels from tolerant humans; studies to date have been confined to animal models.

In summary, while a role of impairment of the bioconverting enzyme in the development of tolerance, is supported by a number of studies in which tolerance is limited to nitrates, ie. is nitrate-specific, and is associated with impaired nitrate bioconversion, considerable controversy exists in this area. Furthermore, human data is limited, raising concerns about potential inter-species differences.

1.8.3.2.4 The "superoxide hypothesis"

In 1995, Munzel et al(1995b) examined the mechanism of in vivo nitrate tolerance using a rabbit model. Induction of tolerance with 3 days of transdermal GTN significantly reduced aortic relaxations to GTN, but also impaired responses to acetylcholine, suggesting significant cross-tolerance. Using the technique of lucigenin chemiluminescence(Gyllenhammar 1987), they demonstrated that tolerance was associated with a 2-fold increase in vascular O_2^- levels. This increase was attenuated by removal of the endothelium and by the flavoprotein inhibitor, diphenyleneiodonium (DPI), suggesting the source of the increased O_2^- was endothelial NAD(P)H oxidase. Treatment with liposome-entrapped superoxide dismutase also normalized O_2^- levels and restored the maximal relaxation in response to GTN. They therefore proposed that tolerance was partly due to inactivation of nitrate-derived NO by increased vascular O_2^-.

Subsequent investigations by the same group using the same model, have expanded on the findings of this study. The source of the increased vascular O_2^- appears to be partly NAD(P)H oxidase(Munzel, *et al.* 1996d), which is inhibited by co-administered hydralazine. Consistent with studies suggesting that angiotensin II can stimulate NAD(P)H oxidases(Berry, *et al.* 2000; Griendling, *et al.* 1994; Rajagopalan, *et al.* 1996), co-administration of an angiotensin receptor antagonist also normalized O_2^- levels and relaxant responses to GTN and acetylcholine(Kurz, *et al.* 1999). As the renin-angiotensin system is thought also to play a role in pseudo-tolerance (discussed below), these findings have blurred the distinction between cellular tolerance and pseudo-tolerance, suggesting they may share some common mechanisms.

The group also raised doubts about the relevance of in vitro tolerance induction(Munzel, *et al.* 1999), after providing evidence that it does not affect NAD(P)H oxidase activity or O_2^- production, suggesting different mechanisms may be involved in in vitro and in vivo tolerance. In the same study, they also demonstrated that in vitro treatment with an inhibitor of superoxide dismutase, diethyldithiocarbamate (DETC)(Misra 1979), mimicked the effects of in vivo tolerance(Munzel, *et al.* 1999). Most recently(Munzel, *et al.* 2000a), they have shown that GTN tolerance is associated with increased expression of a dysfunctional endothelial NO synthase (eNOS) leading to increased O_2^- formation and reduced NO bioavailability, via a protein kinase C dependent mechanism. However, this finding

leaves a number of currently unanswered questions, such as the relative importance of eNOS and NAD(P)H oxidase as O_2^- sources, the potential for alteration of eNOS function via changes in redox state on supplementation with tetrahydrobiopterin (an eNOS cofactor) or the basis of the failure to demontrate more marked cross-tolerance to eNOS-dependent vasodilators (such as acetylcholine) relative to authentic NO/NO donors.

A variety of experimental studies provide support for this hypothesis. Bassenge's group has demonstrated enhanced oxidative stress in association with both in vitro(Dikalov, *et al.* 1998b), and in vivo(Fink, *et al.* 1999) GTN tolerance, which was suppressed by concomitant vitamin C or carvedilol, a beta-blocker with antioxidant activity. Another study(Bauer, *et al.* 1991a), found that hydralalzine attenuated haemodynamic tolerance in vivo, but had no effect on in vitro tolerance. A recent study suggested continuous therapy with GTN may increase abnormal vasoconstrictor responses in human coronary arteries(Caramori, *et al.* 1998)

Previous investigators have also found similar impaired relaxation to nitrovasodilators and endothelium-dependent dilators, following incubation with 10 mM DECT(Cherry, *et al.* 1990; Mugge, *et al.* 1991; Omar, *et al.* 1991). However, there is some doubt concerning the specificity of the mechanism of action of DETC, when used at high concentrations. Firstly, it appears to also inhibit contractile function(Mugge, *et al.* 1991; Omar, *et al.* 1991), possibly related to direct toxic cellular damage(Kelner, *et al.* 1989), and secondly, it may directly scavenge NO(Vedernikov, *et al.* 1992).

On the other hand, many experimental studies argue against a primary role of increased NO clearance by O_2^- in nitrate tolerance. As discussed above, several in vivo studies have found minimal cross-tolerance to non-nitrate sources of NO, including the endothelium(Bauer, *et al.* 1991b; Berkenboom, *et al.* 1988; De la Lande, *et al.* 1999b; Du, *et al.* 1992; Du, *et al.* 1991; Shaffer, *et al.* 1992; Stewart, *et al.* 1987). Other in vitro studies have demonstrated that, while endothelium-dependent relaxation is impaired by acute elevation of O_2^-, nitrate-induced vascular relaxation is unaffected(Hussain, *et al.* 1996; Ignarro, *et al.* 1987a). Two recent in vivo animal

studies(Laight, *et al.* 1998; Ratz, *et al.* 2000) have also dissociated nitrate tolerance from oxidative stress. In the first, nitrate tolerance was unaffected by anti-oxidants, despite reduction in markers of oxidative stress(Laight, *et al.* 1998). In the second, DPI exerted tolerance-independent effects on GTN sensitivity (arguing against a role of NAD(P)H) and tolerance was unaffected by a superoxide scavenger(Ratz, *et al.* 2000).

The final issue of relevance as regards the validity of the superoxide hypothesis as a true mechanism of nitrate tolerance is the large number of clinical trials assessing the effect of hydralazine, anti-oxidants and ACE-inhibitors on nitrate tolerance. Unfortunately, results of trials with all agents have been mixed.

The results of Munzel's findings with hydralazine(1996d), prompted speculation that the benefits seen in the V-HeFT studies with ISDN-hydralazine combination(Cohn, *et al.* 1986; Cohn, *et al.* 1991), may have been due to prevention of tolerance(Elkayam, *et al.* 1998). However, clinical haemodynamic trials have been conflicting(Gogia, *et al.* 1995; Parker, *et al.* 1997).

With regard to antioxidants, several studies inducing nitrate tolerance over 3 days showed that co-administration of various anti-oxidants, including vitamin C and E(Bassenge, *et al.* 1998; Watanabe, *et al.* 1997; Watanabe, *et al.* 1998a; Watanabe, *et al.* 1998b; Watanabe, *et al.* 1998c; Watanabe, *et al.* 1998d), appeared to reduce the extent of tolerance. However, a more recent study, inducing tolerance over 5 days, found no effect of vitamin C(Milone, *et al.* 1999b).

By comparison, far more studies have assessed the effects of ACE-inhibition on nitrate tolerance, but the results are no less conflicting. In predominantly haemodynamic studies, several investigators have shown that ACE-inhibition can prevent or retard nitrate tolerance(Berkenboom, *et al.* 1999; Cotter, *et al.* 1998; Katz, *et al.* 1991; Mehra, *et al.* 1992; Muiesan, *et al.* 1993; Munzel, *et al.* 1996a). On the other hand, several studies have demonstrated no effect of ACE-inhibition(Dakak, *et al.* 1990; Dupuis, *et al.* 1990a; Milone, *et al.* 1999a; Parker, *et al.* 1993). Interpretation of these studies is complicated by several factors. Firstly, the sulphydryl-containing ACE-inhibitors have been shown to potentiate nitrate effects(Meredith, *et al.* 1993; Metelitsa,

et al. 1992), and hence may exert tolerance-independent effects. Secondly, the dose of ACE-inhibitor employed may be critical(Munzel, *et al.* 1998). Lastly, angiotensin II may be involved in multiple processes potentially attenuating nitrate effects (see pseudotolerance section below), hence the effects of ACE-inhibition on tolerance are difficult to dissect.

1.8.3.2.5 Other possible mechanisms

In 1986, Ahlner et al(1986), noted that dipyridamole, which (interalia) is an inhibitor of phosphodiesterase, the enzyme responsible for metabolizing cGMP, could reverse in vitro nitrate tolerance. They postulated that tolerance could be partly due to elevated phosphodiesterase activity. Subsequent study in humans however, did not support this theory(Torfgard, *et al.* 1993).

More recently, Zierhut et al(1996), demonstrated that in vivo tolerance could be prevented by co-administration of a protein-kinase C inhibitor. In support of this observation, Munzel's group found that exposure to a protein-kinase C inhibitor in vitro could reverse ex vivo tolerance(Munzel, *et al.* 2000a). Thus, activation of protein kinase C by nitrates, may be involved the development of cellular tolerance, possibly via one of the mechanisms discussed above.

1.8.4 Pseudotolerance

Pseudotolerance may be defined as progressive attenuation of net vasodilator effect of organic nitrates due to processes that exert opposing pharmacological effects to the nitrate-NO pathway. These include activation of circulating neurohumoral factors, leading to increased vasoconstrictor forces and/or an increase in the sensitivity of vascular smooth muscle to constrictor agents. Thus, there is no implication of decreased activation of soluble guanylate cyclase.

1.8.4.1 Neurohumoral activation

Continuous nitrate therapy has been shown to be associated with variable degrees of activation of several neurohumoral factors. This has been demonstrated with intravenous GTN, transdermal GTN, and ISMN, in patients with coronary artery disease(Jeserich, *et al.* 1995; Munzel, *et al.* 1996b), CHF(Packer, *et al.* 1987), and in normal subjects(Parker 1996; Parker, *et al.* 1991), and includes increases in plasma renin(Dupuis, *et al.* 1990b; Jeserich, *et al.* 1995; Munzel, *et al.* 1996b; Packer, *et al.* 1987; Parker 1996; Parker, *et al.* 1991), vasopressin(Jeserich, *et al.* 1995; Munzel, *et al.* 1996b; Parker, *et al.* 1991), aldosterone(Jeserich, *et al.* 1995; Munzel, *et al.* 1996b; Parker, *et al.* 1991), and catecholamine levels(Parker, *et al.* 1991; Stewart, *et al.* 1986). These changes are also associated with an increase in intravascular volume and a decrease in haematocrit(Dupuis, *et al.* 1990b; Packer, *et al.* 1987; Parker, *et al.* 1991). The possible clinical implications of these effects are discussed in 1.8.4.4.

1.8.4.2 Increased vascular sensitivity to vasoconstrictor agents

In addition to increased elaboration of vasoconstrictors there is increasing evidence that long-term therapy with nitrates also may increase the sensitivity of vascular smooth muscle to some vasoconstrictors. This phenomenon has been seen in several in vivo(Du, *et al.* 1991) and ex vivo(Bauer, *et al.* 1993; Du, *et al.* 1991; Molina, *et al.* 1987; Munzel, *et al.* 1995a; Munzel, *et al.* 2000b; Zierhut, *et al.* 1996) animal studies, and more recently, an increased constrictor response to phenylephrine and angiotensin II has been demonstrated in tolerant humans(Heitzer, *et al.* 1998). In another study, abrupt cessation of intravenous GTN induced rebound coronary artery constriction in dogs(Munzel, *et al.* 1996a). However, this phenomenon has not been found in all studies. Du et al(1992) noted reduced sensitivity to vasoconstrictors in segments of internal mammary artery from patients treated with GTN. In addition, it is uncertain whether the effect occurs with all vasoconstrictors(Munzel, *et al.* 1995a).

The exact cause of this supersensitivity remains unclear, but there is evidence it may be related to increased vascular production of endothelin 1 leading to increased sensitivity of vasoconstrictors via a protein kinase C-mediated mechanism(Munzel, *et al.* 1995a; Zierhut, *et al.* 1996). A recent study also suggested that it can occur despite

incorporation of a nitrate-free period to avoid tolerance(Munzel, *et al.* 2000b). The clinical implications are discussed below.

1.8.4.3 Relationship with true/cellular tolerance

As mentioned briefly above, the recent studies by Munzel's group(Kurz, *et al.* 1999; Munzel, *et al.* 1996d; Munzel, *et al.* 1995b), suggesting a role for angiotensin II-stimulated NAD(P)H oxidase activation in cellular tolerance have blurred the distinctions between cellular tolerance and pseudotolerance. In other words, it is now conceivable that they share a common mechanism, mediated via increased angiotensin II levels. The hypothesis would be that angiotensin II initiates increases in O_2^- formation (leading to true tolerance) and also opposes the effects of NO (pseudotolerance).

1.8.4.4 Clinical implications of pseudotolerance

It is likely that the various aspects of pseudotolerance have differing clinical implications. While increased vasoconstrictor sensitivity may play a role attenuation of clinical efficacy during nitrate therapy(Munzel, *et al.* 1995a; Munzel, *et al.* 1996c), there is now evidence suggesting the two phenomena occur independently(Munzel, *et al.* 2000b). Similarly, there is evidence dissociating neurohumoral activation and increased intravascular volume from the development of tolerance(Dupuis, *et al.* 1990b; Elkayam, *et al.* 1987; Munzel, *et al.* 1996b; Olivari, *et al.* 1983). For example, a large portion of the intravascular fluid shift occurs in the first hour(Dupuis, *et al.* 1990b), whereas haemodynamic tolerance takes considerably longer to occur(Roth, *et al.* 1987). Furthermore, neurohumoral activation and intravascular fluid shifts occur with other nitrovasodilators that do not cause tolerance(Lehmann, *et al.* 1998), suggesting they play little or no part in clinical nitrate tolerance. In addition, while one small study suggested the co-administration of diuretics improved exercise tolerance during nitrate therapy(Sussex, *et al.* 1994), subsequent larger studies showed no effect(Parker, *et al.* 1996; Parker, *et al.* 1992). Overall, therefore, the contribution of pseudotolerance to attenuation of clinical efficacy during nitrate therapy may be less important than that of cellular tolerance.

However, there is considerable evidence that the aspects of pseudotolerance comprising increased vasoconstrictor forces and/or increased vasoconstrictor sensitivity can exert clinically important detrimental effects, in the form of rebound phenomena when nitrate therapy is withdrawn. These are manifested as an increased risk of angina/ischaemia during the nitrate-free period (DeMots, *et al.* 1989; Ferratini, *et al.* 1989; Freedman, *et al.* 1995; Parker, *et al.* 1995a; Pepine, *et al.* 1997), recurrence of ischaemia following sudden cessation of intravenous GTN(Figueras, *et al.* 1991), or rebound haemodynamic effects(Packer, *et al.* 1979; Packer, *et al.* 1981). The original and most spectacular examples of ischaemia precipitated by nitrate withdrawal have been associated with industrial exposure to GTN in munitions manufacture. An associated phenomenon, the "zero-hour phenomenon" refers to impairment of exercise capacity just prior to recommencing nitrate therapy after the nitrate-free period. This has been observed in some(DeMots, *et al.* 1989; Parker, *et al.* 1995a; Pepine, *et al.* 1997), but not all(Parker, *et al.* 1995b), studies of intermittent nitrate therapy for stable angina. To date, there have been no attempts to limit angiotensin II/catecholamine release in order to reduce these phenomena.

1.9 Factors limiting nitrate efficacy- Nitrate resistance

1.9.1 Historical notes

Nitrate resistance refers to primary or de novo impaired responsiveness to the organic nitrates(Abrams 1991). The term was first used (somewhat loosely) in the setting of severe CHF, when several investigators(Armstrong 1987; Armstrong, *et al.* 1980; Elkayam, *et al.* 1987; Elkayam, *et al.* 1985; Kulick, *et al.* 1988; Packer, *et al.* 1986; Roth, *et al.* 1987; Varriale, *et al.* 1991) observed that 25-50% of such patients were unable to achieve significant reductions in pulmonary pressures with nitrate therapy irrespective of dosage. This phenomenon appeared to be related to the presence of elevated right atrial pressure and severe peripheral oedema(Armstrong, *et al.* 1980; Kulick, *et al.* 1988; Packer, *et al.* 1986; Varriale, *et al.* 1991). It was suggested that these might be markers of an impaired ability to vasodilate, due to a combination of

mechanical (high tissue and extravascular pressure) and vasoconstrictor forces(Abrams 1991). The former was supported by the observation that diuretic therapy could improve nitrate responsiveness(Magrini, *et al.* 1980; Varriale, *et al.* 1991). In addition, these studies were performed before it was widely appreciated that diastolic ventricular interaction in patients with severe CHF(Atherton, *et al.* 1997) might reduce the maximal response to vasodilators in terms of reduction of filling pressures, irrespective of vascular sensitivity to NO donors.

More recently however, there is increasing evidence that de novo impaired nitrate vasodilator responsiveness can occur in the absence of severe heart failure (see next section). Whether the two situations share the same pathophysiology remains to be determined.

1.9.2 Evidence for nitrate resistance

As discussed above, impaired response to endothelium-dependent vasodilators ("endothelial dysfunction") has been associated with atherosclerosis, and risk factors for coronary artery disease, and hence, has been used as a surrogate for future cardiovascular risk. It is important to note that many studies in the field have used a single dose of endothelium-independent vasodilator, eliciting a near-maximal response, and therefore are unable to assess changes in sensitivity to these agents unless maximal responses are also reduced markedly.

However, an increasing number of studies examining endothelial function, are also observing a reduced response to endothelium-independent vasodilators, though to a lesser degree, suggesting that the "dysfunction", is not limited to the endothelium, but also affects the underlying vascular smooth muscle. This phenomenon has been observed in patients with atherosclerosis(Liao, *et al.* 1991) coronary artery disease(Celermajer, *et al.* 1992; Forstermann, *et al.* 1988; Nishikawa, *et al.* 1998), or CHF(Carville, *et al.* 1998; Katz, *et al.* 1994), as well as subjects with risk factors for coronary artery disease, including hypercholesterolaemia(Creager, *et al.* 1990; Duffy, *et al.* 1999; Sorensen, *et al.* 1994), hypertriglyceridaemia(Lundman, *et al.* 1997), cigarette smoking(Celermajer, *et al.* 1992; Zeiher, *et al.* 1995), and diabetes(Clarkson, *et al.*

1996; McVeigh, *et al.* 1994; McVeigh, *et al.* 1992; Watts, *et al.* 1996; Williams, *et al.* 1996). Furthermore, there is evidence that responsiveness to endothelium-independent vasodilators is affected by race(Cardillo, *et al.* 1999), and level of fitness(Haskell, *et al.* 1993), and that this phenomenon may be limited to arteries and not seen in veins(Huvers, *et al.* 1997)

In addition, resistance has been demonstrated at the platelet level, in association with stable angina pectoris(Chirkov, *et al.* 1999), and diabetes(Giugliano, *et al.* 1995).

Experimental studies in animal models of provided support for these observations. Isolated arteries from animals with hypercholesterolaemia(Cooke, *et al.* 1991; Miller, *et al.* 1998; Verbeuren, *et al.* 1986) and hypertension(Bauersachs, *et al.* 1998; Laursen, *et al.* 1997; Rajagopalan, *et al.* 1996) have demonstrated reduced vascular responsiveness to nitrovasodilators, and also a NO-independent stimulator of guanylate cyclase(Mulsch, *et al.* 1997).

It is important to note that the issue of endothelium-independent vasodilator resistance remains somewhat controversial, as it has not been demonstrated in many studies. However, evidence supporting its existence continues to accumulate. Clearly, the phenomenon could have important clinical implications in patients with coronary artery disease or hypertension. This has recently been demonstrated by Schachinger et al(Schachinger, *et al.* 2000), who observed that an impaired vasodilator response to intracoronary GTN, was associated with a significantly higher risk of cardiovascular events over a 7 year period.

1.9.3 Possible mechanisms of nitrate resistance

Experimental studies are only beginning to shed light on the possible mechanism(s) underlying this more generalized vasodilator dysfunction. Studies in both animal models(Laursen, *et al.* 1997; Rajagopalan, *et al.* 1996) and humans(Chirkov, *et al.* 1999; Giugliano, *et al.* 1995) have linked resistance to vasodilators to increased O_2^- generation. The predominant source of the incremental O_2^- appears to be NAD(P)H oxidase(Rajagopalan, *et al.* 1996; Wang, *et al.* 1998), possibly stimulated by

angiotensin II(Rajagopalan, *et al.* 1996; Zhang, *et al.* 1999). It has been suggested that the incremental O_2^- leads to desensitization of soluble guanylate cyclase(Kojda, *et al.* 1998a; Mulsch, *et al.* 1997). Similarly, oxidized low-density lipoprotein has also been shown to inhibit vascular smooth muscle and cGMP response to nitrovasodilators in vitro(Galle, *et al.* 1992).

However, other studies have dissociated vasodilator dysfunction from increased O_2^- generation(Bauersachs, *et al.* 1998; Miller, *et al.* 1998) and provided evidence for reduced guanlyate cyclase expression(Bauersachs, *et al.* 1998) or activity(Weisbrod, *et al.* 1997).

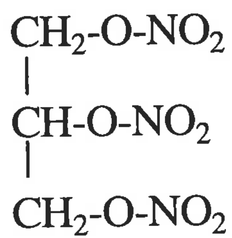
1.10 Scope of present study

The primary aim of the experiments described in the thesis was to investigate the mechanism(s) of nitrate tolerance in human vessels, predominantly via pharmacological studies in organ baths. Additional methodologies included examination of GTN bioconversion by vessels using gas chromatography and measurement of vascular O_2^- production using lucigenin-enhanced chemiluminescence.

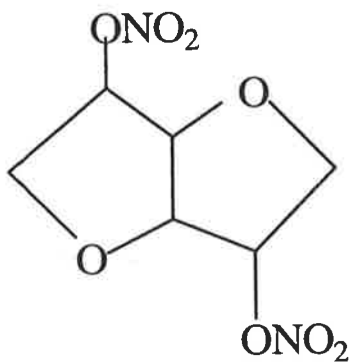
Chapters 3 to 6 describe investigations concerning nitrate tolerance. Chapter 3 is a randomized study of potential GTN tolerance induction in patients undergoing elective CABG. Vessels (IMA and SV) obtained at operation were studied for tolerance to GTN and cross-tolerance to other sources of NO. Also studied were bioconversion of GTN to its dinitrate metabolites following brief exposure to GTN and O_2^- generation. Chapter 4 then describes a series of experiments attemping to modulate vascular GTN tolerance and GTN reactivity, to provide further insights into the mechanism of tolerance induction and GTN action. Chapter 5 describes the relationship between long-term therapy with organic nitrates and subsequent cross-tolerance to GTN in the large vessels. Finally, Chapter 6 addresses the issue of whether increasing sulphydryl availability can prevent or reverse tolerance to GTN in large human vessels.

A secondary aim of the experiments in this thesis was to evaluate the determinants of GTN responsiveness of large vessels from human subjects not

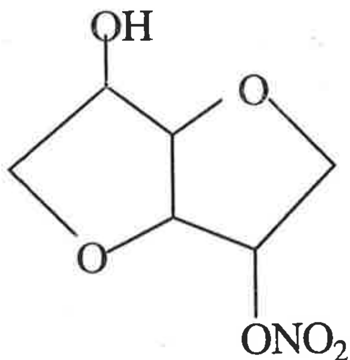
previously exposed to nitrate. These experiments were made possible by the large amount of control data from the tolerance studies and are described in Chapter 7.



Nitroglycerin



Isosorbide dinitrate



Isosorbide 5-mononitrate

Figure 1.1
Structures of several organic nitrates in widespread clinical use.

Table 1.1

Currently available preparations of organic nitrates in widespread use.

Glyceryl trinitrate (nitroglycerin)	Sublingual tablets or spray, buccal tablets cutaneous ointment or transdermal discs intravenous injection
Isosorbide dinitrate	Sublingual and oral tablets (including sustained release), intravenous injection
Isosorbide 5-mononitrate	Oral tablets (including sustained release)
Erythrityl tetranitrate	Sublingual and oral tablets
Pentaerythrityl tetranitrate	Tablets (including sustained release)

Table 1.2

Pharmacokinetics of some commonly used nitrate preparations.

Nitrate	*Prep*	*Major metabolite*	*Bioavailability (%)*	*VD (L/kg)*	*half-life (H)*
Glyceryl trinitrate (nitroglycerin)	IV	1,2-GDN 1,3-GDN 1-GMN 2-GMN	<10	1.1	0.02
Isosorbide dinitrate	Oral	IS 2-MN IS 5-MN	25	3.5	1.2
Isosrbide 5-mononitrate	Oral	IS	>90	0.7	4.5
Nicorandil	Oral	SG86	75	1.4	1.0

VD = apparent volume of distribution (liters/kg); SG86 = *N*-(2-hydroxyethyl) nicorandil.

2. Methods and materials

2.1 Methods

The work in this thesis consists of ex vivo studies in human blood vessels, using a combination of pharmacological and biochemical methodologies. This chapter provides general descriptions of the methods used. Further specific details can be found in Chapters 3 to 7.

2.1.1 Subjects

All study participants were patients undergoing elective coronary bypass surgery for mild to moderate stable angina pectoris. Patients with severe angina pectoris (consistent with Canadian Cardiac Society Class IV) were excluded. Other exclusion criteria were concomitant therapy with sulphydryl-containing medications (eg. captopril, gold salts, and penicillamine), vitamin supplement ingestion, and/or the presence of haemodynamically significant valvular heart disease. Patients with previous adverse reaction to organic nitrates were also excluded from the tolerance induction studies (Chapters 3,4 and 6).

The study was approved by the Human Ethics Committees of the North Western Adelaide Health Service and The Royal Adelaide Hospital. All patients involved in the study gave informed consent.

2.1.2 Experimental Protocols

In all cases, prophylactic anti-anginal agents other than nitrates were continued unchanged up until the day of surgery. Aspirin was withdrawn >7 days prior to surgery. In some experiments described in Chapters 3,4 and 6 the subjects' nitrate therapy was altered; these protocols are detailed in the relevant chapters.

During the operation the left internal mammary artery (IMA) was dissected free from the chest wall. Lengths of saphenous vein (SV) were harvested simultaneously. Following careful measurement and preservation of the conduit lengths required for grafting, the discarded segments of IMA and/or proximal SV were placed in ice-cold (4°C) Krebs solution previously gassed with 5% CO_2 in O_2 and transported to the laboratory. The delay from the operating theatre to the laboratory was approximately 15 minutes. The vessels were then placed in dishes filled with fresh oxygenated Krebs solution at 4°C, carefully dissected out from the surrounding connective tissue and cut into 2-3 mm long segments. The number of segments of IMA and SV obtained from each patient varied from 0-6 and 0-8 respectively. Only segments proximal to the IMA bifurcation were used, since post-bifurcation segments have previously been demonstrated to display different reactivity(He, *et al.* 1994).

2.1.2.1 Assessment of Vascular Reactivity

The vascular segments were suspended under tension in 15 ml organ baths containing Krebs solution and gassed with 5% CO_2 in O_2 at 37 °C. Isometric tension was recorded via two stainless steel wires through the lumen, one of which was fixed and the other attached to a Grass FT03 transducer. In some experiments (see Chapter 4), the endothelium was removed by gentle abrasion with forceps prior to mounting.

In the first series of experiments (Chapter 3), the IMA segment resting tension was normalised for the internal diameter as previously described by He et al(1988). This technique involves stretching each IMA segment in progressive steps to determine its length-tension curve. A computer iterative fitting technique is then used to determine the internal circumference of each segment at a pressure of 100 mm Hg. The segment is then released to the equivalent of 90% of the internal circumference at 100 mm Hg, and this degree of passive tension is maintained throughout the whole experiment. Mean resting tension determined in this way was found to be 2.03 grams (g) (see Chapter 3). Hence, in all other studies following Chapter 3, IMA resting tension was set at exactly 2 g. SV segment resting tension was set at 1 g for all experiments, since this tension gave optimal contractions to KCl solution (120mM) in exploratory experiments.

Measurement of Vasoconstrictor Responses

Following mounting under the appropriate tension, the segments were equilibrated for 60 minutes, during which the bathing solution was changed at least twice. Segments were then exposed to KCl solution (120mM). Segments contracting <1 g were discarded.

Thirty minutes after KCl exposure segments were contracted with increasing concentrations of noradrenaline (NA: 0.01-10 μM, 0.5 log unit increments). From the resultant NA cumulative concentration response (CR) curve, the maximum contractile response (E_{max}) and concentration of NA inducing 50% of E_{max} (EC_{50}) were determined. NA was selected as the vasoconstrictor following exploratory experiments showing consistent contractile responses in both IMA and SV, and a rapid relaxation phase following washout. Two other vasoconstrictors, phenylephrine and U46619 (9,11-dideoxy-11*a*,9*a*-epoxy-methano-prostaglandin F_{2a}) were initially considered in these exploratory studies. However, phenylephrine produced less consistent and weaker contractions than NA in the IMA, as previously shown by other workers(Du, *et al.* 1992; Weinstein, *et al.* 1989), and U46619 was found to have a very long relaxation timee following washout. Hence, NA was used as the contractile agent in all experiments.

The vasoconstrictor response to NA, predominantly reflecting α-adrenoceptor stimulation, can potentially be modulated by stimulation of β-adrenoceptors, neuronal catecholamine uptake and/or extraneuronal catecholamine uptake. Hence, exploratory experiments were performed to determine whether NA responses in human IMA and SV were altered by agents blocking these three processes (using propranolol 10 μM, desipramine 30 nM and hydrocortisone 10 μM, respectively). In relation to this issue, a previous study has demonstrated that desipramine potentiates the contractile response to NA in human SV, but that hydrocortisone has no effect(Janssens, *et al.* 1983). Also of relevance, another study suggested that there are few beta adrenoceptors in human IMA(He, *et al.* 1989). Consistent with these observations, we found that propranolol and desipramine potentiated the contractile response to NA in SV, but not in IMA.

Hence, these two agents were routinely added to SV segments 15 minutes prior to any exposure to NA.

Measurement of Vasodilator Responses

Following washout of after the CR curve to NA was elicited, segments were allowed to return to baseline tension and equilibrated for a further 45 minutes. During this period some segments were exposed to various agents to examine the effect on GTN-induced relaxation. These experiments are described in detail in Chapter 4.

After equilibration, segments were preconstricted with the concentration of NA sufficient to produce 70% of maximum tension in the IMA and 50% of maximum tension in the SV, as derived from the preceding NA CR curve. This modest degree of pre-constriction was used in SV segments, since greater degrees of constriction resulted in rapid decay of the contractile tension, making it more difficult to establish a steady state of contraction.

Once the contractile response had reached a plateau, each segment was exposed to increasing concentrations of one of three vasodilators, GTN (0.001-10 µM and 0.01-10 µM for IMA and SV segments respectively), SNP (0.001-10 µM and 0.01-10 µM for IMA and SV segments respectively), or the endothelium-dependent calcium ionophore A23187 (0.01-3 µM for both IMA and SV segments). Again, the resultant cumulative CR curves were used to derive E_{max} and EC_{50} values for these agents. As discussed in Section 1.3.3, GTN has been shown to have a biphasic vasorelaxant response(Malta 1989); in the experiments in this thesis, the parameters E_{max} and EC_{50} were derived from the full CR curve. In general, the tolerance induction studies (Chapter 3) examined responses to all three vasodilators, to assess both tolerance (to GTN) and cross-tolerance (to SNP or A23187), whereas the experiments described in Chapters 4 to7 were confined to examining GTN responses. Time from harvest of the vessels to assessment of relaxant responses was held constant at 3 hours 15 minutes, with the exception of the experiments involving exposure to various modulating agents described in Chapter 4.

In most experiments, segments were then assessed for the presence of an intact endothelium, as defined by relaxation to the endothelium-dependent vasodilator A23187 following preconstriction with NA. Studies have previously demonstrated that endothelium-dependent relaxation is far greater in IMA than SV(Luscher, *et al.* 1988; Yang, *et al.* 1991). Consistent with these previous findings, the endothelium was considered to be intact if A23187 induced greater than 50% relaxation in IMA, and greater than 30% relaxation in SV segments.

2.1.2.2 Measurement of GTN Bioconversion - Tissue GTN/GDN Assay

In some experiments, segments of SV were used to assess bioconversion of GTN to its dinitrate metabolites (1,2-and 1,3-GDN). These segments were weighed and equilibrated in fresh Krebs solution gassed with 5% CO_2 in O_2 at 37 ° C. During equilibration, some segments were exposed to various agents to examine the effect on GTN bioconversion (see Chapters 4 and 6 for details).

Approximately 3 hours after removal from the patient, segments were individually placed in test-tubes containing 1 ml Krebs at 37 ° C and allocated to incubate with either 1 μM GTN (final concentration) or GTN vehicle for 2 minutes. The segments were then rinsed for 5 seconds in ice-cold Krebs before snap freezing in liquid nitrogen. Segments were then stored at -80 ° C until assay.

GTN, 1,2-GDN and 1,3-GDN were extracted from the segments using a modification of the two-step extraction procedure of Bennett et al(1992). Frozen segments were placed into glass extraction tubes containing internal standard (1,4-dinitrooxy-butan-2-ol) and extracted twice with 5 ml of hexane to separate the GTN from its metabolites; hexane extracts were then combined. The segment was then extracted with 5 ml methyl tert-butyl ether containing the internal standard, to isolate the 1,2-GDN and 1,3-GDN. Organic phases were dried with anhydrous sodium sulfate, concentrated to approximately 200 μl under a stream of nitrogen and stored at -20 ° C until assay. Standard curves were prepared by spiking 1 ml of Krebs with GTN (1-50 pmole), 1,2-GDN (0.4-10 pmole) and 1,3-GDN (0.1-5 pmole) and extracted as for the segments.

GTN, 1,2-GDN and 1,3-GDN were separated using a Varian 3300 gas chromatograph with an electron capture detector and a BP-1 (100% dimethyl polysiloxane) capillary column (25m x 0.53mm id, 1 µm film) from SGE (Ringwood, Vic, Australia). Column temperature was programmed at 125 °C; this was followed by thermal cleaning between each 1 µl injection. The injector temperature was 125 °C and the detector temperature was 250 °C. Hydrogen was the carrier gas (4ml/minute) and nitrogen was the makeup gas (20 ml/minute). Under these conditions the retention times for GTN, 1,2-GDN, 1,3-GDN and the internal standard were 6.2, 6.5, 7.9 and 9.3 minutes respectively. Peak height ratios of GTN, 1,2-GDN and 1,3-GDN to internal standard were used for quantitation. The intra-assay coefficient of variation was ≤11% (n=5) for GTN, 1,2-GDN and 1,3-GDN at concentrations of 1, 0.4 and 0.1 pmol respectively and the interassay assay coefficient of variation was <10% (n=9) at concentrations of 20, 4 and 2 pmoles GTN, 1,2-GDN and 1,3-GDN respectively.

2.1.2.3 Measurement of Vascular Superoxide (O_2^-) Production

In some experiments, segments of IMA and SV were used to determine vascular O_2^- production via lucigenin-enhanced chemiluminescence as described by Ohara et al(1993). Each segment was first equilibrated in Krebs solution gassed with 5% CO_2 in O_2 at 37 °C and was then placed in Krebs-HEPES buffer at 37 °C for 30 minutes before measurement of O_2^-. The segment was then placed in 0.5 ml Krebs-HEPES buffer containing 250 µM lucigenin at 37 °C in a Picolite luminometer (Packard) and luminescence counts were measured every minute for 15 minutes. The segment was then weighed. Background counts were determined for 15 minutes before adding the segment; they were then subtracted from the luminescence counts and the results expressed as counts/min/mg. In most experiments, two segments from each patient were studied at 90 and 120 minutes post-harvest and the results meaned to obtain a single value.

In some experiments in Chapter 4, the effect of inhibiting endogenous superoxide dismutase (SOD) with diethyldithiocarbamic acid (DETCA) on O_2^- generation was studied. Before measuring O_2^- generation as described above, some

segments were exposed to 1 mM DETCA for 30 minutes followed by 30 minutes washout; other segments were incubated with DETCA vehicle only.

During the course of this thesis, evidence emerged that high concentrations of lucigenin can contribute to superoxide generation via redox cycling(Li, *et al.* 1998; Skatchkov, *et al.* 1999). This phenomenon appears to occur if concentrations of 20 μM or greater are utilized. Therefore, to exclude distortion of the results due to this artefactual generation, a number of additional experiments were also performed using 10 μM lucigenin (see Chapter 3).

2.2 Data Analysis

The results are expressed as mean ± SEM, unless specified. Vascular contractile and relaxant responses are analysed using the parameters concentration eliciting half the maximum response (EC_{50} expressed in log units) and maximum response (E_{max}). GTN and dinitrate content are expressed in pmole/mg. Lucigenin-enhanced chemiluminescence is expressed as counts/min/mg. A P value of less than 0.05 was considered statistically significant in all analyses. Further details of the statistical analyses used are discussed in each chapter.

2.3 Materials

N-acetylcysteine, (-)-Arterenol bitartrate salt, calcium ionophore A23187, diethyldithiocarbamic acid sodium salt (DETCA), desipramine hydrochloride, HEPES sodium salt, bis-N-methylacridinium nitrate (lucigenin), dl-propranolol hydrochloride and sodium nitroprusside (SNP) were purchased from Sigma (St Louis, MO, USA). 1 *H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) was purchased from Tocris Cookson, (Langford, Bristol,UK). Diphenyleneiodonium sulphate (DPI) was purchased from Colour Your Enzyme (Ontario, Canada). Glyceryl trinitrate (GTN) was purchased from David Bull Laboratories (Mulgrave, Vic, Australia). The 1,2-glyceryl dinitrate and 1,3-glyceryl dinitrate were purchased from Radian International (Austin, TX, USA). Hexane was residue analysis grade and was purchased from Fluka (Buchs,

Switzerland) and methyl tert-butyl ether was Omnisolv grade and was purchased from EM Science (Gibbstown, NJ).

The internal standard 1,4-dinitrooxy-butan-2-ol was prepared from 1,4-dibromo-butan-2-ol by Dr G. Phillipov(Aldrich, Milwaukee, WI) by displacing the bromo groups using silver nitrate and subsequently purifying it by silica-gel column chromatography.

The Krebs and Krebs-HEPES solutions were gassed with carbogen (95% O_2, 5% CO_2) and were of the following composition (mM) Krebs: NaCl (118), KCl (3.89), KH_2PO_4 (1.18), $NaHCO_3$ (25), $MgCl_2$ (1.05), $CaCl_2$ (2.34), EDTA (0.01), glucose (5.56), pH 7.4 and Krebs-HEPES: NaCl (99), KCl (4.69), $CaCl_2$ (1.87), $MgCl_2$ (1.20), K_2HPO_4 (1.03), $NaHCO_3$ (25), Na-HEPES (20) and glucose (11.1), pH 7.4. KCl (120 mM) solution was obtained by replacing NaCl in Krebs solution with iso-osmolar KCl.

3. Nitrate Tolerance: Mechanism(s) of GTN Tolerance Induction in Vivo

3.1 Introduction

As discussed in Chapter 1, there is overwhelming evidence that attenuation of the clinical effects of most or all organic nitrates occurs with continuous therapy. However, a number of aspects of this process remain controversial or uncertain. Firstly, the rate and extent to which true tolerance is induced in vessels of humans treated continuously with GTN is unknown. Secondly, the relative contribution(s) of impaired nitrate bioconversion, incremental O_2^- generation and/or desensitization of guanylate cyclase to true tolerance remains controversial. The experiments described in this chapter aim to shed light on these issues.

Several studies have shown that measurable attenuation of the anti-ischaemic, haemodynamic and direct vasodilator effects of nitrates in human subjects occurs within 24 hours (see Section 1.8.2). Zimrin et al(1988) observed that exercise time to angina was prolonged by intravenous GTN (mean dose 52 micrograms/min) acutely, but did not differ from placebo after a 24 hour infusion. Several groups(James, *et al.* 1985; Parker, *et al.* 1984a; Reichek, *et al.* 1984; Sullivan, *et al.* 1985; Thadani, *et al.* 1986) have found similar results with transdermal GTN at doses varying from 5-25 mg/24 hours. Continuous infusion of intravenous GTN at a dose sufficient to lower pulmonary capillary wedge pressure by 30% or 10 mmHg acutely, produces loss of this effect within 12 hours(Elkayam, *et al.* 1987). Haemodynamic tolerance to transdermal GTN also appears to occur within 18 hours(Jordan, *et al.* 1986; Packer, *et al.* 1986). Lastly, Boesgard et al(1994b) demonstrated attenuation of the effect of GTN on venous volume in humans after a 23 hour infusion at a comparatively low rate (7 micrograms/minute).

Importantly however, the majority of human studies to date have examined tolerance in vivo, and therefore cannot dissect out the relative contributions of true tolerance and pseudotolerance (see 1.8.4) to this process. Only two studies(Du, *et al.* 1992; Torfgard, *et al.* 1995) have used ex vivo methodology to assess the degree of true

tolerance in isolated human vessels. These workers found significant tolerance in vascular segments from patients treated with intravenous(Torfgard, *et al.* 1995) or transdermal(Du, *et al.* 1992) GTN. However, these studies were non-randomized, and the duration of treatment was prolonged in some subjects. Hence the extent to which true tolerance develops in human vessels following short-term treatment (24 hours) with GTN remains unknown.

As discussed in detail in 1.8.3 considerable controversy surrounds the mechanism(s) by which true tolerance to nitrates develops. Several theories have been proposed (see Section 1.8.3.2), but the major categories are **(a) impaired nitrate bioconversion resulting in diminished NO release (b) increased NO clearance, mediated via increased generation of O_2^- and/or (c) desensitization of guanylate cyclase**. Critically, the supporting evidence for these mechanisms is derived almost entirely from animal studies. Definitive evidence from studies in human subjects is lacking.

An important component of the evidence from animal studies has been the extent of cross-tolerance to non-nitrate sources of NO, or in other words, to what extent tolerance is nitrate-specific. This is a critical issue, since the absence of cross-tolerance implies that tolerance in nitrate-specific and favours mechanism (a) above, whereas the presence of cross-tolerance would favour mechanism (b) or (c). As discussed in 1.8.3.2.3, while most studies inducing tolerance in isolated vessels in vitro have shown no cross-tolerance to non-nitrate sources of NO, the results from ex vivo tolerance induction studies have been conflicting(Berkenboom, *et al.* 1988; Du, *et al.* 1991; Molina, *et al.* 1987) (De la Lande, *et al.* 1999b; Laursen, *et al.* 1996a; Munzel, *et al.* 1996d; Munzel, *et al.* 1995b). Importantly however, there are few studies to date which assess cross-tolerance in human subjects. The only previous study on isolated vessels from tolerant subjects(Du, *et al.* 1992) found no cross-tolerance to SNP or acetylcholine following treatment with transdermal GTN. Similarly, a lack of ex vivo cross-tolerance between GTN and SNP has been demonstrated at the level of platelet aggregation in humans(Chirkov, *et al.* 1997).

Further supporting evidence for mechanism (a) comes from animal studies showing that tolerance in vivo is associated with impaired bioconversion of GTN to 1,2-GDN(Fung, *et al.* 1986b; Torfgard, *et al.* 1992) or NO(Agvald, *et al.* 1999; Forster, *et al.* 1991; Husain, *et al.* 1994). However, not all workers have found impaired nitrate bioconversion in association with tolerance(Laursen, *et al.* 1996b). To date, there have been no studies in human vessels assessing the effect of in vivo tolerance on nitrate bioconversion.

While direct evidence supporting mechanism (b) above (see section 1.8.3.2.4) has come largely from one group of workers(Munzel, *et al.* 1999; Munzel, *et al.* 1996d; Munzel, *et al.* 1995b), these findings have been indirectly supported by several studies demonstrating an inhibitory effect of DETCA on GTN effects(Cherry, *et al.* 1990; Mugge, *et al.* 1991; Omar, *et al.* 1991), as well as some studies demonstrating a beneficial effect of antioxidants on some measures of nitrate tolerance(Bassenge, *et al.* 1998; Watanabe, *et al.* 1997; Watanabe, *et al.* 1998a; Watanabe, *et al.* 1998b; Watanabe, *et al.* 1998c; Watanabe, *et al.* 1998d). However, the importance of increased vascular O_2^- in the development of nitrate tolerance in humans is currently unknown.

3.2 Experimental Protocol

This component of the thesis was a randomized study of potential in vivo GTN tolerance induction in patients awaiting elective coronary artery bypass grafting. Participants were selected using the criteria described in Chapter 2. After informed consent was obtained, patients were randomized to receive either no nitrate therapy (control group) or a continuous intravenous infusion of GTN at 10 µg/minute via non-adsorptive tubing (nitrate group) for 24 hours prior to surgery. All other nitrate therapy was held during this period, unless indicated for medical reasons, in which case the patient was withdrawn from the study.. All other prophylactic anti-anginal agents were continued unchanged, as summarized in Table 3.1. Patients were withdrawn from the study if they developed recurrent angina, hypotension (systolic BP<90 mmHg) or headache unresponsive to paracetamol, or if surgery was postponed for other reasons. The infusion of GTN was continued unchanged throughout the operation. During the

operation, discarded segments of distal left IMA and/or proximal SV were collected as described in Chapter 2.

3.2.1 Vascular Reactivity Studies

Segments of IMA and SV were mounted in organ baths as described in Chapter 2. Mean normalized resting tension was 2.03 g and 2.04 g for control and tolerant IMA segments, respectively. Following equilibration contractile responses to KCl solution and increasing concentrations of NA were determined as described in Chapter 2.

Segments were allocated to one of the vasodilators GTN, SNP or A23187 and following submaximal preconstriction with NA, cumulative CR curves to these agents were elicited as described in Chapter 2. All segments were then assessed for endothelium-dependent relaxation; segments without intact endothelium were not used for analysis. Time from harvest of vessels to assessment of relaxant responses was held constant at 3 hours 15 minutes.

3.2.2 GTN Bioconversion Studies

Other segments of SV were allocated to measurement of GTN and dinitrate metabolites as described in detail in Chapter 2. These studies were performed at approximately 3 hours post-harvest to coincide with the vascular reactivity studies. Segment content of GTN and the dinitrate metabolites were compared between study groups. During the course of the study, it emerged that there was a significant inverse correlation between tissue GTN concentration and segment weight (p=0.02; Figure 3.12), suggesting that the content might be partly determined by the rate diffusion of GTN into the SV segments. Hence, the ratio (expressed as log units) of 1,2-GDN content to GTN content was also compared between groups.

3.2.3 Vascular O_2^- Generation

These studies were performed using IMA segments only, as exploratory investigations revealed far greater variability within control SV (48±10; n=10) than in IMA (32±4; n=10) luminescence counts. Further patients were randomized as described above and IMA segments were obtained at operation for determination of O_2^- generation via lucigenin-enhanced chemiluminescence as described in Chapter 2.

As discussed previously, during the course of the study evidence emerged that high concentrations of lucigenin (≥ 20 μM) can contribute to O_2^- generation via redox cycling(Li, *et al.* 1998; Skatchkov, *et al.* 1999). Hence, a further series of experiments was performed using 10 μM lucigenin.

3.2.4 Data Analysis

Data are expressed as mean ± SEM, unless otherwise indicated. The vascular relaxant responses are compared using the parameters log EC_{50} and E_{max} via an unpaired t-test. Tissue GTN and dinitrate content are expressed in pmole/mg and compared using an unpaired t-test. Lucigenin-enhanced chemiluminescence is expressed as counts/min/mg and compared using an unpaired t-test. The further series examining the effect of 10 μM lucigenin vs 250 μM lucigenin in control and tolerant vessels was analysed using two-way ANOVA.

3.3 Results

3.3.1 Patient Characteristics

The clinical characteristics of the patients studied in the experiments on vascular reactivity and GTN bioconversion are summarized in Table 3.1. The control and nitrate groups were well balanced. Specifically, there were similar numbers of patients in each group with the known risk factors for coronary artery disease, similar numbers receiving ACE inhibitors or nitrates prior to entry into the study, and at operation similar numbers of each bypass conduit were used in each group. Five patients were withdrawn from the study prior to surgery. Two patients randomized to receive GTN

developed significant (but reversible) hypotension, one patient in the control group experienced angina, and one patient from each group had the operation postponed for reasons unrelated to the study.

The clinical characteristics of the cohort of patients randomized for examination of vascular O_2^- generation were similar to those of the vascular reactivity and GTN bioconversion cohort and are shown in Table 3.2; again there were no significant differences between control and nitrate groups. Specifically, there was no disparity as regards prior nitrate therapy, ACE inhibitor or statin therapy, or of the known risk factors for coronary artery disease.

3.3.2 Vasoconstrictor Responses

The contractile responses to KCl (120 mM) and NA are illustrated in Figures 3.1, 3.2, and 3.3. E_{max} and EC_{50} values are shown in Table 3.3. In general the contractile responses were far larger in the SV than the IMA. Comparison of the control and nitrate groups revealed no significant differences in the contractile responses, though responses tended to be reduced in the tolerant segments (Figures 3.1, 3.2, 3.3; Table 3.3).

3.3.3 Vasodilator Responses

The mean concentrations of NA required to preconstrict the segments were identical in both patient groups (1.1±0.1μM and 0.4±0.1μM for IMA and SV, respectively).

<u>Comparison of IMA and SV CR curves</u>

In both IMA and SV segments, relaxations to GTN, SNP and A23187 were characterized by sigmoid CR curves (Figures 3.4 to 3.9).

In general, the SV segments were less sensitive to the three vasodilators compared to the IMA segments(see Table 3.4). Although E_{max} values were similar, log EC_{50} values for GTN and SNP were greater in segments of SV than segments of IMA.

In the case of A23187, maximum relaxation was considerably lower in SV as compared with IMA (42% vs 79%).

IMA Responses

Compared with segments from the control group, segments from the nitrate group were less responsive to GTN (Figure 3.4) with an approximately 5-fold increase in EC_{50} and a reduction in E_{max} from 97% to 85% (Table 3.4). Statistically, the differences in both parameters were highly significant ($p<0.01$). In contrast, the responses to SNP and A23187 did not differ significantly (Figures 3.5 and 3.6; Table 3.4). There was a trend towards a reduction in E_{max} to A23187 in the nitrate group, but this did not reach statistical significance ($p=0.06$).

SV Responses

Similarly, the SV segments from the nitrate group were less responsive to GTN (Figure 3.7) with an approximately 3-fold increase in EC_{50} and a reduction in E_{max} from 97% to 84% (Table 3.4). Again however, responses to SNP or A23187 did not differ (Figures 3.8 and 3.9; Table 3.4).

3.3.4 GTN Bioconversion

GTN bioconversion was studied in SV from 8 patients in the control group and 7 patients in the nitrate group. Mean segment weight (36±7 vs 37±9 mg) and GTN content (0.54±0.06 vs 0.50±0.07 pmole/mg) were similar in both groups (Figures 3.10 and 3.11).

SV tissue content of 1,2-GDN was much greater than that of 1,3-GDN in both groups (Figure 3.13). Tissue content of 1,2-GDN was significantly lower in segments from the nitrate group compared to control segments (0.10±0.01 vs 0.16±0.02 pmole/mg; $p=0.012$), whereas 1,3-GDN content was similar in both groups (Figure 3.13). Similarly, the log of the ratio of 1,2-GDN to GTN was lower in segments from the nitrate group compared to the control segments (-0.70 ± 0.05 vs -0.52 ± 0.07).

3.3.5 Vascular O_2^- Generation

Lucigenin-enhanced chemiluminescence counts using 250µM lucigenin were approximately 70% greater ($p<0.001$) in the segments from the nitrate group compared to control segments (Figure 3.14). With 10µM lucigenin, chemiluminescence (Figure 3.15) counts were reduced relative to 250µM lucigenin, but the proportional difference between the nitrate and control groups remained similar (24±3 vs 9±1 counts/min/mg). Two-way ANOVA revealed significant main effects for both lucigenin concentration, $F=28.7$, $p<0.001$ and nitrate therapy, $F=31.8$, $p<0.001$; there was no significant interaction between lucigenin concentration and nitrate treatment.

3.4 Discussion

This study is the first to examine the relative role(s) of impaired nitrate bioconversion and incremental O_2^- generation in the induction of in vivo GTN tolerance in human vessels. The results can be summarized as follows:- (1) 24 hours of therapy with intravenously infused GTN at 10 µg/minute in patients with stable angina induces a moderate degree of tolerance to GTN in both IMA and SV (5-fold and 3-fold reduction in sensitivity respectively) which is not accompanied by significant cross-tolerance to two non-nitrate sources of NO (SNP and A23187). (2) In the tolerant SV, GTN bioconversion to 1,2-GDN is impaired, whereas bioconversion to 1,3-GDN is unchanged. (3) Tolerance induction in the IMA is associated with a significant increase in O_2^- generation as measured via lucigenin-enhanced chemiluminescence.

In vivo induction of nitrate tolerance in the IMA has previously been reported by Du et al(1992) using similar ex vivo methodology, following more prolonged prior nitrate therapy administered in a non-randomized fashion. Boesgaard et al(1994b) demonstrated attenuation of the GTN effect on venous volume in humans after a 23 hour infusion of GTN at a rate slightly lower than that used in this study, although this may have included some component of pseudotolerance. Ex vivo tolerance to GTN in isolated human saphenous vein has not previously been reported. Therefore, an important implication of the results of this study are that true tolerance to GTN is induced in human vessels within 24 hours. Furthermore, this occurs at an infusion rate

that is at the lower end of most therapeutic protocols(Horowitz 1992). In view of results of previous acute studies(Horowitz, *et al.* 1983) demonstrating that this infusion rate is associated with relatively minor haemodynamic effects, the current findings suggest that a degree of true tolerance induction is unavoidable at doses required to achieve therapeutic effects.

Previously, the role of impaired nitrate bioconversion in tolerance induction has been investigated largely by two methods:- by the extent of cross-tolerance to other NO-mediated vasodilators which do not utilize the nitrate bioconversion pathway(Henry, *et al.* 1989c) and by assessment of bioconversion of GTN(Fung, *et al.* 1986b; Torfgard, *et al.* 1992). The current experiments used both methods to determine the contribution of impaired GTN bioconversion to true tolerance in human vessels.

The overwhelming majority of in vitro pharmacological studies on isolated vessels have shown minimal cross-tolerance to non-nitrate sources of NO(Berkenboom, *et al.* 1988; Hasegawa, *et al.* 1999; Henry, *et al.* 1989a; Hinz, *et al.* 1998; Kowaluk, *et al.* 1990a; Kowaluk, *et al.* 1987; Miller, *et al.* 2000; Mulsch, *et al.* 1988; Mulsch, *et al.* 1989a; Mulsch, *et al.* 1989b; Slack, *et al.* 1988; Van de Voorde, *et al.* 1987). However, these studies have generally induced tolerance by exposing isolated vessels to high concentrations of GTN for a short duration. This method of tolerance induction has been criticized(Munzel, *et al.* 1999; Munzel, *et al.* 1996c) as it may exclude factors that are important to tolerance induction in vivo. Specifically it excludes circulating neurohumoral factors such as angiotensin II, which potentially play a role in the development of true tolerance(Kurz, *et al.* 1999). Indeed, Munzel at al(Munzel, *et al.* 1999) recently demonstrated that in vivo tolerance resulted in significant cross-tolerance to acetyl choline, whereas in vitro tolerance did not. Hence, the relevance of in vitro tolerance induction studies has been questioned(Harrison, *et al.* 1993; Munzel, *et al.* 1999).

The data from in vivo animal studies are somewhat more conflicting. Several studies examining isolated vessels(Berkenboom, *et al.* 1988; De la Lande, *et al.* 1999b; Du, *et al.* 1991) have demonstrated that ex vivo GTN tolerance is associated with no or minimal cross-tolerance to non-nitrate sources of NO. Similar results have been

obtained in intact animals by workers examining in situ coronary artery dilation(Stewart, *et al.* 1987) or haemodynamic indices(Bauer, *et al.* 1991b; Shaffer, *et al.* 1992). However, several other studies using animal models(Laursen, *et al.* 1996a; Molina, *et al.* 1987; Munzel, *et al.* 1999; Munzel, *et al.* 1996d; Munzel, *et al.* 1995b) have reported varying degrees of cross-tolerance to non-nitrate sources of NO. In this regard, the possibility of inter-species differences cannot be excluded.

Few previous studies have assessed cross-tolerance in humans. Studies inducing GTN tolerance in isolated human saphenous veins in vitro(Berkenboom, *et al.* 1988; Kuhn, *et al.* 1989), found no evidence of cross-tolerance. However, these studies are open to criticism regarding the method of tolerance induction, as discussed above. Sutsch et al(Sutsch, *et al.* 1997) demonstrated that dilation of dorsal hand veins with a non-nitrate NO donor (linsidomine) remains intact in the presence of GTN tolerance. However, this study, while important cannot assess the relative contributions of true tolerance and pseudotolerance to the observed impairment of GTN response. Du et al(1992) focussed on true tolerance by examining ex vivo tolerance to GTN in isolated human internal mammary artery, and found no cross-tolerance to SNP or acetylcholine. Similarly, ex vivo studies of GTN tolerance induction at the level of platelet aggregation in humans, have shown no cross-tolerance between GTN and SNP(Chirkov, *et al.* 1997).Thus, the lack of significant cross-tolerance to other NO-mediated vasodilators found in the current study is in agreement with the few previous analogous human studies.

The current study demonstrates for the first time in humans that true GTN tolerance is associated with impairment of the enzymatic bioconversion of GTN to 1,2-GDN. As discussed in the Introduction (Section 1.3) GTN is a pro-drug which undergoes largely enzymatic bioconversion to yield dinitrates (1,2-GDN or 1,3-GDN) and NO, which is responsible for its pharmacological effects. Evidence from animal studies(Bennett, *et al.* 1989; McGuire, *et al.* 1998)suggests that conversion of GTN to 1,2-GDN predominates in vascular tissue, representing the "mechanism-based" pathway responsible for generation of NO. Consistent with this, we found that levels of 1,2-GDN were much higher than levels of 1,3-GDN in both control and tolerant SV segments exposed to GTN. Animal studies have demonstrated that GTN to 1,2-GDN

bioconversion is impaired in association with tolerance induction in vitro(Bennett, *et al.* 1989) or in vivo(Fung, *et al.* 1986b; Torfgard, *et al.* 1992). Furthermore, other studies(Agvald, *et al.* 1999; Forster, *et al.* 1991; Husain, *et al.* 1994) have demonstrated that nitrate tolerance in intact animals is associated with diminished NO generation from organic nitrates, but not from non-nitrate NO donors, such as SNP or 3-morpholino-sydnonimine. The current study demonstrates that diminished GTN bioconversion also occurs in human vessels in association with in vivo tolerance induction. Taken together, the lack of cross-tolerance to non-nitrate sources of NO, and the impairment of bioconversion of GTN to 1,2-GDN support the primary role of impaired bioconversion in true tolerance to NTG in human vessels, and that increased clearance of nitrate-derived NO and decreased guanylate cyclase sensitivity have a minimal contribution.

This study also demonstrates for the first time in humans that nitrate tolerance is associated with increased vascular O_2^- production. An alternative theory proposed recently(Munzel, *et al.* 1995b) is that GTN tolerance involves increased clearance of NO via increased O_2^-, the latter being generated primarily as a result of angiotensin II-induced activation of NAD(P)H-oxidase(Munzel, *et al.* 1996d). In the current study, measurement of lucigenin-enhanced chemiluminescence using both 250μM and 10μM lucigenin suggested that tolerant IMA segments have a greater potential for superoxide generation than control segments, the difference being similar to that reported previously by Munzel et al(1995b) in rabbit aortae. These experiments also confirmed the previous observation of some(Li, *et al.* 1998; Skatchkov, *et al.* 1999) but not all(Berry, *et al.* 2000) studies that a higher concentration of lucigenin contributes to chemiluminescence via redox cycling.

Several previous studies in animals(Berkenboom, *et al.* 1999; Kurz, *et al.* 1999; Munzel, *et al.* 1996a) and humans(Cotter, *et al.* 1998; Katz, *et al.* 1991; Mehra, *et al.* 1992; Muiesan, *et al.* 1993) have suggested that nitrate tolerance can be prevented by co-administration of ACE-inhibitors, possibly by preventing the activation of vascular NAD(P)H-oxidase and the resultant increased O_2^- generation(Berkenboom, *et al.* 1999; Kurz, *et al.* 1999; Munzel, *et al.* 1996a). In this regard, while the present study was not

designed to specifically address this issue, the degree of tolerance did not vary between those patients receiving or not receiving ACE-inhibitors.

The results of this section do not allow us to make any conclusions regarding the source of the incremental O_2^-. As mentioned above, animal studies have suggested it is derived from NAD(P)H-oxidase(Munzel, *et al.* 1996d). A recent study in human IMA(Berry, *et al.* 2000) suggested that both xanthine oxidase and NAD(P)H-oxidase contributed to basal levels of O_2^- generation. However, additional studies are required to investigate the source(s) of the elevatedl O_2^- production in tolerant human vessels. Furthermore, the current study does not investigate the role of the incremental O_2^- generation in nitrate tolerance. This issue is further addressed in Chapter 4.

This study has several limitation. We examined only one GTN dosing regimen and induced only a moderate degree of tolerance. The previous animal studies which showed significant cross-tolerance to non-nitrate NO sources have in general used higher GTN regimens and reported greater degrees of tolerance(Laursen, *et al.* 1996a; Munzel, *et al.* 1995b). The mechanisms of nitrate tolerance may vary with the extent of tolerance induction; hence, we cannot exclude the possibility that a greater degree of tolerance induction might have been associated with cross-tolerance.

In addition, we have assumed that the three sources of NO examined in the study act largely via activation of soluble guanylate cyclase; if this were not the case, a more cautious interpretation of the results would be warranted. This issue is studied further in Chapter 4.

Lastly, the study did not attempt to assess the contribution of the neurohumoral component of "pseudotolerance" to attenuation of nitrate efficacy. However, the magnitude of the vasoconstrictor responses to noradrenaline was examined. In contrast to previous animal studies(De la Lande, *et al.* 1999b; Munzel, *et al.* 1995b), in the present study vasoconstrictor responses tended to be reduced in the tolerant human vessels, though this did not quite reach statistical significance. Interestingly, the only previous ex vivo study in human vessels(Du, *et al.* 1992) also observed a reduced vasoconstrictor response in tolerant vessels. A greater change in sensitivity to

vasoconstrictor agents may have become manifest with a greater degree of tolerance induction. Furthermore, it is possible that the presence of β-adrenoceptor antagonist and inhibitor of reuptake may have altered the observed responses to NA. It is also possible that changes in vasoconstrictor responsiveness may have been agonist specific and thus might have been missed since only NA responses were examined.

In summary, this study demonstrates that true GTN tolerance is induced in human vessels within 24 hours of therapy with a relatively low dose of intravenously infused GTN, and that this is largely (or wholly) due to impaired enzymatic GTN bioconversion. GTN tolerance is also associated with increased vascular O_2^- generation, the role of which is further examined in Chapter 4.

Table 3.1

Patient Characteristics: Vascular Reactivity and GTN Bioconversion.

	Control Group (n=15)	Nitrate Group (n=15)
Age (years)	56 ± 3	64 ± 3
Sex (M : F)	12 : 3	10 : 5
Extent of CAD (1:2:3)	0 : 7 : 8	1 : 6 : 8
Conduit used (IMA:SV)	12 : 11	13 : 9
Prior nitrate therapy	6	7
Concomitant therapy:-		
ß-adrenoceptor antagonist	8	9
L-calcium channel blocker	4	5
ACE inhibitor	9	6
Statin	5	4
Coronary risk factors:-		
Hypercholesterolemia	10	10
Hypertension	6	8
Smoking	5	5
Diabetes	2	3
Family history	7	4

Values are number of patients or mean ± SEM. CAD indicates coronary artery disease.

Table 3.2

Patient Characteristics: IMA Vascular Superoxide.

	Control Group (n=10)	Tolerant Group (n=11)
Age (years)	63 ± 3	63 ± 3
Sex (M : F)	9 : 1	9 : 2
Prior nitrate therapy	4	6
Concomitant therapy:-		
ß-adrenoceptor antagonist	5	4
L-calcium channel blocker	5	7
ACE inhibitor	3	2
Statin	6	9
Coronary risk factors:-		
Hypercholesterolaemia	7	9
Hypertension	4	3
Smoking	2	4
Diabetes	2	1

Values are number of patients or mean ± SEM.

Table 3.3

Reactivity of Internal Mammary Artery (IMA) and Saphenous Vein (SV) Segments to KCl (120mM) and Noradrenaline.

	No of Subjects		Log EC_{50} (M)		E_{max} (g)	
	Control	Nitrate	Control	Nitrate	Control	Nitrate
IMA						
KCl	12	13			2.4 ± 0.2	1.9 ± 0.2
NA	12	13	-6.3 ± 0.1	-6.5 ± 0.1	2.6 ± 0.2	2.3 ± 0.2
SV						
KCl	11	9			4.9 ± 0.5	4.2 ± 0.7
NA	11	9	-6.5 ± 0.1	-6.4 ± 0.1	5.3 ± 0.5	4.1 ± 0.5*

Values are mean ± SEM.
$P > 0.05$ for all control group vs nitrate group comparisons.
* $p = 0.06$ for control group vs nitrate group.

Table 3.4

Reactivity of Internal Mammary Artery (IMA) and Saphenous Vein (SV) Segments to GTN, SNP and A23187.

	No of Subjects		Log EC_{50} (M)		E_{max} (%)	
	Control	Nitrate	Control	Nitrate	Control	Nitrate
IMA						
GTN	9	10	-7.7 ± 0.1	-7.0 ± 0.1*	97 ± 2	85 ± 2*
SNP	8	8	-7.0 ± 0.1	-7.0 ± 0.1	98 ± 1	95 ± 1
A23187	8	8	-7.5 ± 0.2	-7.4 ± 0.2	79 ± 4	69 ± 3#
SV						
GTN	9	8	-7.0 ± 0.1	-6.5 ± 0.1*	97 ± 1	84 ± 4*
SNP	8	7	-6.8 ± 0.2	-6.8 ± 0.1	98 ± 1	96 ± 2
A23187	7	6	-7.4 ± 0.1	-7.3 ± 0.1	42 ± 5	38 ± 4

Values are mean ± SEM.
* $p < 0.01$ for control group vs nitrate group.
# $p = 0.06$ for control group vs nitrate group.

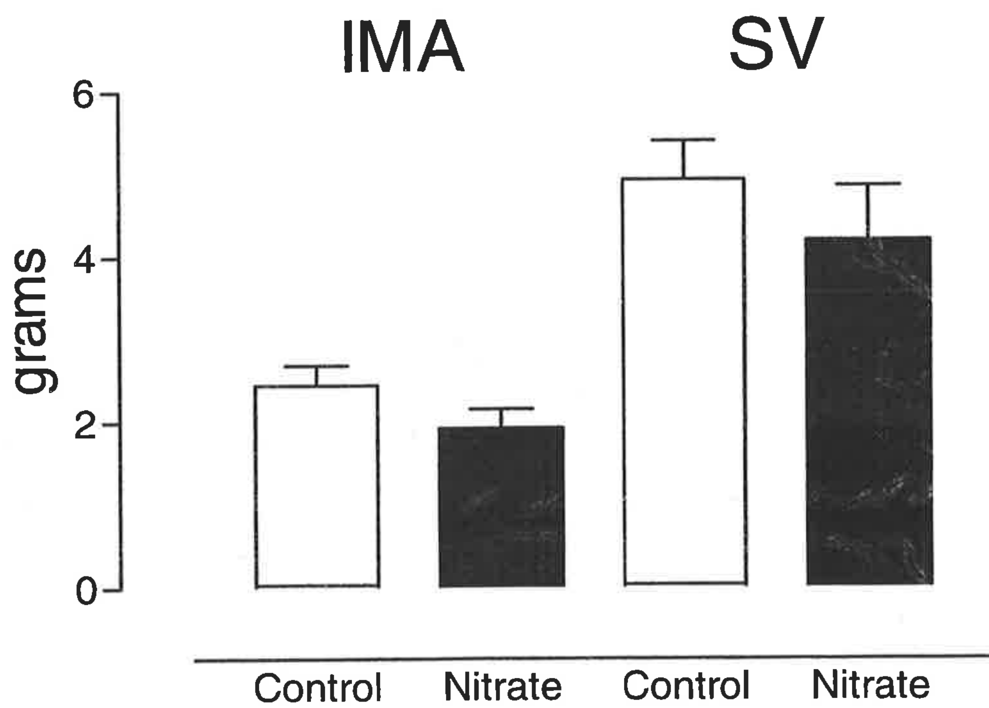


Figure 1.
Responses of IMA and SV to KCl solution (120 mM). There were no significant differences between control and nitrate goups (n=9-13 each group).

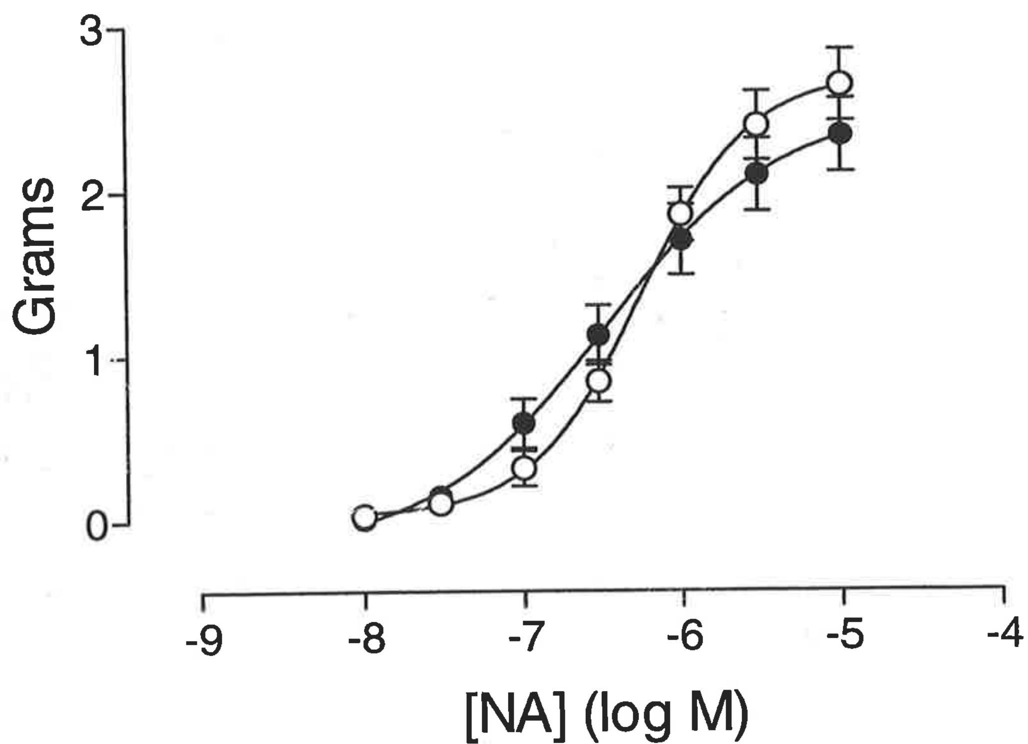


Figure 3.2
Effect of GTN treatment on responses of IMA to noradrenaline. Open symbols indicate control group (n=12) and closed symbols, nitrate group (n=13). The curves were not significantly different.

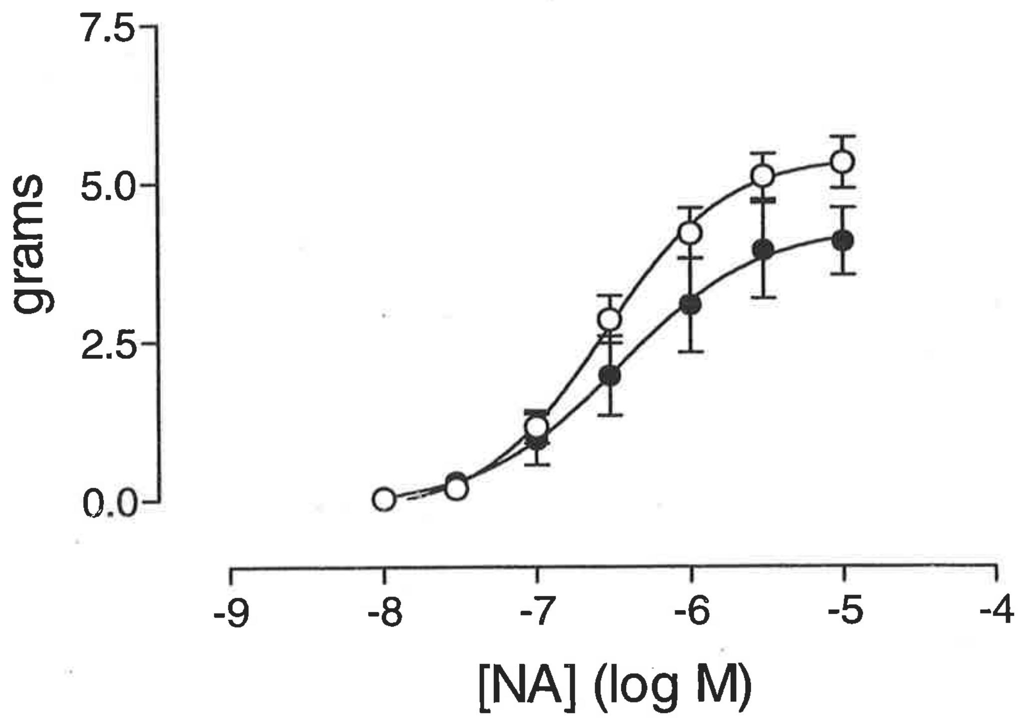


Figure 3.3
Effect of GTN treatment on responses of SV to noradrenaline. Open symbols indicate control group (n=11) and closed symbols, nitrate group (n=9). p = 0.06 for control vs nitrate E_{max} values.

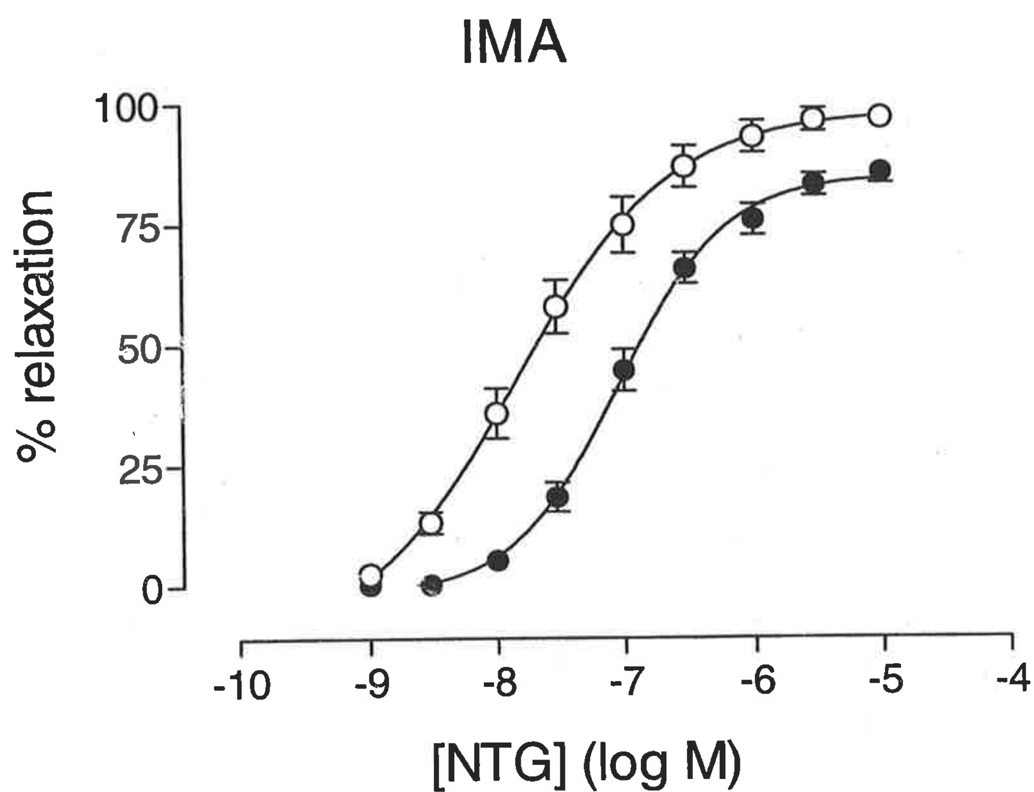


Figure 3.4
Effect of GTN treatment on responses of IMA to GTN. Open symbols indicate control group (n=9) and closed symbols, nitrate group (n=10). Segments from the nitrate group were significantly less responsive compared to segments from the control group (see Table 3.4).

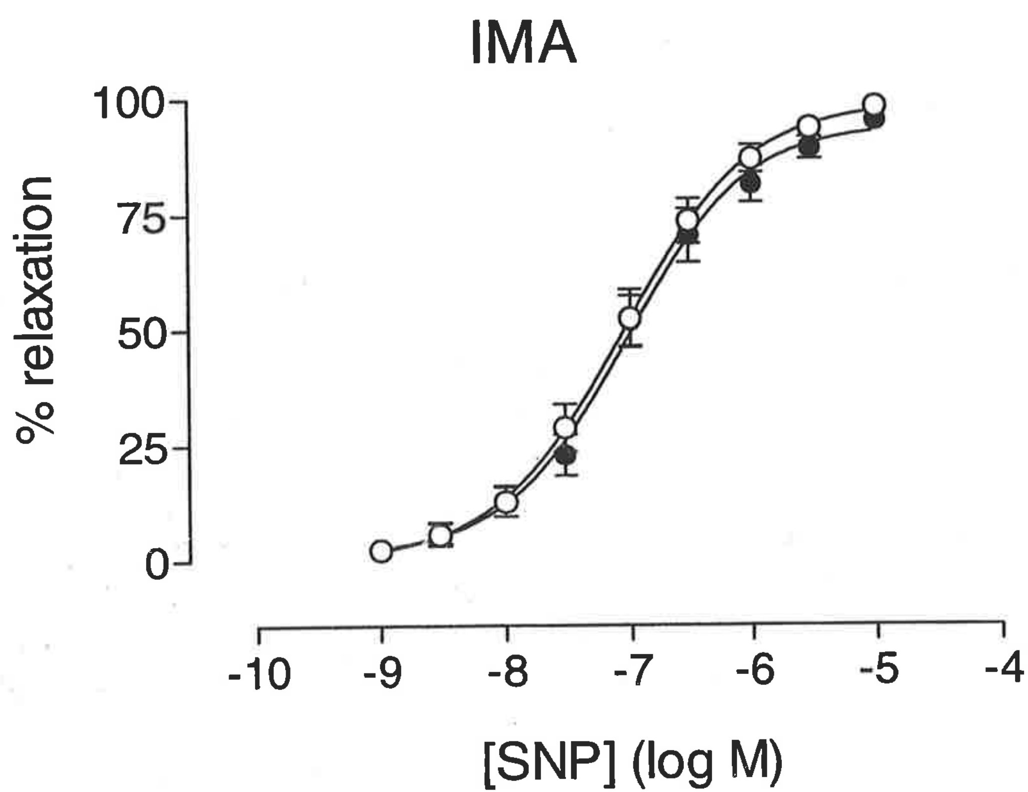


Figure 3.5
Effect of GTN treatment on responses of IMA to sodium nitroprusside. Open symbols indicate control group (n=8) and closed symbols, nitrate group (n=8).

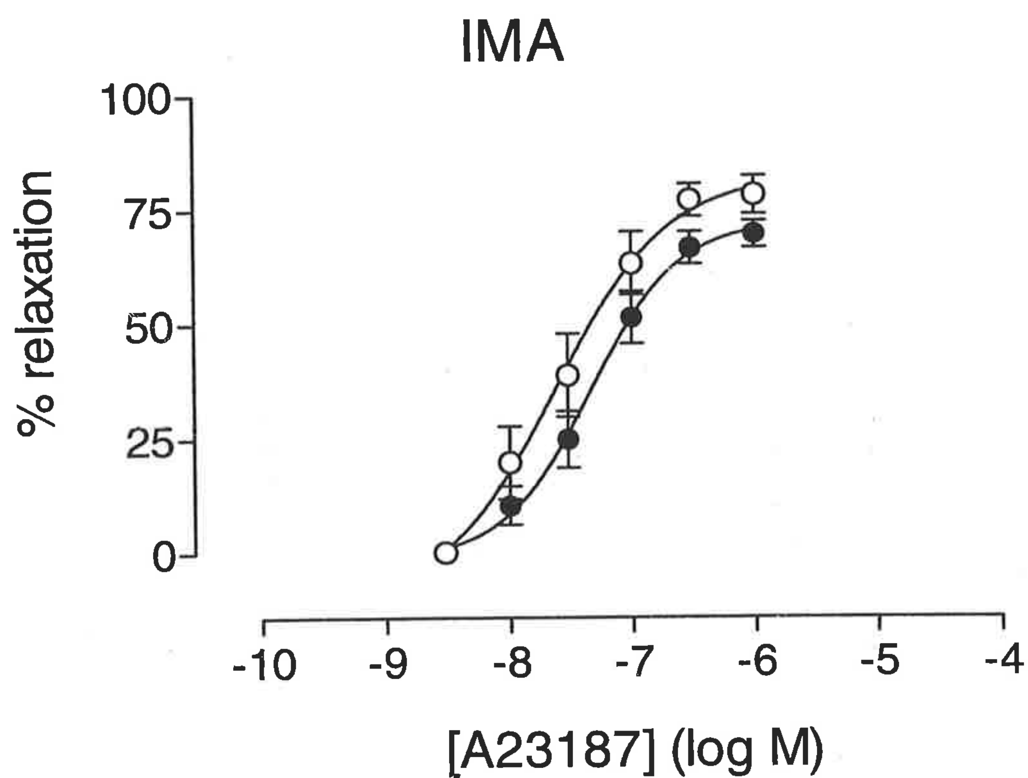


Figure 3.6
Effect of GTN treatment on responses of IMA to A23187. Open symbols indicate control group (n=8) and closed symbols, nitrate group (n=8). p = 0.06 for control vs nitrate E_{max} values.

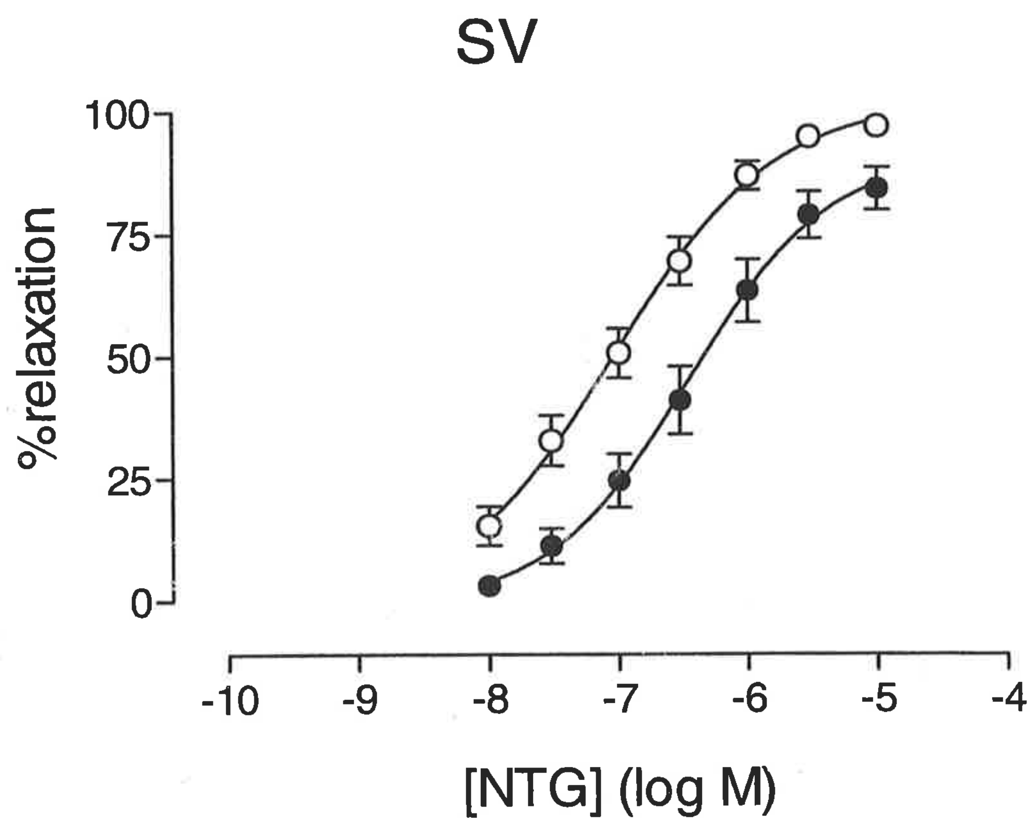


Figure 3.7
Effect of GTN treatment on responses of SV to GTN. Open symbols indicate control group (n=9) and closed symbols, nitrate group (n=8). Segments from the nitrate group were significantly less responsive compared to segments from the control group (see Table 3.4).

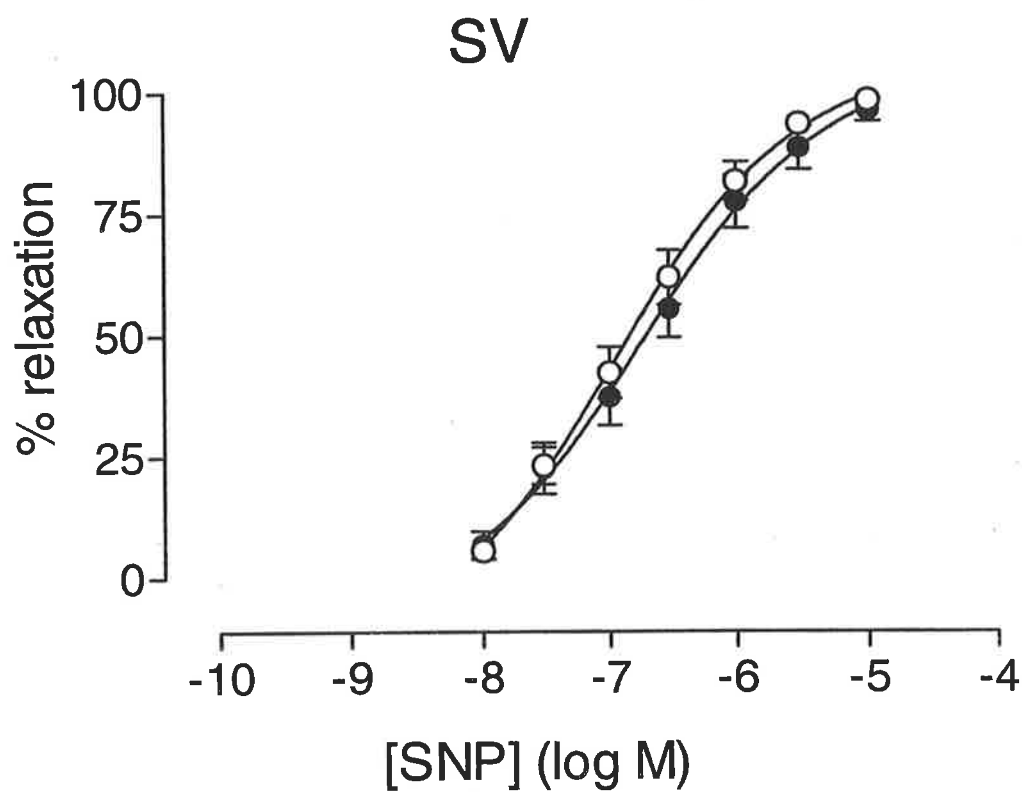


Figure 3.8
Effect of GTN treatment on responses of SV to sodium nitroprusside. Open symbols indicate control group (n=8) and closed symbols, nitrate group (n=7).

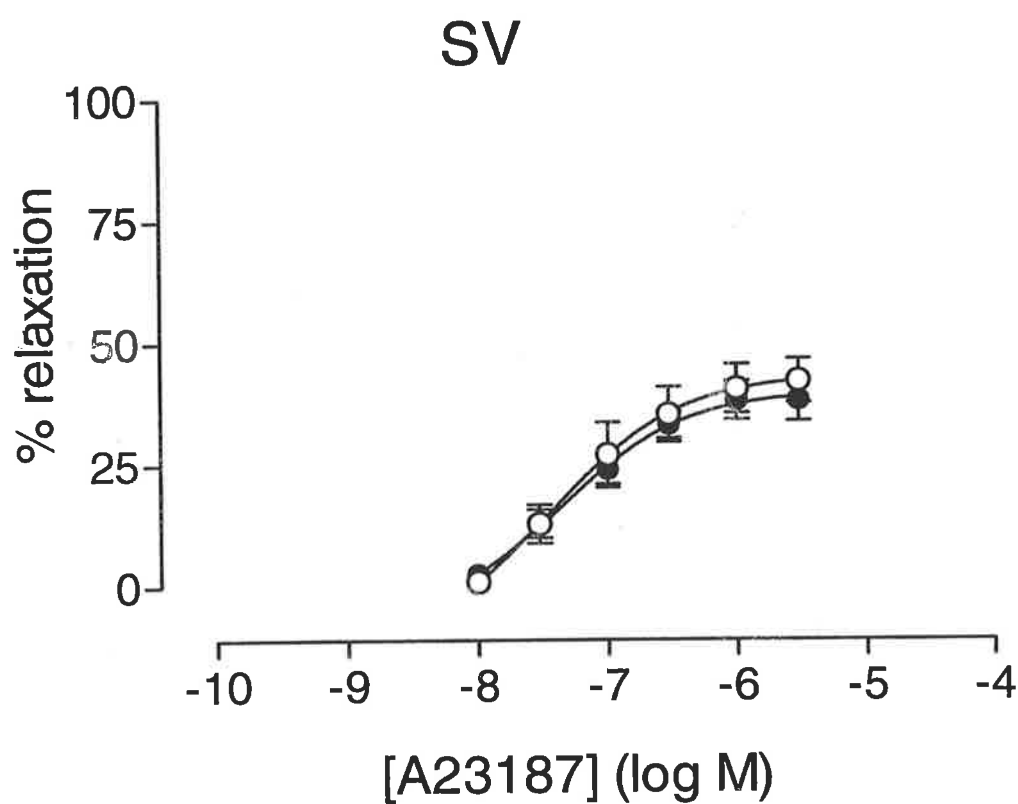


Figure 3.9
Effect of GTN treatment on responses of SV to A23187. Open symbols indicate control group (n=7) and closed symbols, nitrate group (n=6).

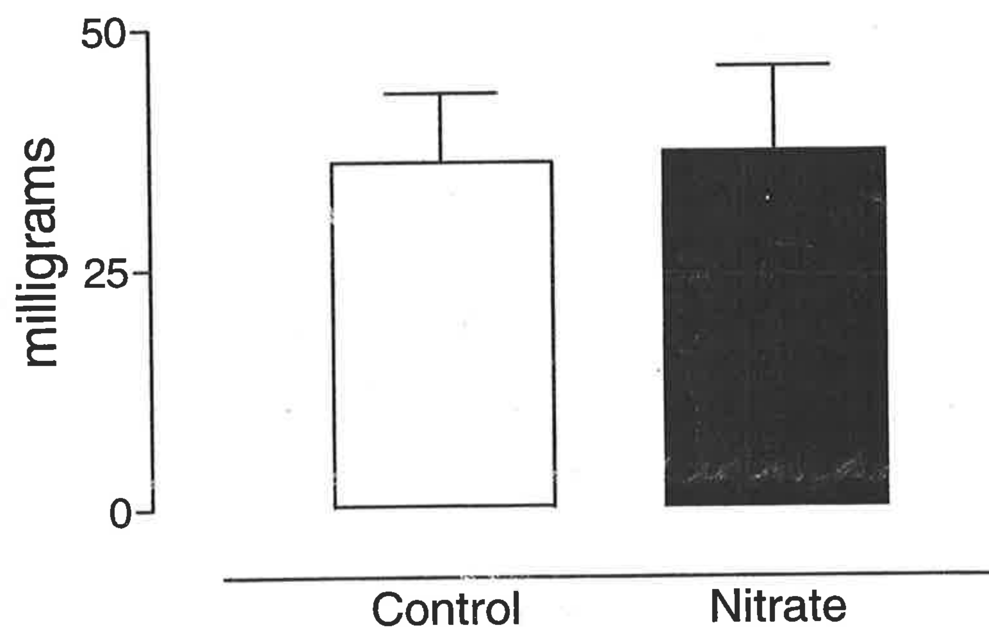


Figure 3.10
Weight of SV segments from control group (n=8) and nitrate group (n=7).

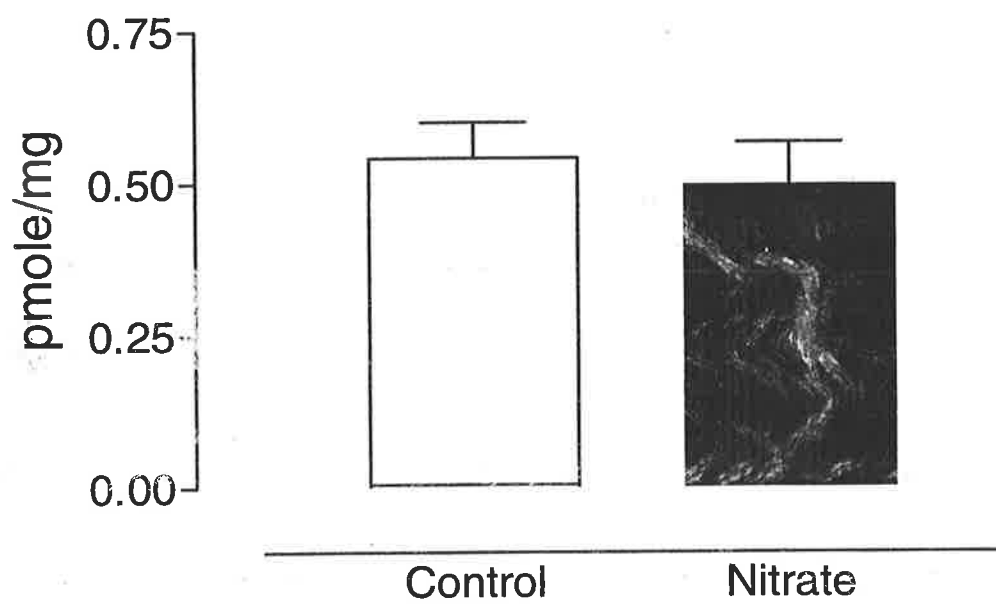


Figure 3.11
SV content of GTN after incubation of segments from control and nitrate groups with 1.0 μM GTN. $p = 0.6$ for control group (n=8) vs nitrate group (n=7).

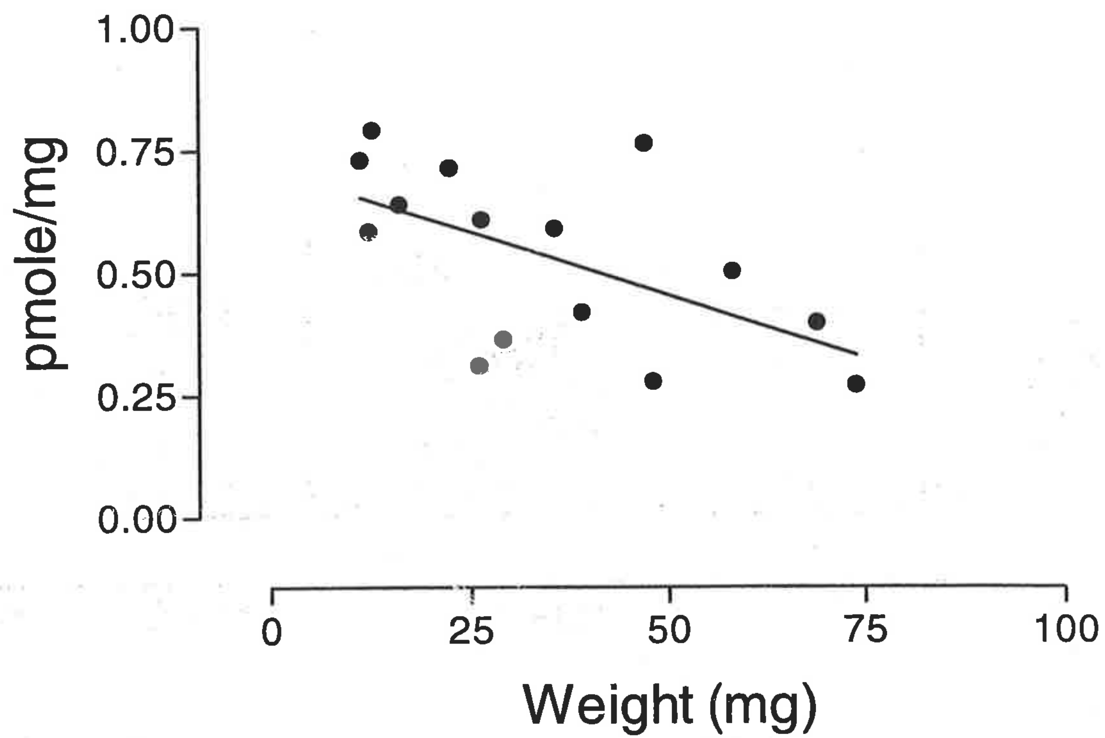


Figure 3.12
Correlation between the weight of the segments of SV and the GTN concentration following incubation with GTN (1.0μM: 2 minutes).
$p = 0.02$, $r = -0.58$

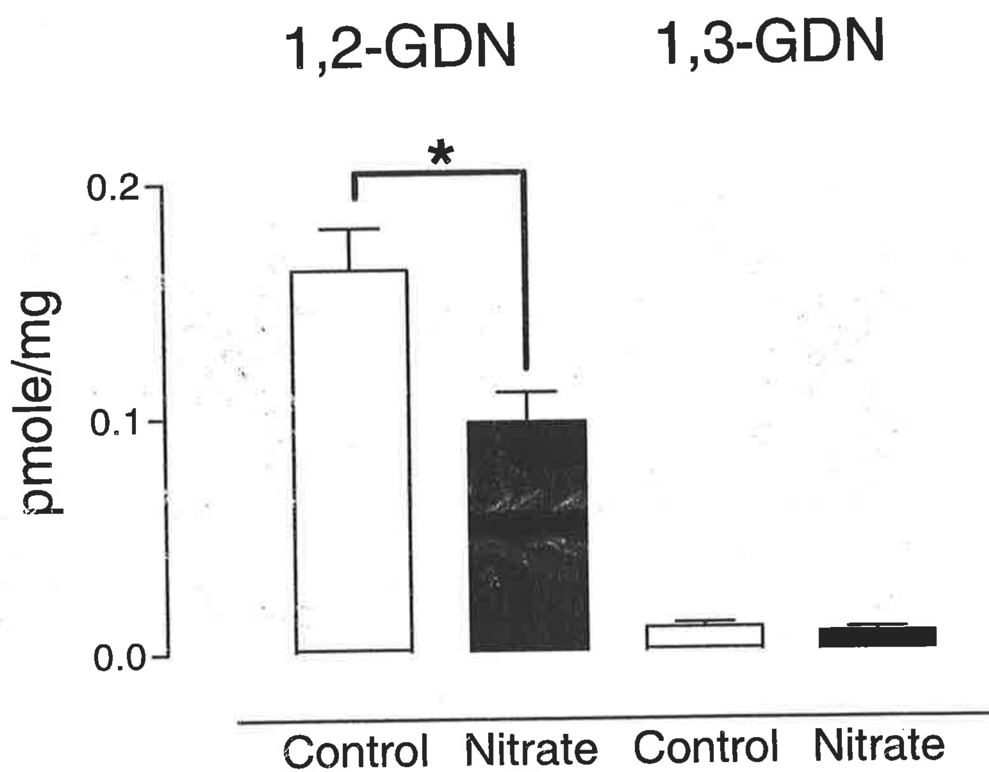


Figure 3.13
SV content of dinitrate metabolites (1,2-GDN and 1,3-GDN) after incubation of segments from control (n=8) and nitrate (n=7) groups with 1 μM GTN.
* $p = 0.012$ for control group vs nitrate group.

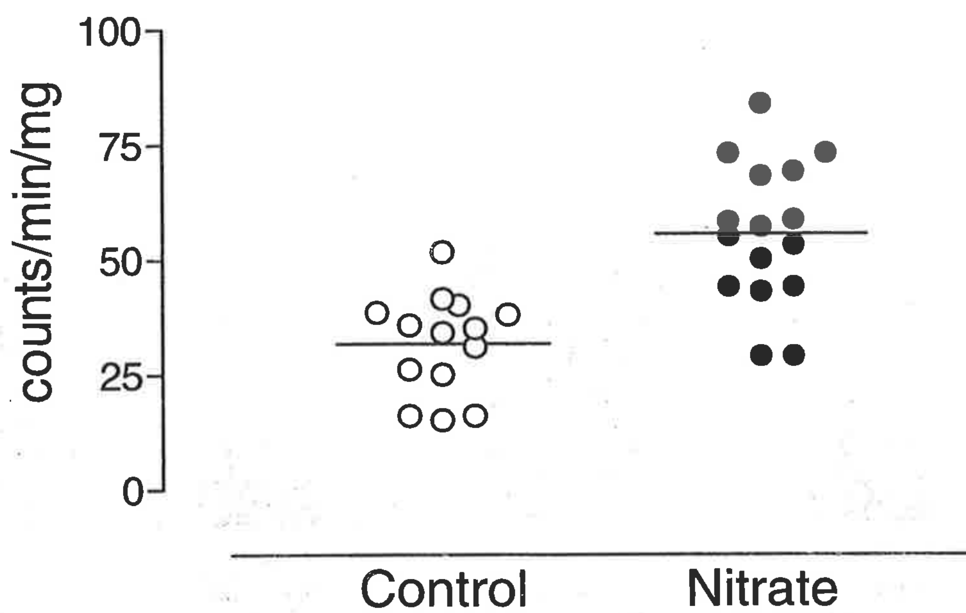


Figure 3.14
Lucigenin-enhanced chemiluminescence for IMA segments from control and nitrate groups. $p < 0.001$ for control vs nitrate group.

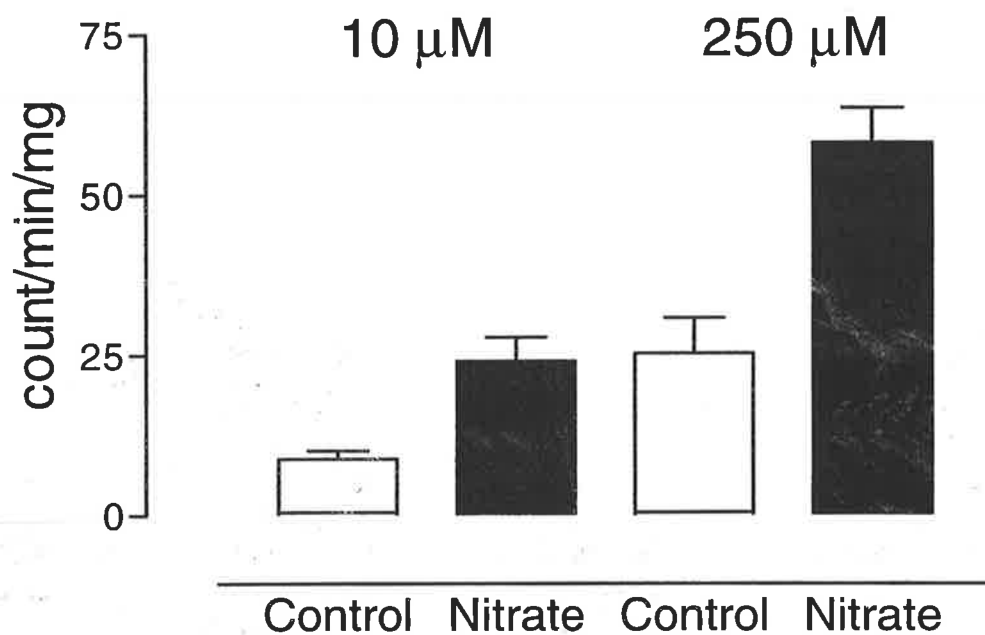


Figure 3.15
Effect of different lucigenin concentrations on lucigenin-enhanced chemiluminescence for IMA segments from control and nitrate groups (both n=5).
$p < 0.001$ for effects of lucigenin concentration and nitrate therapy (two-way ANOVA).
p = NS for interaction between lucigenin concentration and nitrate therapy.

4. Nitrate Tolerance: In Vitro Modulation of GTN Effect and GTN Tolerance

4.1 *Effect of Diethyldithiocarbamate on GTN Vasorelaxant Responses*

4.1.1 Introduction

As discussed in Chapter 1 (section 1.8.3.2), Munzel and co-workers (1995b) recently proposed a novel mechanism underlying the development of nitrate tolerance in vivo. Supporting evidence for this "superoxide hypothesis" was derived from experiments examining the effect of modulating O_2^- generation or O_2^- clearance on nitrate tolerance. Induction of GTN tolerance in New Zealand White rabbits with continuous transdermal GTN for 3 days was associated with a twofold increase in O_2^- generation from tolerant aortae(Munzel, *et al.* 1995b). Pretreatment with liposome-entrapped superoxide dismutase (SOD) in vitro effectively normalized both O_2^- generation and responses to GTN. It was proposed that tolerance was due to inactivation of nitrate-derived NO by increased vascular O_2^-. Further studies by the same group provided support for these initial findings. Pretreatment of aortae from tolerant rabbits with a O_2^- scavenger, tiron also normalized O_2^- generation(Munzel, *et al.* 1995b) and restored the vasorelaxant effect of GTN(Munzel, *et al.* 1999), whereas pretreatment of control vessels with an inhibitor of SOD, diethyldithiocarbamate (DETCA), increased O_2^- generation and attenuated the vasorelaxant responses to GTN to a similar degree to that observed in in vivo tolerance(Munzel, *et al.* 1999). Co-administration of hydralazine(Munzel, *et al.* 1996d) or an angiotensin II receptor antagonist, losartan(Kurz, *et al.* 1999) during GTN exposure also normalized O_2^- generation and prevented GTN tolerance.

Several other studies have provided support for this hypothesis. Other workers also demonstrated that in vivo GTN tolerance induction in canine(Fink, *et al.* 1999) and rabbit(Dikalov, *et al.* 1999) models was associated with increased oxidant stress, and that co-administration of the anti-oxidant vitamin C prevented tolerance. Beneficial effects on tolerance have also been seen with co-administration of hydralazine(Bauer, *et al.* 1991a; Unger, *et al.* 1993) in rat models. However, Laight et al(1998) also in a rat

model, found that pretreatment with a variety of anti-oxidants had no effect on nitrate tolerance. Similarly Ratz et al(2000) were unable to demonstrate reversal of tolerance in rat aortae by tiron.

Studies of the effect of co-administration of anti-oxidants on tolerance induction in humans have also produced conflicting results. While several studies have reported a beneficial effect of a variety of agents with anti-oxidant effects, including hydralazine(Gogia, *et al.* 1995), vitamin C(Bassenge, *et al.* 1998; Watanabe, *et al.* 1998b; Watanabe, *et al.* 1998d), vitamin E(Watanabe, *et al.* 1997) and carvedilol(Watanabe, *et al.* 1998a; Watanabe, *et al.* 1998c), others have been unable to demonstrate an effect on tolerance(Milone, *et al.* 1999a; Milone, *et al.* 1999b; Parker, *et al.* 1997).

Lastly, several other workers have also studied the effect of modulating vascular O_2^- generation or O_2^- clearance on the vasorelaxant responses to GTN in isolated vessels. Again, the results have been conflicting. Studies(Cherry, *et al.* 1990; Omar, *et al.* 1991) examining the effect of DETCA have found an inhibitory effect on GTN relaxation. However at the concentrations used in these studies, DETCA also appears to have inhibitory effects on contractile function(Mugge, *et al.* 1991; Omar, *et al.* 1991), suggesting that its cellular effects may not be limited to inhibition of SOD. In addition, DETCA has also been demonstrated to scavenge NO directly at high concentrations(Vedernikov, *et al.* 1992).

In contrast, other studies examining the effects of alternative sources of O_2^-, such as pyrogallol(Abrahamson, *et al.* 1992; Ignarro, *et al.* 1987b) or xanthine/xanthine oxidase(Hussain, *et al.* 1996) have found that GTN vasorelaxant responses are relatively unaffected by incremental O_2^-.

The effect of elevated O_2^- levels on GTN relaxant responses has not been studied in human vessels. To date, the effect of elevated O_2^- levels on GTN bioconversion in the vascular smooth muscle has also not been studied. As we have previously demonstrated that 24 hours treatment with intravenous GTN is associated with increased O_2^- generation in segments of human IMA (Chapter 3), the aim of these

experiments was to determine the effect of incremental O_2^- levels on (1) vasorelaxant responses to GTN and (2) GTN bioconversion in human vessels.

4.1.2 Experimental Protocol

Experiments were conducted using segments of IMA and SV obtained from patients undergoing CABG as outlined in Chapter 2. Patients receiving prophylactic nitrate therapy were excluded from these experiments.

4.1.2.1 Effect of DETCA on Vasoconstrictor Responses

Reports of impairment of both contractile and relaxant responses in vessels following incubation with DETCA 10 mM for 30 minutes(Cherry, *et al.* 1990; Omar, *et al.* 1991) prompted studies examining the effect of DETCA on the contractile responses of segments of IMA to NA. Following mounting and equilibration in organ baths as described in Chapter 2, IMA segments were allocated to incubation with either 1 mM DETCA, 10 mM DETCA or Krebs alone for 30 minutes, followed by a 30 minute washout period with Krebs solution. During the washout period, solutions were changed a minimum of four times in all experiments. Cumulative concentration responses curves to NA were then obtained as described in Chapter 2.

4.1.2.2 Effect of DETCA on IMA O_2^- Generation and Relaxant Responses to GTN

IMA segments from nitrate-free patients (n=6) were allocated to study of either O_2^- generation (using lucigenin 250 µM) or GTN reactivity in an identical manner to that described in Chapter 2 (and performed simultaneously).

Prior to measurement of O_2^- generation or determination of the relaxant response to GTN, some segments from each patient were exposed to 1 mM DETCA for 30 minutes followed by 30 minutes washout with Krebs solution, as above. Other segments were incubated with DETCA vehicle only.

4.1.2.3 Effect of DETCA on GTN Bioconversion

SV segments from a small number of patients (n=3) were used to assess GTN bioconversion as described in Chapter 2. Beforehand, segments from each patient were allocated to 30 minutes exposure to either 1 mM DETCA or DETCA vehicle followed by 30 minutes washout with Krebs solution, as described above.

4.1.2.4 Data Analysis

Data are expressed as mean ± SEM. Vascular relaxant responses are compared using the parameters log EC_{50} and E_{max} via paired t-test. Lucigenin-enhanced chemiluminescence is expressed as counts/min/mg and compared using paired t-test. In the GTN bioconversion experiments, GTN and dinitrate content are expressed in pmole/mg and compared using paired t-test.

4.1.3 Results

4.1.3.1 Vasoconstrictor Responses

The IMA contractile responses to NA are illustrated in Figure 4.1. Incubation with 10 mM DETCA for 30 minutes caused a significant reduction in the maximum contractile response to NA (Table 4.1; $p<0.05$). In contrast, NA responses following incubation with 1 mM DETCA were similar to control segments (Table 4.1).

4.1.3.2 IMA O_2^- Generation and Relaxant Responses to GTN

Incubation of IMA segments with 1 mM DETCA resulted in an approximately three-fold increase in lucigenin chemiluminescence counts over control segments ($p<0.01$; Figure 4.2). However, relaxant responses of IMA segments to GTN were unchanged (Figure 4.3; Table 4.1).

4.1.3.3 GTN Bioconversion

SV content of 1,2 GDN and 1,3 GDN are shown in Figure 4.4. Tissue content of GTN and its metabolites were similar following incubation with DETCA or with vehicle.

4.1.4 Discussion

This study is the first to examine the effect of increased O_2^- levels on GTN relaxant responses and bioconversion of GTN in human vessels. The results can be summarized as follow. (1) Incubation with 10 mM DETCA impairs contractile responses to NA in IMA, whereas 1 mM DETCA does not. (2) Incubation with 1 mM DETCA induces a significant (3-fold) increase in vascular O_2^- levels in segments of human IMA with (3) no effect on relaxant responses of segments of IMA to GTN. (4) Incubation of segments of SV with 1 mM DETCA does not affect bioconversion of GTN to 1,2-GDN.

The finding of impaired contractile response following incubation with the higher concentration of DETCA (10 mM) has been demonstrated in previous studies in bovine arteries(Cherry, *et al.* 1990; Mugge, *et al.* 1991). It remains uncertain whether this is related to an effect on the cellular contractile apparatus or represents a more non-specific process, perhaps due to direct cellular toxicity(Kelner, *et al.* 1989). However, until this mechanism is elucidated, studies examining the effects of this higher concentration of DETCA on relaxant responses should probably be interpreted with caution.

In contrast to previous studies in bovine arteries(Omar, *et al.* 1991) and rabbit aortae(Munzel, *et al.* 1999), we found no effect of DETCA on responses of human IMA to GTN, despite increasing vascular O_2^- levels 3-fold. This may have been due to the lower DETCA incubating concentration, or conceivably due to inter-species differences. Nonetheless, the results are consistent with several previous studies(Abrahamson, *et al.* 1992; Hussain, *et al.* 1996; Ignarro, *et al.* 1987b) suggesting

that NO derived from GTN is relatively impervious to inactivation by incremental O_2^- levels. The results also demonstrate that any direct scavenging of NO by DETCA(Vedernikov, *et al.* 1992) at the concentration used in this study is minimal; any such effect would have been surprising given the period of washout prior to GTN exposure.

These findings have important implications with regard to the mechanism(s) of nitrate tolerance. The recently proposed "superoxide hypothesis"(Munzel, *et al.* 1996c) suggests that tolerance may be due to increased vascular O_2^- generation, resulting in increased inactivation of nitrate-derived NO. In Chapter 3, we have demonstrated that tolerance to GTN in human vessels is associated with increased vascular O_2^- generation. However, the results of the current experiments with DETCA (where an even greater elevation of O_2^- levels was induced) suggest that increased NO clearance by O_2^- has a minimal contribution to nitrate tolerance. The results are also consistent with the lack of cross-tolerance to other sources of NO found in the previous Chapter.

In addition, the finding that 1,2-GDN contents were similar in control and DETCA treated vessels, although based on small numbers, suggests that bioconversion of GTN in human vessels is not sensitive to acute increases in redox stress. This finding also has implications with regard to nitrate resistance, as discussed further in Chapter 7.

The results of this study do not completely exclude a role for the observed increase in O_2^- levels in tolerance induction. It is conceivable that a more prolonged elevation of O_2^- levels (>24 hours) in vivo might affect NO clearance or impair the NO-vasorelaxation pathway at some point. Similarly, it is possible that changes in responsiveness to nitrates during prolong elevation of O_2^- generation may be mediated via effects on peroxynitrite ($ONOO^-$). No convenient method exists for measurement of $ONOO^-$ in vivo, and the current study did not attempt surrogate assays for $ONOO^-$ such as nitrotyrosine assay. In addition, it remains possible that bioconversion of GTN might be affected by exposure to excess O_2^- or $ONOO^-$ for a period greater than 30 minutes.

In summary however, the study demonstrates that acute elevation of O_2^- levels has minimal effect on GTN-induced vasodilation and GTN bioconversion in human vessels.

Table 4.1

Effect of DETCA on responses of IMA to noradrenaline and GTN.

	Log EC_{50} (M)	E_{max}
NA (n=4)		
Control	-6.2 ± 0.2	2.2 ± 0.1
DETCA 1 mM	-6.3 ± 0.2	2.2 ± 0.3
DETCA 10 mM	-5.9 ± 0.3	0.7 ± 0.2 *
GTN (n=5)		
Control	-7.9 ± 0.1	97 ± 1
DETCA 1 mM	-7.8 ± 0.1	97 ± 1

Values are mean ± SEM. E_{max} expressed as g for NA and % for GTN.
* $p<0.05$ for control vs DETCA 10 mM segments

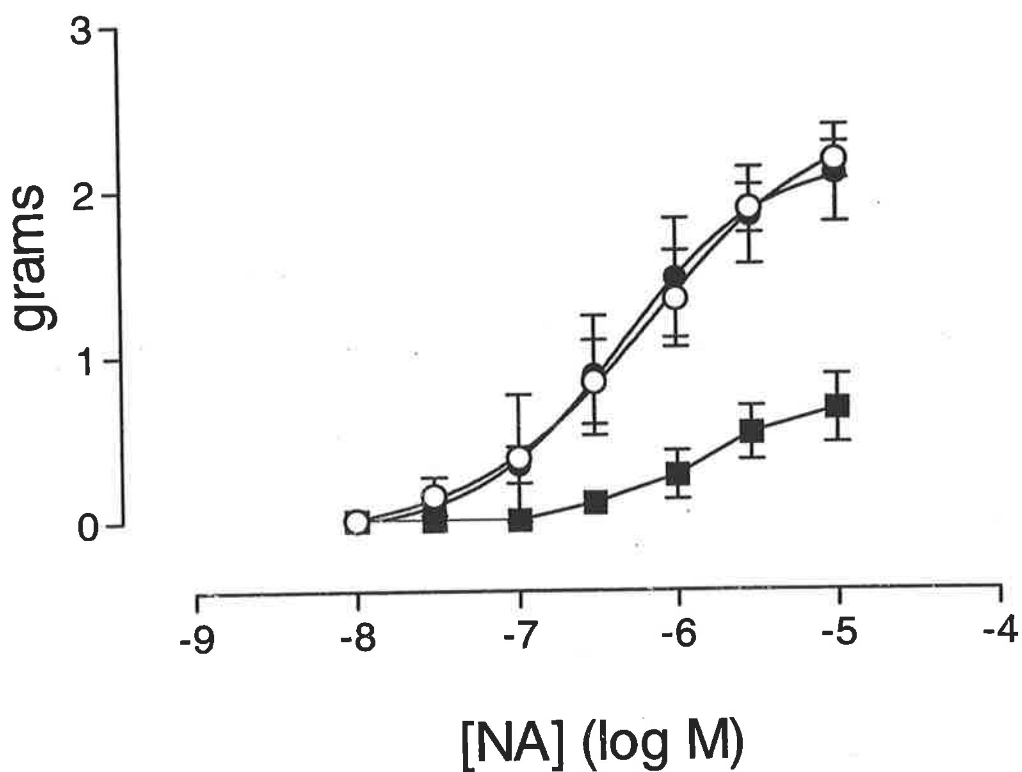


Figure 4.1
Effect of DETCA on responses of IMA (n=4 each group) to noradrenaline. Open circles indicate control segments, closed circles indicate segments exposed to DETCA (1 mM) and closed squares indicate segments exposed to DETCA (10 mM).

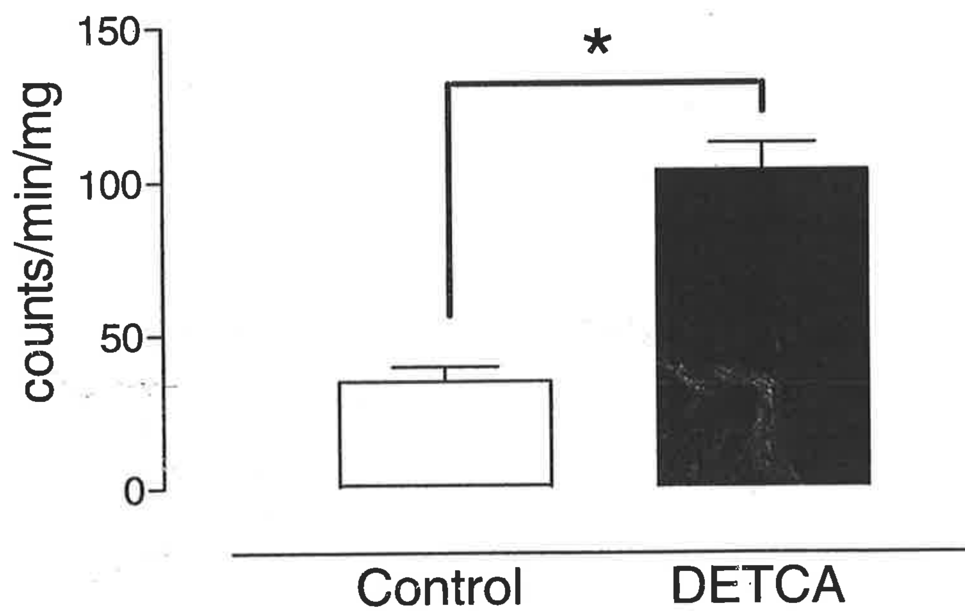


Figure 4.2
Effect of DETCA (1 mM) on lucigenin-enhanced chemiluminescence in IMA segments from nitrate-free patients (n=5).
* $p<0.01$ for control vs DETCA-exposed segments.

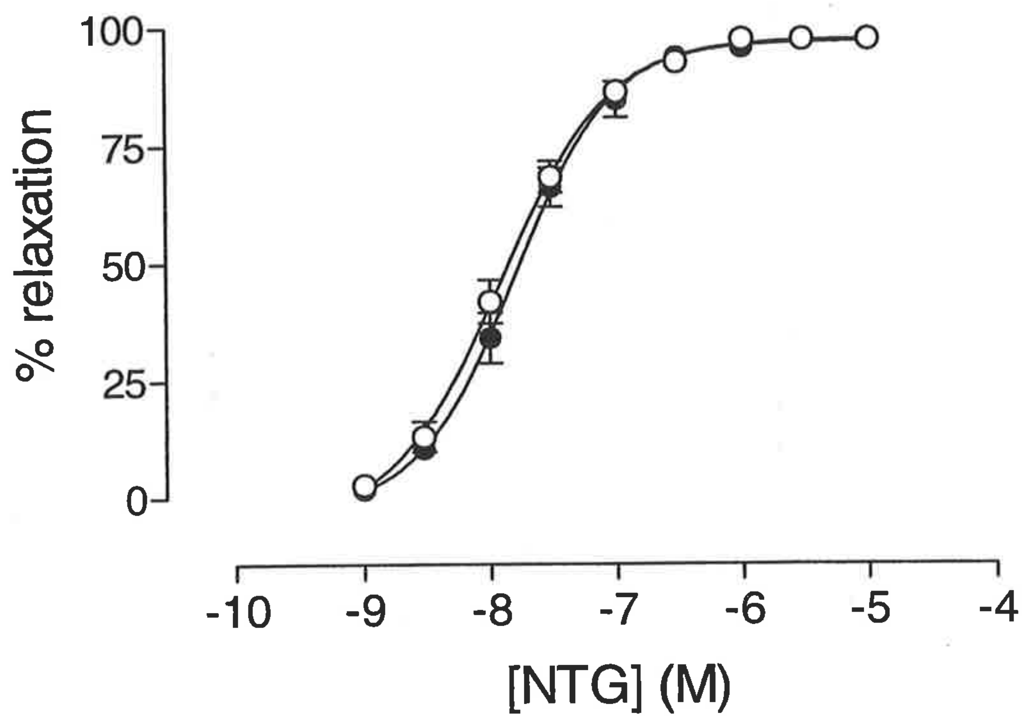


Figure 4.3
Effect of DETCA (1 mM) on GTN responses of IMA segments from nitrate-free patients (n=5). Open circles indicate control segments, closed circles indicate segments exposed to DETCA (1 mM).

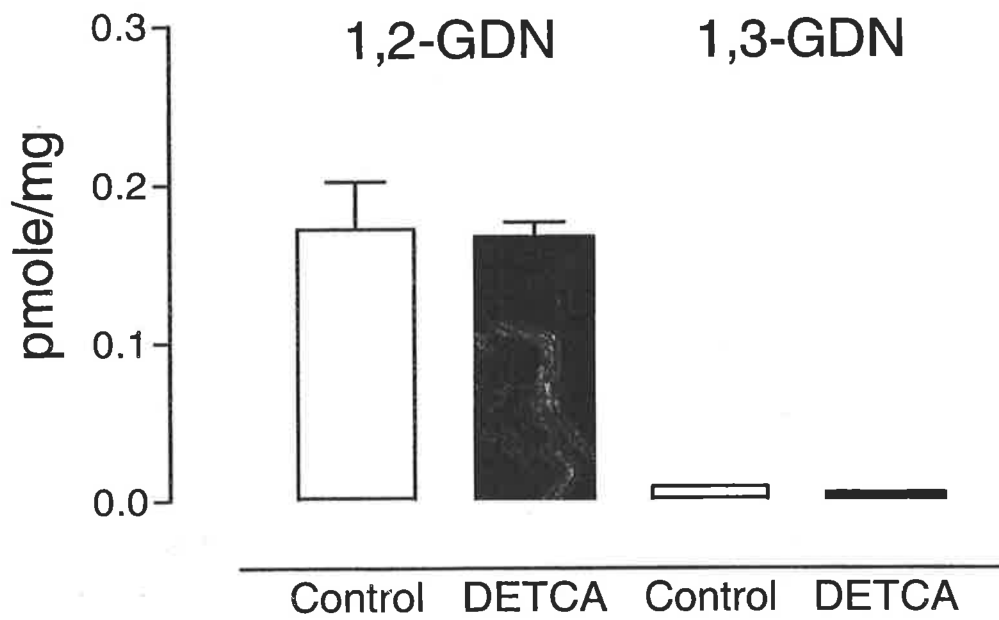


Figure 4.4
Effect of incubation with DETCA (1 mM: 30 minutes) on SV content of dinitrate metabolites (n=3 each group).

4.2 Effect of Diphenyleneiodonium Sulfate in GTN Vasorelaxant Responses

4.2.1 Introduction

The aim of the following experiments is to examine the effect of diphenyleneiodonium (DPI) on responses of human vessels to GTN. DPI is an inhibitor of a number of flavoprotein-containing vascular enzymes, including the cytochrome P450-NADPH/cytochrome P450 reductase system(McGuire, *et al.* 1998), NAD(P)H oxidase(Griendling, *et al.* 1994), and endothelial NO synthase(Stuehr, *et al.* 1991). Of particular relevance to the issue of nitrate tolerance are the effects of DPI on cytochrome P450 reductase and NAD(P)H oxidase because altered activity of these enzymes may be involved in changes in nitrate bioconversion or in O_2^- generation, respectively. However, previous studies in animal models have produced conflicting results.

Studies by McGuire et al in isolated rat aortae(1994; 1998) have shown that DPI (0.3 μM) causes impairment of GTN-induced relaxation, which correlates with decreased bioconversion of GTN to 1,2-GDN and decreased GTN-induced cGMP accumulation. DPI also inhibited the haemodynamic response to GTN in an in vivo rat model(McGuire, *et al.* 1998), suggesting that the target enzyme of DPI was involved in "mechanism-based" bioconversion of GTN. These findings raised the possibility that DPI and nitrate tolerance might affect a common target enzyme. However, a more recent study by the same workers(Ratz, *et al.* 2000) found that DPI inhibited GTN-induced relaxation to a similar degree in aortae from both tolerant and non-tolerant rats, suggesting that DPI does not target the process responsible for the development of GTN tolerance.

De la Lande et al(1996a) demonstrated a similar inhibitory effect of DPI (0.3 μM) on GTN-induced relaxation in rat aortae, but in contrast, found a mild potentiating effect in isolated bovine coronary arteries. In the initial paper proposing the "superoxide hypothesis" of nitrate tolerance(Munzel, *et al.* 1995b), Munzel et al found that DPI normalized O_2^- generation in aortae from tolerant rabbits, suggesting the source of the

incremental O_2^- generation was NAD(P)H oxidase. Since normalization of O_2^- generation by liposome-entrapped SOD was associated with reversal of nitrate tolerance in the same model, the possibility that DPI might also increase sensitivity to GTN and/or reverse tolerance existed, thus providing a possible explanation for the observations of De la Lande et al(1996a) in bovine coronary artery.

Overall therefore, the experimental data to date are conflicting and suggest that DPI can exert differing (and opposite) effects on GTN vasorelaxation in different species. The study by Ratz et al(Ratz, *et al.* 2000) suggests DPI does not share a common target with nitrate tolerance, at least in the rat, however work by Munzel suggests otherwise(Munzel, *et al.* 1996d; Munzel, *et al.* 1995b). To date there have been no studies examining the effect of DPI on GTN-induced relaxation in human vessels.

4.2.2 Experimental Protocol

Experiments were conducted using IMA and SV segments obtained from patients undergoing CABG as outlined in Chapter 2. Patients taking prophylactic nitrate therapy were not excluded; analysis of the responses to GTN was stratified according to prior nitrate therapy. Segments were mounted in organ baths and following equilibration contractile responses to KCl solution and NA were obtained as described in detail in Chapter 2.

4.2.2.1 Effect of DPI on GTN Relaxant Responses

Segments of IMA and SV from each patient were allocated to incubation with either DPI (1.0 μM) or DPI vehicle for 30 minutes before eliciting a cumulative concentration response curve to GTN as described in Chapter 2. DPI was not washed out before assessing GTN responses.

4.2.2.2 Data Analysis

Data are expressed as mean ± SEM. The vascular relaxant responses are compared using the parameters log EC_{50} and E_{max} via paired t-test. To exclude the possibility that an effect of DPI might be attenuated in tolerant vessels, a further analysis of the data excluding patients exposed to nitrates was performed.

4.2.3 Results

4.2.3.1 GTN Relaxant Responses

GTN responses were studied in segments of IMA and SV obtained from 9 patients and 7 patients respectively. Of these, 3 patients in each group were taking prophylactic nitrates prior to operation.

Relaxant responses of IMA and SV segments to GTN are illustrated in Figures 4.5 and 4.6. Incubation of segments of both vessels with DPI resulted in a small inhibitory effect on sensitivity to GTN, as assessed by log EC_{50}, which was not statistically significant (Table 4.2).

When the responses of IMA segments from nitrate-free patients (n=6) to GTN were considered, the inhibitory effect of DPI remained non-significant ($p=0.12$; Figure 4.7), with regard to log EC_{50}. However, the relaxant response to the lower concentrations of GTN were significantly impaired (Figure 4.7). Responses of segments from patients on nitrates were unaffected by DPI, though this observation is based on small numbers (n=3).

4.2.4 Discussion

The major findings of these experiments are that the flavoprotein inhibitor DPI has a small (non-significant) inhibitory effect on the relaxant response to GTN in isolated human IMA and SV.

Previous studies(De la Lande, *et al.* 1996a; McGuire, *et al.* 1994; Ratz, *et al.* 2000) have demonstrated a marked inhibitory effect (approximately 10-15 fold) of DPI on GTN-induced relaxation in rat aortae. Further study by McGuire et al in intact rats(1998), found that pretreatment with DPI significantly inhibited both the haemodynamic effect of GTN and the appearance of 1,2-GDN in the plasma, suggesting that formation of 1,2-GDN was related to "mechanism-based" bioconversion of GTN and that the bioconverting enzyme was sensitive to DPI. Since DPI inhibited the vascular NADPH oxidase-cytochrome P450 reductase(McGuire, *et al.* 1998), it was proposed that this enzyme system was responsible for "mechanism-based" bioconversion of GTN. A more recent study(Ratz, *et al.* 2000) demonstrated that this impairment of this enzyme system was not responsible for nitrate tolerance in intact rats.

Paradoxically, a vascular NAD(P)H oxidase, which is also inhibited by DPI, has been proposed to generate increased O_2^- in association with nitrate tolerance in vivo(Munzel, *et al.* 1996d; Munzel, *et al.* 1995b), resulting in increased inactivation of nitrate-derived NO. Hence, DPI might be expected to exert a potentiating effect on GTN via this mechanism. Indeed, this was found to be the case in isolated bovine coronary artery(De la Lande, *et al.* 1996a). This observation raised the possibility that inter-species differences exist with regard to the enzyme system responsible for "mechanism-based" bioconversion of GTN and the effect of DPI.

The current study raises the possibility of a minor component of GTN action in human vessels that can be inhibited by DPI. However, the shift to the right in GTN sensitivity was small (indeed only half that seen following the induction of tolerance in Chapter 3) and non-significant, indicating a large amount of "mechanism-based" bioconversion was independent of mechanisms affected by DPI. Therefore, the current experiments provide evidence against an important role of the NADPH oxidase-cytochrome P450 reductase system in the bioconversion of GTN to NO in human vessels..

The study is limited by the fact that we did not measure O_2^- generation or GTN bioconversion. It is conceivable that the lack of significant effect of DPI might be due

to a combination of effects on different enzyme systems, resulting in both reduced O_2^- generation and reduced GTN bioconversion. Formal studies in tolerant vessels would also have helped clarify this issue. Sensitivity to GTN was unaffected by DPI in the patients on chronic nitrate therapy, but the number of patients studied was small. Previous studies(Ratz, *et al.* 2000) in rats have argued against a role for DPI sensitive enzymes in nitrate tolerance.

In addition, we not did examine the effect of varying the concentration of DPI; we cannot exclude the possibility that we may have seen a more pronounced effect with a higher concentration. Indeed, the animal studies demonstrating inhibition of NAD(P)H-derived O_2^- generation used concentrations of 100 μM(Munzel, *et al.* 1995b). Nonetheless, the concentration utilized was supramaximal for GTN-induced relaxation in rat aorta(McGuire, *et al.* 1994). Furthermore, a 10-fold higher concentration failed to affect GTN responses in bovine coronary artery(De la Lande, *et al.* 1996a).

Table 4.2

Effect of DPI on responses of IMA and SV segments to GTN.

	No of Subjects	Log EC_{50} (M)	E_{max} (%)
IMA			
Control	9	-7.7 ± 0.1	95 ± 2
DPI 1μM	9	-7.4 ± 0.1	98 ± 1
SV			
Control	7	-6.9 ± 0.1	95 ± 1
DPI μM	7	-6.7 ± 0.1	95 ± 2

Values are mean ± SEM. $p>0.05$ for DPI-exposed segments vs control segments.

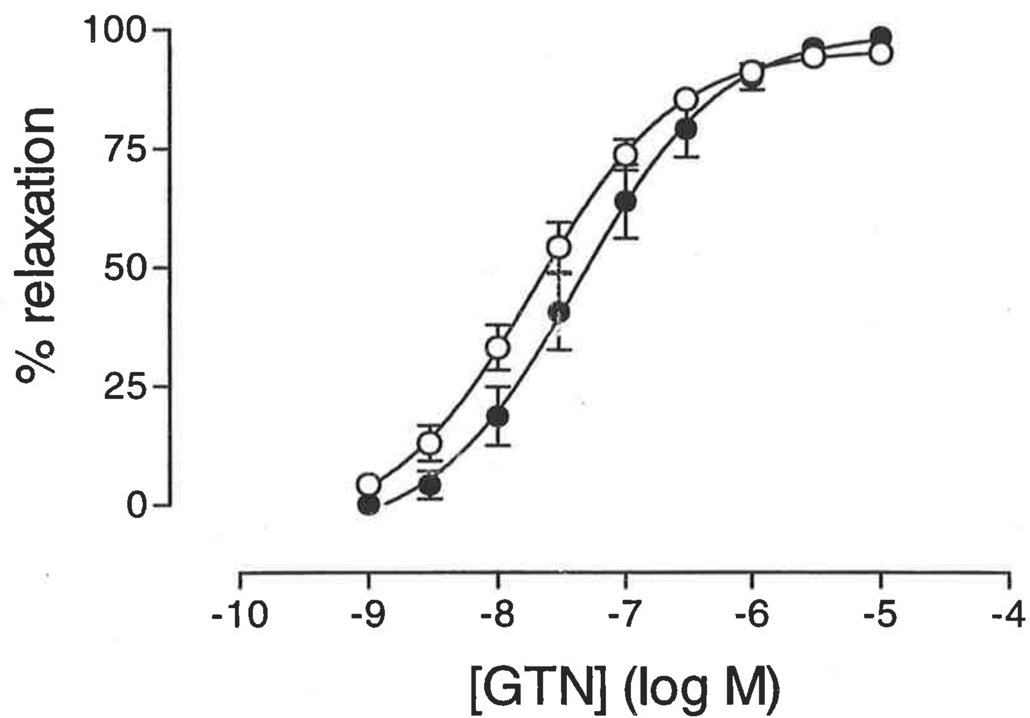


Figure 4.5
Effect of DPI on responses of IMA (n=9) to GTN. Open circles indicate control segments, closed circles indicate segments exposed to DPI (1 μM).

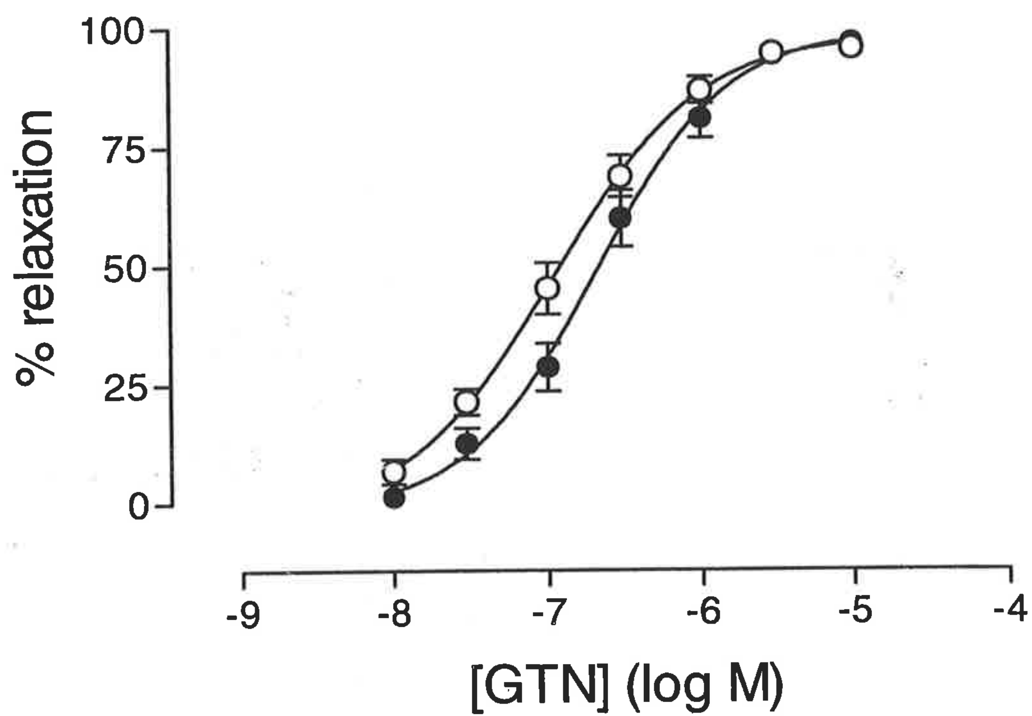


Figure 4.6
Effect of DPI on responses of SV segments (n=7) to GTN. Open circles indicate control segments, closed circles indicate segments exposed to DPI (1 μM).

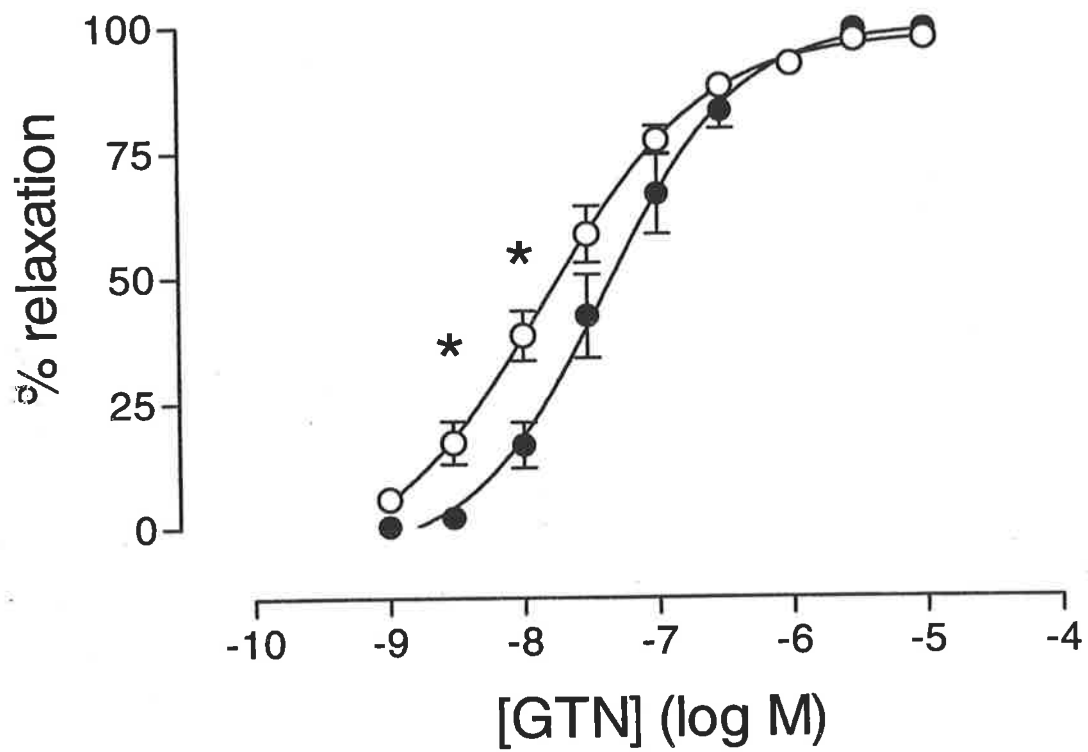


Figure 4.7
Effect of DPI on responses of IMA segments from nitrate-free patients (n=6) to GTN. Open circles indicate control segments, closed circles indicate segments exposed to DPI (1 μM). * $p<0.05$.

4.3 Effect of removal of the endothelium on GTN relaxant responses in nontolerant and tolerant human vessels

4.3.1 Introduction

The objectives of the following experiments are to (1) determine the effect of removal of the endothelium on GTN relaxant responses in human vessels and (2) examine the role of the endothelium in the development of nitrate tolerance in human vessels.

There is evidence that vascular responsiveness to organic nitrates is affected by the extent of release of endogenous NO from the endothelium. Several studies have demonstrated that the sensitivity of isolated vessels to GTN is increased when the endothelium is denuded, or in the presence of inhibition of NO synthase(Alheid, *et al.* 1987; Luscher, *et al.* 1989; Moncada, *et al.* 1991; Pohl, *et al.* 1987; Shirasaki, *et al.* 1985). These observations may have important implications regarding enhanced efficacy of exogenous NO donors in the presence of endothelial dysfunction. They may also provide support for the concept of end-product inhibition of GTN bioconversion, which was recently put forward as a possible explanation for the differing sensitivities of arteries and veins to GTN(Kojda, *et al.* 1998b), although the increase in sensitivity appears to also affect non-nitrate NO donors.

However, these studies have limitations which warrant discussion. Firstly, in vitro removal of the endothelium may not be an accurate surrogate for the complex pathophysiological processes accompanying endothelial dysfunction in vivo. Indeed a recent study failed to observe an increase in sensitivity to GTN in the presence of endothelial dysfunction in humans(Anderson, *et al.* 1996). Secondly, the observations of a supersensitivity to GTN in the presence of endothelial removal or inhibition of NO synthase(Alheid, *et al.* 1987; Moncada, *et al.* 1991) is currently limited to studies in isolated vessels from animals.

Recent studies(Laursen, *et al.* 1996b; Munzel, *et al.* 2000a; Munzel, *et al.* 1995b) have also implicated the endothelium in the development of nitrate tolerance. Using a model of in vivo tolerance induction in rabbits, Munzel et al(1995b) found that

removal of the endothelium markedly improved the relaxant response of aortae from tolerant animals to GTN and also attenuated the observed increase in O_2^- generation induced by GTN. A similar effect of endothelium denudation on in vivo nitrate tolerance has been reported in an in rat model(Laursen, *et al.* 1996b). In a more recent study(Munzel, *et al.* 2000a), Munzel's group demonstrated that treatment with GTN was associated with enhanced expression of a dysfunctional form of endothelial NO synthase that formed O_2^- rather than NO. It was suggested that the dysfunctional NO sythase lead to incremental O_2^- production, which contributed to reduced nitrate-derived NO bioavailability and hence played a role in nitrate tolerance.

However, data exist suggesting tolerance induction is largely independent of the endothelium. Specifically(Berkenboom, *et al.* 1999; De la Lande, *et al.* 1999b; Ferdinandy, *et al.* 1995) have found considerable tolerance to GTN despite removal of the endothelium. Furthermore, the degree of tolerance seen in rats exposed to 2 days continuous transdermal GTN is similar in both endothelium-intact and endothelium-denuded vessels(De la Lande, *et al.* 1999b). To date, the effect of endothelium removal on GTN tolerance has not been studies in human vessels.

4.3.2 Experimental Protocol

Subjects were patients undergoing elective CABG as described in Chapter 2. Two patient groups were studied, a nitrate-free group and a nitrate tolerant group; the patients' nitrate therapy was adjusted as described in Chapter 3. IMA segments obtained at operation were mounted in organ baths and contractile responses to KCl solution and NA were obtained as described in detail in Chapter 2. Prior to mounting some segments from each patient were allocated to have the endothelium removed by gentle rubbing with forceps.

4.3.2.1 Effect of Endothelial Removal on Responses to GTN

Cumulative relaxant response curves to GTN were performed simultaneously in endothelium-intact and endothelium-denuded segments from each patient. All segments

were then assessed for the presence of endothelium using A23187; relaxation less than 20% was taken to indicate successful removal of the endothelium.

4.3.2.2 Data Analysis

Data are expressed as mean ± SEM. Vascular relaxant responses are compared using the parameters log EC_{50} and E_{max} via two-way ANOVA.

4.3.3 Results

Segments of IMA were obtained from a total of 10 patients (5 patients in each of the nitrate-free and nitrate-tolerant groups). Contractile responses to NA were similar in endothelium-intact and endothelium-denuded segments of IMA; log EC_{50} and E_{max} values are shown in Table 4.3.

4.3.3.1 GTN Relaxant Responses

Relaxant responses of IMA segments from nitrate-free and tolerant patients are illustrated in Figures 4.8 and 4.9. In endothelium-intact vessels, the log EC_{50} and E_{max} values (Table 4.4) for the two groups of patients were comparable to those obtained in the randomized tolerance induction experiments described previously (see Chapter 3). In segments from both groups of patients, responses to GTN were unchanged by removal of the endothelium (Figures 4.8 and 4.9; Table 4.4).). Two-way ANOVA revealed a significant main effect for nitrate therapy ($F= 20.6$; $p<0.001$) but not for endothelium removal, and no interaction between the two variables was apparent.

4.3.4 Discussion

These experiments are the first to examine (1) whether removal of the endothelium induces supersensitivity to GTN in human vessels and (2) the role of the endothelium in the mechanism of nitrate tolerance in humans. The major findings are (1) removal of the endothelium from segments of IMA from nitrate-free patients does

not alter sensitivity to GTN and (2) the reduction in sensitivity to GTN induced in segments of IMA by 24 hours intravenous GTN at 10 μg/min is unaffected by removal of the endothelium.

The lack of supersensitivity to GTN found in these experiments is in conflict with previous animal experiments(Alheid, *et al.* 1987; Luscher, *et al.* 1989; Moncada, *et al.* 1991; Pohl, *et al.* 1987; Shirasaki, *et al.* 1985). There are several possible explanations for this discrepancy. Firstly, the method used to denude the endothelium may not have removed it completely. Relaxant responses less than 20% to the endothelium-dependent vasodilator A23187 were considered to represent adequate removal. Hence, a small amount of endothelium may have remained intact. Secondly, Moncada(1991) proposed that the increased sensitivity seen in the denuded vessels from (normal) animals was due to removal of basal endogenous NO release. Since the study population consisted only of patients with coronary artery disease, it is conceivable that the basal endogenous NO release was already impaired, despite a relaxant response to A23187 in vitro. Lastly, the discrepancy between the animal and human studies may be due to inter-species differences. Consistent with the latter two possibilities, a recent study found that sensitivity to GTN was unaffected by the presence of endothelial dysfunction in humans(Anderson, *et al.* 1996), although it is important to note that endothelial dysfunction in vivo does not necessarily equate to endothelial removal in vitro.

In the current experiments in patients receiving a 24 hour infusion of intravenous GTN, the endothelium-denuded segments of IMA displayed a similar degree of GTN tolerance compared to segments of IMA with intact endothelium. This was in contrast with a previous experiment in rabbits(Munzel, *et al.* 1995b). In the latter study, a large reduction in the extent of tolerance was observed following removal of the endothelium and this observation formed part of the evidence for an important role of endothelium-derived O_2^- in the induction of nitrate tolerance. However, a number of other studies in rats have demonstrated that tolerance induction is unaffected by removal of the endothelium(Berkenboom, *et al.* 1999; De la Lande, *et al.* 1999b; Ferdinandy, *et al.* 1995). The findings of the current study demonstrate that this is also

the case in human vessels, suggesting that inactivation of GTN-derived NO by incremental O_2^- from the endothelium has a minimal contribution to tolerance.

The results of this study do not completely exclude a role of the endothelium in the development of nitrate tolerance. The GTN regimen used in the current study induced only a moderate degree of tolerance, in comparison to the study by Munzel et al(1995b). We cannot exclude the possibility that induction of a greater degree of tolerance may have unmasked an endothelium-dependent component. However, the aforementioned rat studies(Berkenboom, *et al.* 1999; De la Lande, *et al.* 1999b)employed longer tolerance induction regimens and found similar results to ours. Secondly, although the experimental protocol following induction of tolerance was similar to that of Munzel, it remains possible that a more prolonged absence of a putative endothelium-derived factor might have a beneficial effect on tolerance via mechanisms other than inactivation of NO (eg altered GTN bioconversion).

In summary however, the current study suggests that, in patients with coronary artery disease and stable angina pectoris, the endothelium has minimal effect on GTN sensitivity and the induction of tolerance to GTN.

Table 4.3

Effect of endothelium removal on responses of IMA segments to NA.

	No of Subjects	Log EC_{50} (M)	E_{max} (grams)
E+	10	-6.3 ± 0.1	2.9 ± 0.6
E-	10	-6.4 ± 0.1	2.4 ± 0.4

Values are mean ± SEM.
E+ indicates Endothelium-intact, E- indicates Endothelium-denuded segments.
$p>0.05$ for E+ vs E- comparisons.

Table 4.4

Effect of endothelium removal on relaxant responses of IMA from nitrate-free and tolerant patients to GTN.

	No of Subjects		Log EC_{50} (M)		E_{max} (%)	
	Nitrate-free	Tolerant	Nitrate-free	Tolerant	Nitrate-free	Tolerant
E+	5	5	-7.5 ± 0.1	-7.0 ± 0.1*	99 ± 1	84 ± 4*
E-	5	5	-7.5 ± 0.1	-6.9 ± 0.1*	99 ± 1	80 ± 6*

Values are mean ± SEM.
E+ indicates endothelium-intact, E- indicates endothelium-denuded segments
*$p<0.001$ for effect of nitrate therapy and p=.NS for effect of endothelium removal (two-way ANOVA). There was no interaction between the two variables.

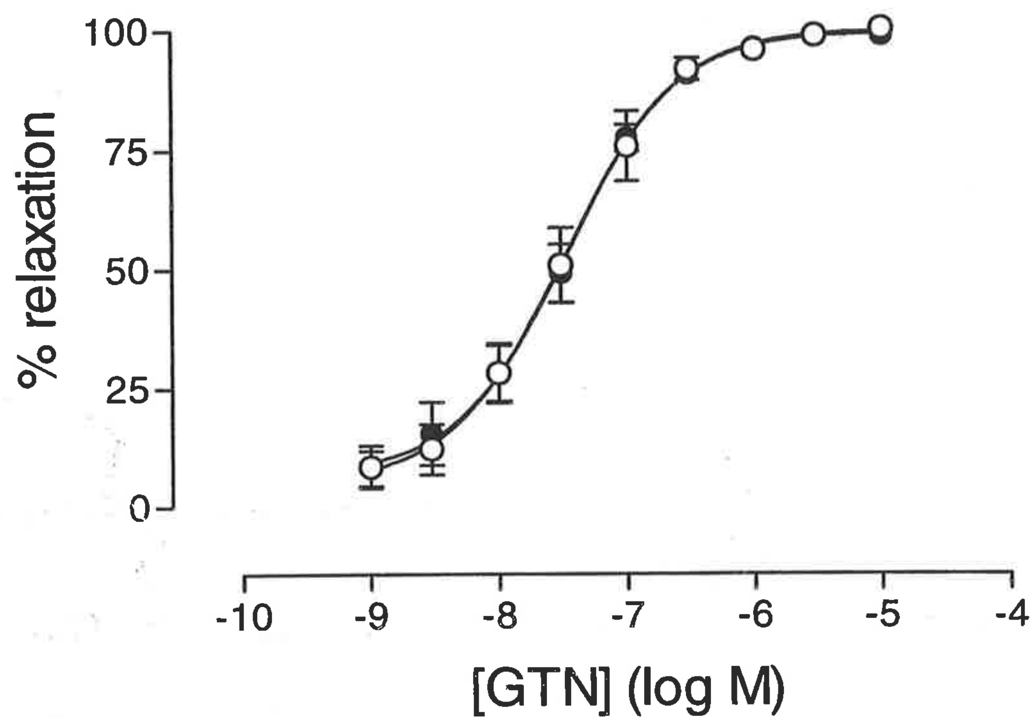


Figure 4.8
Effect of endothelium removal on relaxant responses of IMA from nitrate-free patients (n=5). Open symbols indicate endothelium-intact segments, closed symbols endothelium-denuded segments.

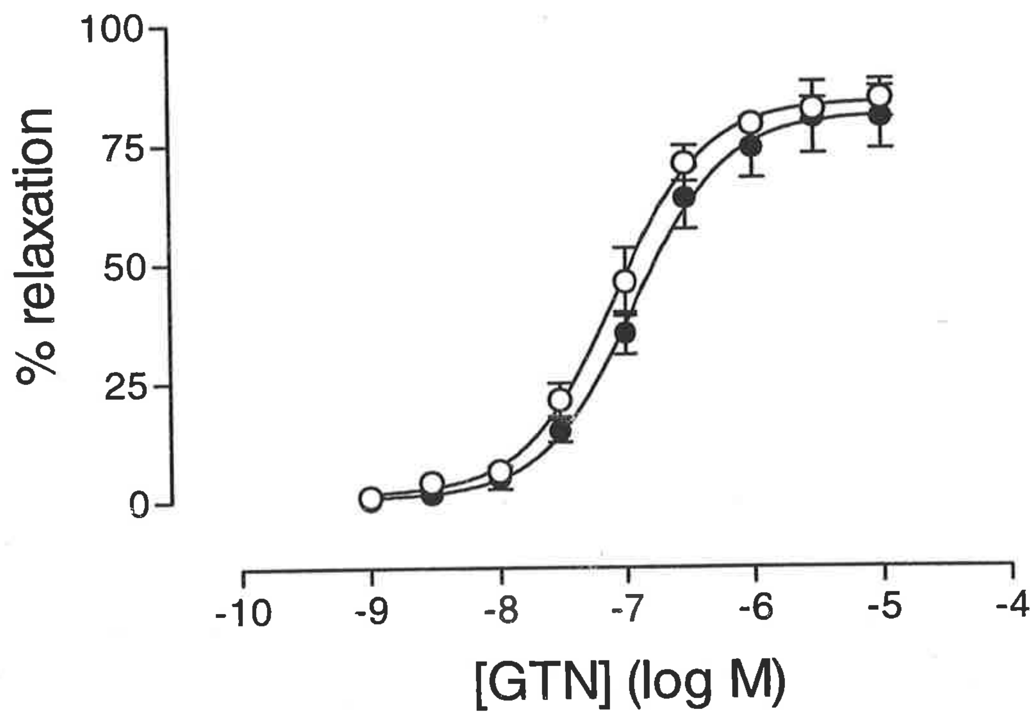


Figure 4.9
Effect of endothelium removal on relaxant responses of IMA from tolerant patients (n=5). Open symbols indicate endothelium-intact segments, closed symbols endothelium-denuded segments.

4.4 Effects of Soluble Guanylate Cyclase Inhibition on Vasodilator Responses to NO Donors in Human Vessels

4.4.1 Introduction

In Chapter 3, the mechanism underlying the development of nitrate tolerance is investigated in isolated human vessels by comparing the effects of GTN with those of two non-nitrate sources of NO. The critical assumption on which interpretation of the results is based is that all three sources of NO act largely via activation of soluble guanylate cyclase. The aim of the following experiments is to test this assumption by examining the effects of an inhibitor of this enzyme (1 *H*-[1,2,4]oxadiazole[4,3-*a*]quinoxalin-1-one; ODQ) in isolated human vessels.

ODQ was recently introduced as a specific inhibitor of soluble guanylate cyclase(Garthwaite, *et al.* 1995) and has since been used to differentiate between the cGMP-dependent and -independent effects of NO and NO donors in isolated animal vessels(Brunner, *et al.* 1996; De la Lande, *et al.* 1999a; Feelisch, *et al.* 1999; Olson, *et al.* 1997). These studies have confirmed that ODQ dose-dependently inhibits cGMP generation in response to NO and NO donors, but raised two important issues.

Firstly, it appears that ODQ may not be completely specific for soluble guanlyate cyclase. Feelisch et al(1999) found that pretreatment of rat aortae with ODQ (3 μM) induced a 2-log-order shift to the right of the concentration response curve for NO and several other NO donors. However, the inhibitory effect on responses to GTN, SNP and acetylcholine was greater (3- to 5-log-order), suggesting that ODQ may interfere with the mechanism of action of these agents independently of soluble guanylate cyclase. Secondly, it has been shown by other investigators that, despite effective prevention of any increase in cGMP by ODQ, higher concentrations of NO or NO donors can still cause complete relaxation of vascular tissue(Brunner, *et al.* 1996; Feelisch, *et al.* 1999; Weisbrod, *et al.* 1998). Hence, mechanisms independent of cGMP production, such as direct activation of K_{ca} channels(Bolotina, *et al.* 1994) may contribute to the vasodilator action of NO/NO donors at high concentrations.

Importantly, these issues appear to be of relevance only at concentrations of NO and NO donors above 10^{-5} M, which are greater than those used in the current studies.

The aim of the following experiments was to determine the effects of ODQ on the relaxant responses to GTN, sodium nitroprusside (SNP) and the calcium ionophore, A23187 in human vessels.

4.4.2 Experimental Protocol

Subjects were patients undergoing elective CABG selected on the criteria described in Chapter 2. The patients' nitrate therapy was held on the day of operation. IMA and SV segments obtained at operation were mounted in organ baths and contractile responses to KCl solution and NA were obtained.

4.4.2.1 Effect of ODQ on Relaxant Responses

Cumulative concentration response (CR) curves to one of the three vasodilators, GTN, SNP or A23187 were performed as described in Chapter 2. Beforehand, some segments of IMA and SV from each patient were allocated to incubation with ODQ (10 μM) for 30 minutes. This concentration of ODQ was chosen as it has previously been shown to completely abolish the vasodilating effects of these agents up to a concentration of 10^{-5} M in rat aorta(Feelisch, *et al.* 1999). Responses of SV to A23187 were not assessed since they had been found to be poor in previous experiments (Chapter 3).

4.4.2.2 Data Analysis

The data are expressed as mean ± SEM. The effect of ODQ on vascular relaxant responses is assessed using the parameters log EC_{50} and E_{max} via paired t-test.

4.4.3 Results

In both IMA and SV segments, relaxations to GTN, SNP and A23187 were characterized by sigmoid CR curves similar to those seen in Chapter 3.(Figure 4.10 to 4.14).

4.4.3.1 Effect of ODQ on Relaxant Responses

In both IMA and SV segments, ODQ markedly inhibited the relaxant response to GTN (Figures 4.10 and 4.13). This was manifest largely as a highly significant reduction in E_{max}, from 97% to 13% and 96% to 16% in IMA and SV segments, respectively (Table 4.5; both $p<0.001$), with apparent loss of the characteristic sigmoid CR curve.

ODQ exerted a similar inhibitory effect on relaxant responses of IMA and SV segments to SNP (Figure 4.11 and 4.14). In IMA segments the E_{max} was reduced from 97% to 12% and in SV segments, from 98% to 19% (Table 4.5; both $p<0.001$)).

ODQ also inhibited the response of IMA segments to A23187 (Figure 4.12), although to a lesser degree (reduction of E_{max} from 74% to 35%; $p<0.01$).

4.4.4 Discussion

The major findings of these experiments are that ODQ strongly inhibits the relaxant responses of GTN and SNP in human vessels, while only partly inhibiting those of A23187. The results are largely consistent with those of animal studies on isolated vessels(Brunner, *et al.* 1996; De la Lande, *et al.* 1999a; Feelisch, *et al.* 1999).

Previous studies have demonstrated that ODQ dose-dependently inhibits the vasorelaxation induced by NO and NO donors by activation of soluble guanylate cyclase(Brunner, *et al.* 1996; De la Lande, *et al.* 1999a; Feelisch, *et al.* 1999). Concentrations of 3 µM or greater of ODQ virtually abolish the vasorelaxant and cGMP-accumulating effects of concentrations of 10 µM GTN and SNP in bovine pulmonary artery(Brunner, *et al.* 1996) and rat aorta(Feelisch, *et al.* 1999). The current study demonstrates that a slightly higher concentration of ODQ (10 µM) has a similar

effect on the relaxant responses to GTN and SNP in human vessels. This finding suggests that GTN and SNP work largely (or entirely) via activation of soluble guanylate cyclase; thus the conclusions regarding the lack of cross-tolerance to SNP in tolerant vessels seen in Chapter 3 hold true.

We did not study the effect of concentrations of GTN of SNP greater than 10 μM, since this was the maximum concentration used in the experiments in this thesis. Hence, we cannot determine whether higher concentrations of NO donors can cause vasorelaxation of human vessels independently of activation of soluble guanylate cyclase(Feelisch, *et al.* 1999; Weisbrod, *et al.* 1998).

A further limitation of the study is that the effect of ODQ on authentic NO was not examined. During the course of the study, Feelisch et al(1999) suggested that ODQ (at high concentration) may not be specific for soluble guanylate cyclase, but may also interfere with the release of NO from GTN and SNP, based on evidence of greater inhibition of GTN and SNP than of authentic NO. Thus, we cannot exclude the possibility that a component of the inhibitory effect of ODQ on GTN and SNP was due to impaired NO release. However, this seems unlikely at the concentrations of the NO donors studied, since ODQ has been shown to completely abolish the effects of authentic NO in rat aorta at similar concentrations(Feelisch, *et al.* 1999).

An important finding of the study is that ODQ does not completely inhibit the relaxant response to the endothelium-dependent calcium ionophore A23187. Pretreatment with ODQ reduced the maximum relaxation to A23187 by approximately half (from 74% to 35%), with no significant effect on the log EC_{50}. This is consistent with previous observations in the bovine pulmonary artery(Brunner, *et al.* 1996), but conflicts with results obtained in rat aorta(Feelisch, *et al.* 1999). It suggests that a significant component of the relaxant response to A23187 in human IMA is independent of activation of soluble guanylate cyclase. Possible additional mechanisms of action include direct activation of K_{ca} channels by NO(Bolotina, *et al.* 1994), or NO-independent mechanisms, such as endothelium-derived hyperpolarizing factor. Further studies, in particular using high K^+ media, are required to determine which of these is responsible for this relaxant mechanism of A23187.

This study has important implications for previous experiments in this thesis and indeed other studies examining the effects of NO donors. It demonstrates that GTN and SNP, at the concentrations studied, induce vasodilation of human vessels largely (or entirely) via activation of soluble guanylate cyclase. Hence, the lack of cross-tolerance to SNP observed in the tolerance-induction experiments (Chapter 3) is consistent with the concept that tolerance is nitrate-specific, and is not due to increased inactivation of NO, or desensitization of soluble guanylate cyclase. However, since it appears that a significant component of vasodilation induced by A23187 is independent of soluble guanylate cyclase, we must be more cautious in our interpretation of the lack of cross-tolerance to A23187 seen in Chapter 3. It is conceivable that the presence of a significant degree of cross-tolerance to A23187 may have been masked by additional mechanism(s) of vasodilation independent of activation of soluble guanylate cyclase.

Table 4.5

Effect of ODQ on reactivity of Internal Mammary Artery (IMA) and Saphenous Vein (SV) Segments to GTN, SNP and A23187.

	Number of subjects	E_{max} (%)	
		Control	ODQ
IMA			
GTN	5	97 ± 1	13 ± 4*
SNP	5	97 ± 2	12 ± 3*
A23187	5	74 ± 4	35 ± 5#
SV			
GTN	5	96 ± 3	16 ± 5*
SNP	5	98 ± 2	19 ± 4*

Values are mean ± SEM.
* $p < 0.001$ for control vs ODQ-exposed segments.
# $p < 0.01$ for control vs ODQ-exposed segments.

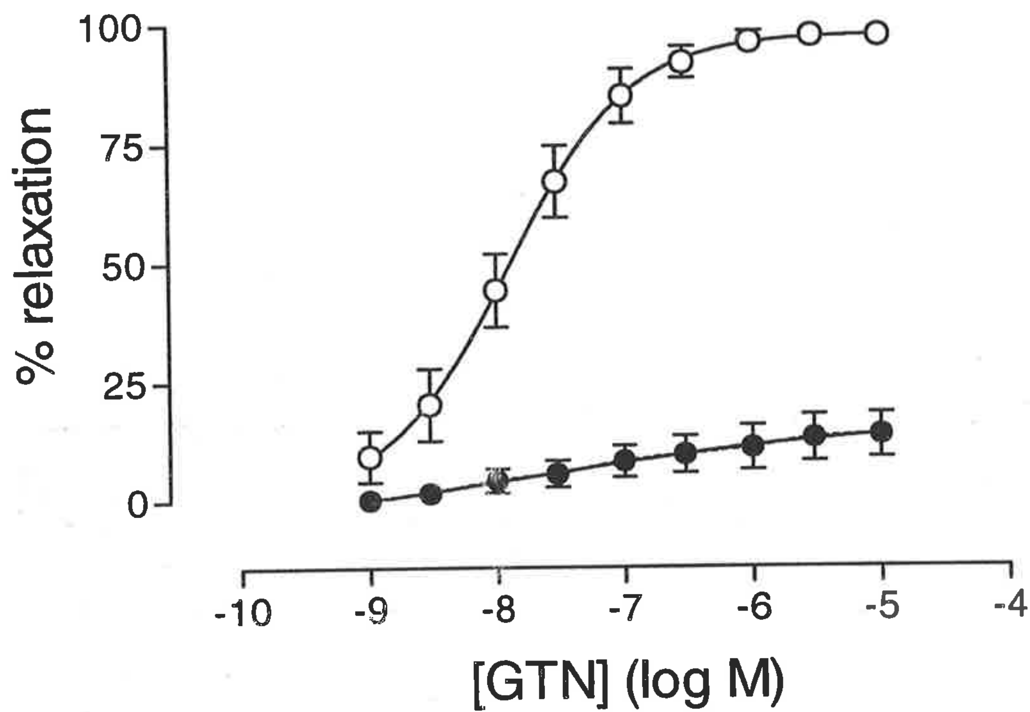


Figure 4.10
Effect of ODQ (10 μM) on responses of IMA segments from nitrate-free patients (n=5) to GTN. Open circles indicate control segments, closed circles indicate segments exposed to ODQ.

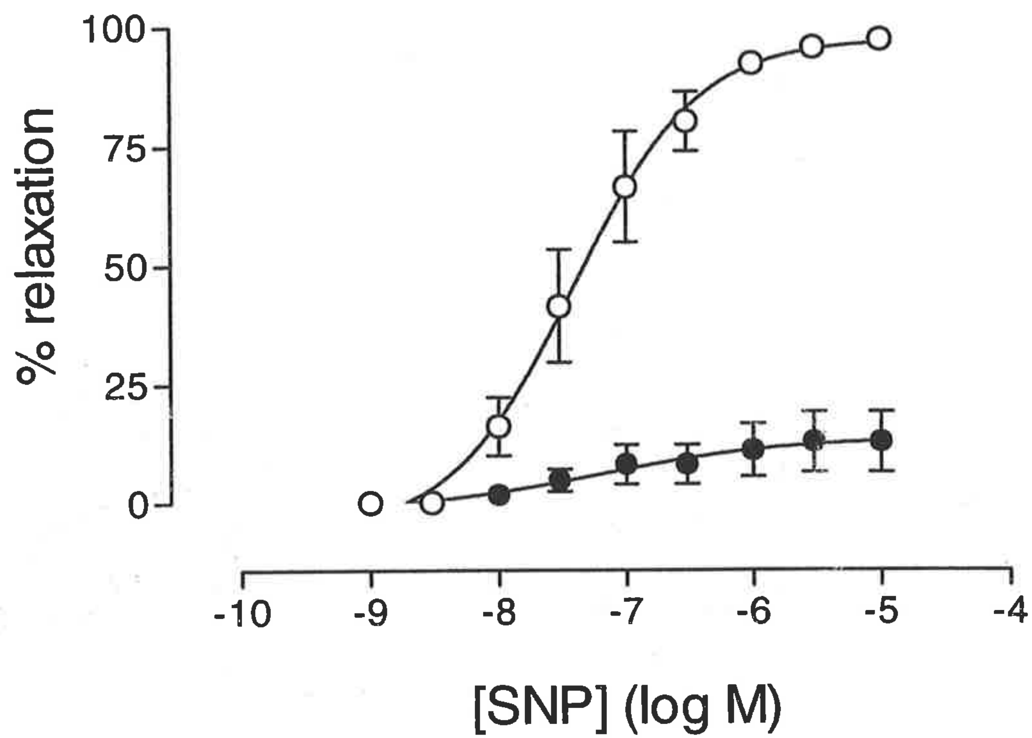


Figure 4.11
Effect of ODQ (10 μM) on responses of IMA segments from nitrate-free patients (n=5) to SNP. Open circles indicate control segments, closed circles indicate segments exposed to ODQ.

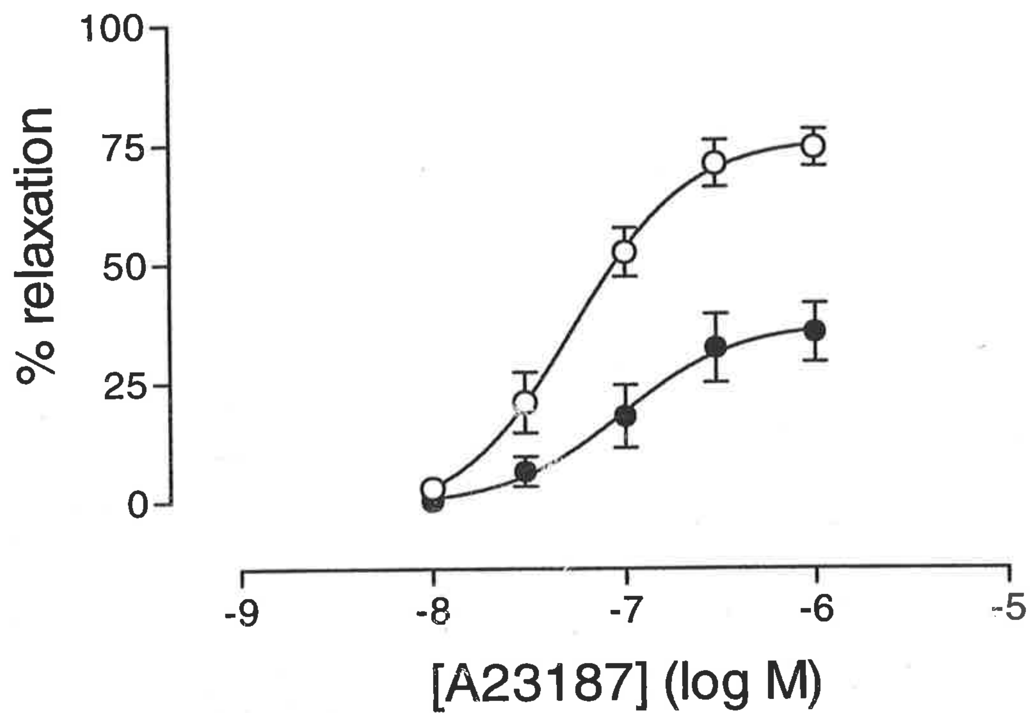


Figure 4.12
Effect of ODQ (10 μM) on responses of IMA segments from nitrate-free patients (n=5) to A23187. Open circles indicate control segments, closed circles indicate segments exposed to ODQ.

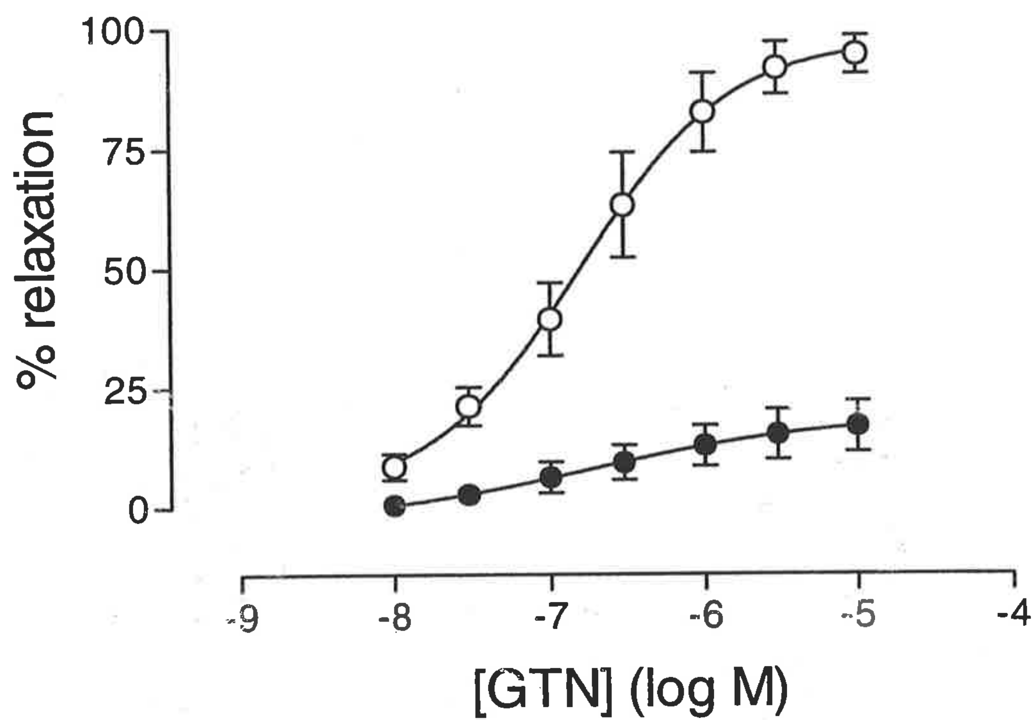


Figure 4.13
Effect of ODQ (10 μM) on responses of SV segments from nitrate-free patients (n=5) to GTN. Open circles indicate control segments, closed circles indicate segments exposed to ODQ.

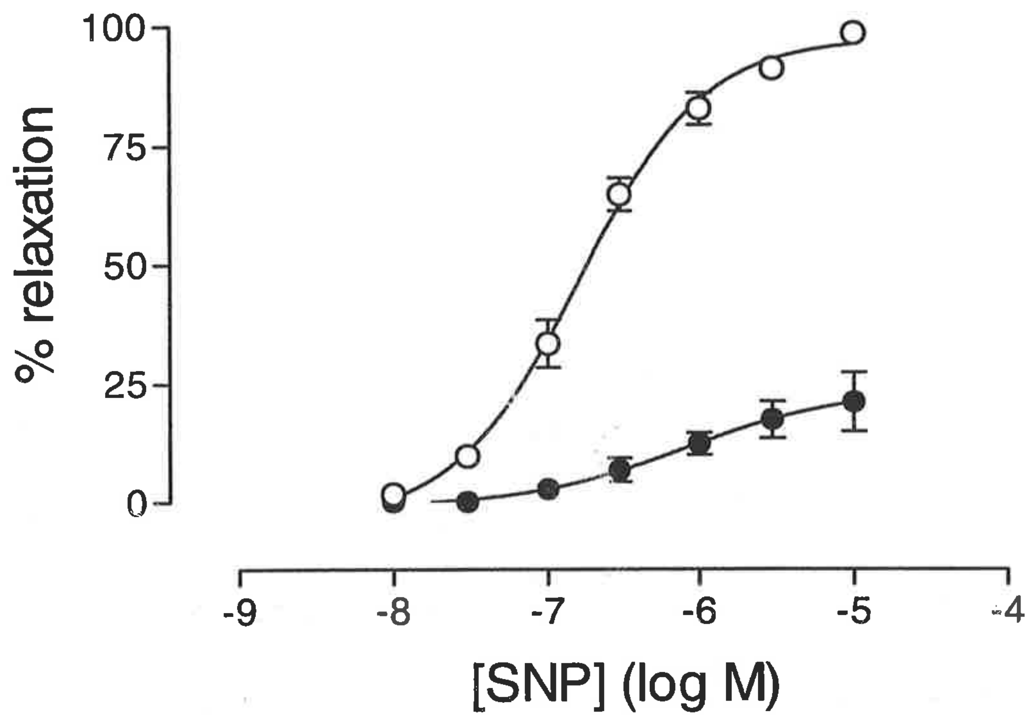


Figure 4.14
Effect of ODQ (10 μM) on responses of SV segments from nitrate-free patients (n=5) to SNP. Open circles indicate control segments, closed circles indicate segments exposed to ODQ.

5. Nitrate Cross-Tolerance: The Relationship of Long-term Oral Nitrate Therapy with ex Vivo Responses of Human Vessels to GTN

5.1 Introduction

The oral preparations of the organic nitrates enjoy widespread use in the treatment of stable angina pectoris and congestive heart failure. In particular, oral isosorbide dinitrate (ISDN) and the sustained-release preparation of oral isosorbide 5-mononitrate (ISMN) are commonly used in the treatment of these conditions in Australia.

However, as discussed in Chapter 1 (Sections 1.6 and 1.8), it took many years for the optimum dosing regimen of these agents to be determined. Following the initial studies highlighting the importance of the development of tolerance to the antianginal effects of continuous transdermal GTN(Crean, *et al.* 1984; James, *et al.* 1985; Parker, *et al.* 1984a; Reichek, *et al.* 1984; Sullivan, *et al.* 1985; Thadani, *et al.* 1986), reports began emerging showing attenuation or loss of the clinical efficacy of standard-preparation ISDN when administered orally 4 or more times a day(Boesgaard, *et al.* 1991a; Boesgaard, *et al.* 1992; Elkayam, *et al.* 1991; Parker, *et al.* 1987a; Parker, *et al.* 1987b; Parker, *et al.* 1985; Wisenberg, *et al.* 1989). This led to the incorporation of a nitrate-free period into the dosing regimen; chronic therapy with oral ISDN two or three times a day, using an eccentric dosing schedule incorporating a space of 14 hours between two doses, was found to provide sustained therapeutic effects(Bassan 1990; Elkayam, *et al.* 1991; Parker, *et al.* 1987a). However, even this regimen which is now commonly used, may produce a degree of tolerance the following day(Bassan 1990; Parker, *et al.* 1987a), and the beneficial effects may only be present for as little a 6 hours of the day(Bassan 1990).

Similarly, while once a day administration of oral sustained-release ISMN can produce sustained beneficial effects in patients with stable angina(Beyerle, *et al.* 1990; Chrysant, *et al.* 1993; Glasser 1997; Wisenberg, *et al.* 1989), more frequent administration rapidly produces tolerance(Jahnchen 1992). Even with once-daily

dosing, partial tolerance is evident with sustained therapy(Beyerle, *et al.* 1990; Chrysant, *et al.* 1993; Wisenberg, *et al.* 1989) and their are conflicting data regarding the minimum dose required to produce sustained clinical effects. While some investigators(Beyerle, *et al.* 1990; Wisenberg, *et al.* 1989) have found sustained beneficial effects with only 50-60 mg ISMN per day, others(Chrysant, *et al.* 1993; Thadani, *et al.* 1987a) have demonstrated complete loss of efficacy at these doses. Indeed, the largest study of once-daily sustained-release ISMN(Chrysant, *et al.* 1993), found that doses of 120 mg or 240 mg were required to maintain clinical benefit.

To date there have been no direct comparisons between the optimum regimens of these two oral nitrate preparations, with regard to clinical efficacy. Hence, the choice of one agent over the other is often determined by other factors such as patient compliance or adverse effects. Similarly, there have been no previous studies comparing the extent of tolerance induction during long-term administration of the two regimens. The only previous study examining ex vivo nitrate tolerance in human vessels, followed short-term exposure to mainly transdermal GTN(Du, *et al.* 1992).

The aim of the current study was to compare the ex vivo GTN vasorelaxant responses of vessels obtained from patients receiving either prophylactic oral nitrates with those from patients not on prophylactic nitrates. This represents a new approach for assessing the relative extent of tolerance induction during long-term therapy. A secondary objective was to compare vascular reactivity to GTN in vessels from patients taking once-daily oral sustained-release ISMN with those from patients taking ISDN three times a day with a 14 hour nitrate-free period.

5.2 Experimental Protocol

5.2.1 Patient Selection

Subjects were selected using the clinical criteria described in Chapter 2. In addition, subjects were required to be either (1) on no prophylactic nitrates, or (2) receiving a stable dose of oral prophylactic nitrate therapy for a minimum of 4 weeks. The patients' usual nitrate therapy was not altered. The nitrate dosage regimens

consisted of either sustained-release ISMN (60-120mg) once-daily at 0730, or ISDN (10-20mg) administered at 0730, 1230 and 1730. In contrast to the experiments described in Chapter 3, patients taking nitrates continued to receive their usual dose up to but not including the day of operation.

5.2.2 Vascular Reactivity Studies

Segments of IMA and/or SV were obtained during the operation, at approximately 9.30 am in each case. Segments were mounted in organ baths and following equilibration, contractile responses to KCl solution and increasing concentrations of NA were determined as described in Chapter 2.

Approximately 3 hours post-harvest (ie. approximately 1230 am), the relaxant responses of the IMA and SV segments to GTN were examined. Hence, in vessels from patients taking long-term nitrate therapy, the relaxant responses to GTN were determined approximately 29 hours after the last dose of ISMN, and 17 hours after the last dose of ISDN. All segments were then assessed for endothelium-dependent relaxation using A23187; segments without intact endothelium were not used for analysis.

5.2.3 Data Analysis

Data are expressed as mean ± SEM unless indicated. Comparison of vascular relaxant responses to GTN between segments obtained from nitrate-free patients and those on nitrate therapy was performed using the parameters log EC_{50} and E_{max} via unpaired t-test. In addition a secondary comparison was made between segments obtained from the sub-groups of patients on ISMN and ISDN, via unpaired t-test.

5.3 Results

The clinical characteristics of the patients from which the IMA and SV segments were obtained are summarized in Tables 5.1 and 5.2, respectively. A greater

number of the patients on nitrate therapy were also taking calcium channel blockers, compared with those patients not on nitrate therapy; the situation was reversed with regard to beta-adrenoceptor blocker therapy. In other respects, the groups were well balanced.

Comparison of the contractile responses to KCl and NA revealed no significant differences between groups in either IMA or SV segments.

5.3.1 Studies in IMA Segments

Vasorelaxant responses to GTN were studied in segments of IMA from 23 patients not receiving nitrate therapy and 13 patients receiving prophylactic oral nitrates. Of this latter group, 7 patients were taking a once-daily dose of oral sustained-release ISMN at 7.30 am (60-120 mg; mean dose 86 mg) and 6 patients were taking 10 mg of oral ISDN three times a day, at 7.30 am, 12.30 am, and 5.30 pm.

The segments of IMA from patients who were receiving oral nitrates were significantly less sensitive to GTN than those from nitrate-free patients (Figure 5.1). Both log EC_{50} and E_{max} parameters were significantly reduced (Table 5.3).

Vascular reactivity of segments of IMA from nitrate-free patients and the sub-groups of patients on ISMN and ISDN are shown in Figure 5.2 and Table 5.3. Segments from patients receiving ISMN were significantly less responsive to GTN than those from patients not on nitrate therapy or receiving ISDN ($P<0.05$; Table 5.3). Responses of segments of IMA from patients receiving ISDN were similar to those from nitrate-free patients.

5.3.2 Studies in SV Segments

Segments of SV from 14 patients not receiving nitrate therapy and 11 patients receiving prophylactic oral nitrates were studied. Of those taking nitrates, 6 patients were taking a once-daily oral dose of sustained-release ISMN at 7.30 am (60-120 mg;

mean dose 80 mg) and 5 patients were taking 10 mg ISDN at 7.30 am, 12.30 am, and 5.30 pm.

Vasorelaxant responses to GTN were also reduced in the segments of SV from patients receiving oral nitrates compared to those from nitrate-free patients (Table 5.3, Figure 5.3), though to a lesser extent than seen in the segments of IMA. This was manifested as a significant reduction in E_{max} from 96% to 92%, whereas log EC_{50} values were similar between groups.

Comparison of the subgroups of patients taking ISDN or ISMN revealed that the segments of SV from patients receiving ISMN were significantly less responsive than those from patients taking ISDN ($P<0.05$); again this was evident as a reduction in E_{max} with minimal difference in log EC_{50} values(Table 5.3, Figure 5.4).

5.4 Discussion

The major findings of these experiments are (1) Vascular responsiveness to GTN is less in IMA and SV removed from patients previously receiving ISDN or ISMN than in those from patients not receiving nitrates. (2) There is some difference in vascular responsiveness between the ISDN and ISMN groups, the with ISMN group being less responsive.

The oral preparations of the organic nitrates are widely used to treat stable angina pectoris and congestive heart failure(Parker, *et al.* 1998). In particular the use of oral ISDN and oral sustained-release ISMN in the treatment of stable angina pectoris is popular in Australia. Standard-preparation ISDN is rapidly absorbed when given orally and has a terminal half-life of just over an hour, and hence must be given every 4 to 6 hours to maintain therapeutic plasma concentrations. Unfortunately, such frequent administration results in a marked attenuation of clinical efficacy during long-term treatment due to nitrate tolerance(Boesgaard, *et al.* 1991a; Boesgaard, *et al.* 1992; Elkayam, *et al.* 1991; Parker, *et al.* 1987a; Parker, *et al.* 1987b; Parker, *et al.* 1985; Wisenberg, *et al.* 1989). Thus a compromise is reached in the form of a three times a day regimen with eccentric dosing incorporating a 14 hour nitrate-free period

overnight(Bassan 1990; Elkayam, *et al.* 1991; Parker, *et al.* 1987a). This regimen appears to produce sustained effects for at least several hours of the day during long-term therapy(Bassan 1990; Parker, *et al.* 1987a), although a small degree of attenuation of effect is evident with the first dose of each day(Parker, *et al.* 1987a). In the current study, the vasoreactivity of vessels obtained from patients receiving this regimen of oral ISDN long-term(10 mg doses) were studied approximately 17 hours after the last dose was taken the previous day. Responses of the segments of IMA and SV to GTN were found to similar to those of patients not receiving prophylactic nitrate therapy. In the present study therefore, there was no evidence of GTN cross-tolerance in either IMA or SV segments 17 hours after the last dose of oral ISDN was taken.

In contrast to oral ISDN, oral sustained-release ISMN has a far longer half-life and has been shown to provide apparently therapeutic plasma concentrations for up to 12 hours a day and lower concentrations during the latter part of the 24 hour period. However, clinical studies have produced conflicting results. While several small studies(Beyerle, *et al.* 1990; Parker 1991; Wisenberg, *et al.* 1989) suggested administration of 50-60 mg oral ISMN once-daily provided sustained improvements in exercise duration without tolerance, another study did not(Thadani, *et al.* 1987a). A larger randomized placebo-controlled trial(Chrysant, *et al.* 1993) found that once-daily doses of 30 mg and 60 mg, while initially producing a beneficial effect on exercise duration in patients with stable angina, were not better than placebo following 7 weeks of therapy, indicating the progressive induction of tolerance. However, doses of 120 mg and 240 mg showed sustained clinical efficacy for up to 12 hours after 7 weeks of therapy. These findings indicate that substantial tolerance can develop during once-daily administration of ISMN. Thus, the finding in the current study of impaired responsiveness of human vessels to GTN, approximately 29 hours after the last dose of oral sustained-release ISMN, is consistent with the previous clinical studies.

While the results of these experiments do not allow us to draw conclusions regarding the relative clinical efficacy of the two agents in the treatment of stable angina pectoris or heart failure, they do have important clinical implications. Firstly, the study suggests that long-term therapy with once-daily ISMN may produce a more prolonged period of nitrate tolerance compared with thrice-daily ISDN. Hence, the

patient who requires frequent sublingual administration of GTN for treatment or prophylaxis of anginal episodes may derive greater benefit from GTN while taking the ISDN regimen as compared with ISMN. A second situation where a patient might derive a similar benefit would be in the setting of unstable angina, where the induction of significant nitrate tolerance might be particularly detrimental.

The study has several limitations. Firstly, although only patients with stable angina pectoris were included, the study was not randomized. The differences seen in the concomitant anti-anginal therapy probably reflects differences in the usual prescribing patterns between the hospitals from which patients were referred. Nonetheless, we cannot exclude the possibility that the results were affected by the differences between the groups. Responses of the vessels to GTN were examined at only one time point and we did not standardize the nitrate-free interval; we may have found a greater degree of tolerance with either agent had the responses been examined at earlier time points. Similarly, only a single dose of ISDN was studied, while the dosage of ISMN varied. In addition, we have no indication of whether the doses studied were of comparable anti-anginal efficacy; we may have been studying non-equivalent doses. It would have been desirable to compare cross-tolerance to SNP to exclude differences in NO resistance. This would have (partly) comtrolled for inter-individual variability and provided further insight into the mechanism underlying the cross-tolerance to GTN. It would also have been interesting to perform simultaneous GTN metabolism studies to correlate GTN cross-tolerance with bioconversion. Lastly, cross-tolerance to GTN does not necessarily equate to tolerance to ISMN or ISDN. Hence the results of this study may only be relevant for patients requiring sublingual GTN.

Table 5.1

Patient Characteristics: Vascular Reactivity of IMA segments

	Nitrate-free Group (n=23)	Chronic Nitrate Group (n=13)
Age (years)	59 ± 10	58 ± 10
Concomitant therapy:-		
ß-adrenoceptor antagonist	15 (65)	6 (46)
L-calcium channel blocker	5 (22)	7 (54)
ACE inhibitor	10 (43)	5 (38)
Statin	17 (74)	9 (69)
Coronary risk factors:-		
Hypercholesterolemia	18 (78)	10 (7)
Hypertension	11(48)	5 (38)
Smoking	6 (26)	3 (23)
Diabetes	6 (26)	3 (23)

Values are number of patients (with % in brackets) or mean ± SD.

Table 5.2

Patient Characteristics: Vascular Reactivity of SV segments

	Nitrate-free Group (n=14)	**Chronic Nitrate Group (n=11)**
Age (years)	58 ± 11	61 ± 7
Concomitant therapy:-		
ß-adrenoceptor antagonist	11 (79)	5 (45)
L-calcium channel blocker	5 (36)	7 (64)
ACE inhibitor	3 (21)	4 (36)
Statin	9 (64)	8 (73)
Coronary risk factors:-		
Hypercholesterolemia	9 (64)	8 (73)
Hypertension	6 (43)	6 (55)
Smoking	6 (43)	4 (36)
Diabetes	2 (14)	1 (9)

Values are number of patients (with % in brackets) or mean ± SD.

Table 5.3

Reactivity of IMA and SV segments to GTN.

	No of Subjects	Log EC_{50} (M)	E_{max} (%)
IMA			
Nitrate-free	23	-7.8 ± 0.1	98 ± 1
Oral nitrates:-	13	-7.5 ± 0.1*	89 ± 3**
ISDN	7	-7.7 ± 0.1	96 ± 2
ISMN	6	-7.4 ± 0.1#	82 ± 5#
SV			
Nitrate-free	14	-6.9 ± 0.1	96 ± 1
Oral nitrates:-	11	-6.8 ± 0.1	92 ± 2*
ISDN	6	-6.9 ± 0.1	96 ± 2
ISMN	5	-6.7 ± 0.1	89 ± 1#

Values are mean ± SEM.
* $p<0.05$ for nitrate-free group vs oral nitrates group (unpaired t-test).
** $p<0.01$ for nitrate-free group vs oral nitrates group (unpaired t-test).
# $p<0.05$ for ISMN group vs ISDN group (unpaired t-test).

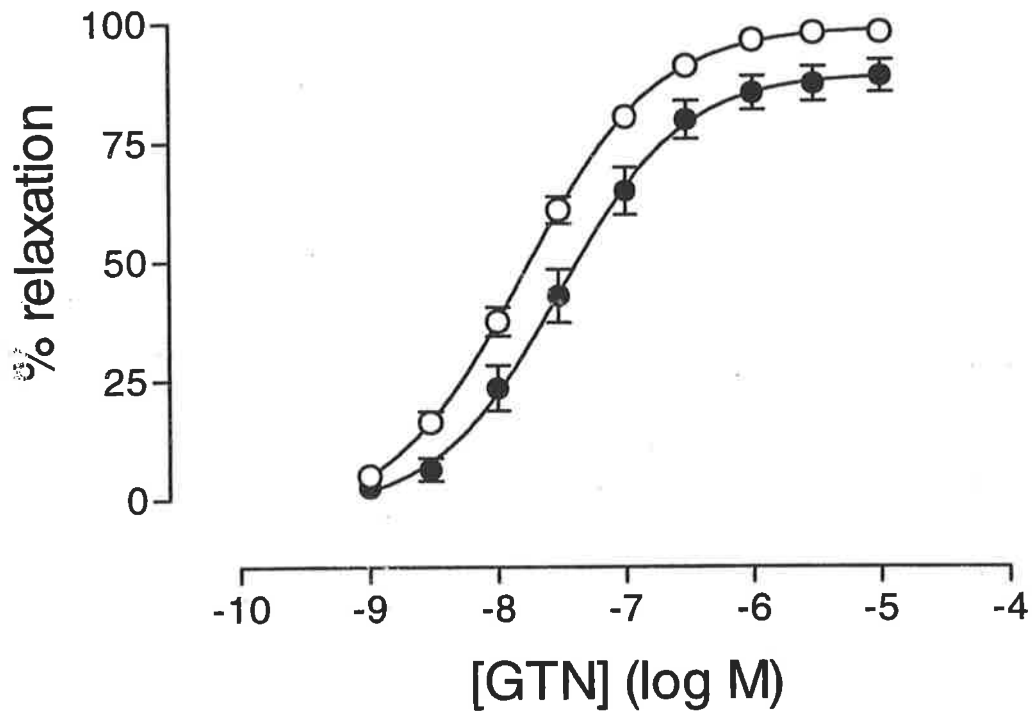


Figure 5.1
Relationship of long-term oral nitrate therapy on responses of segments of IMA to GTN. Open symbols indicate the nitrate-free group and closed symbols, the oral nitrates group. Segments from the oral nitrates group (n=13) were significantly less responsive compared to segments from the control group (n=23).

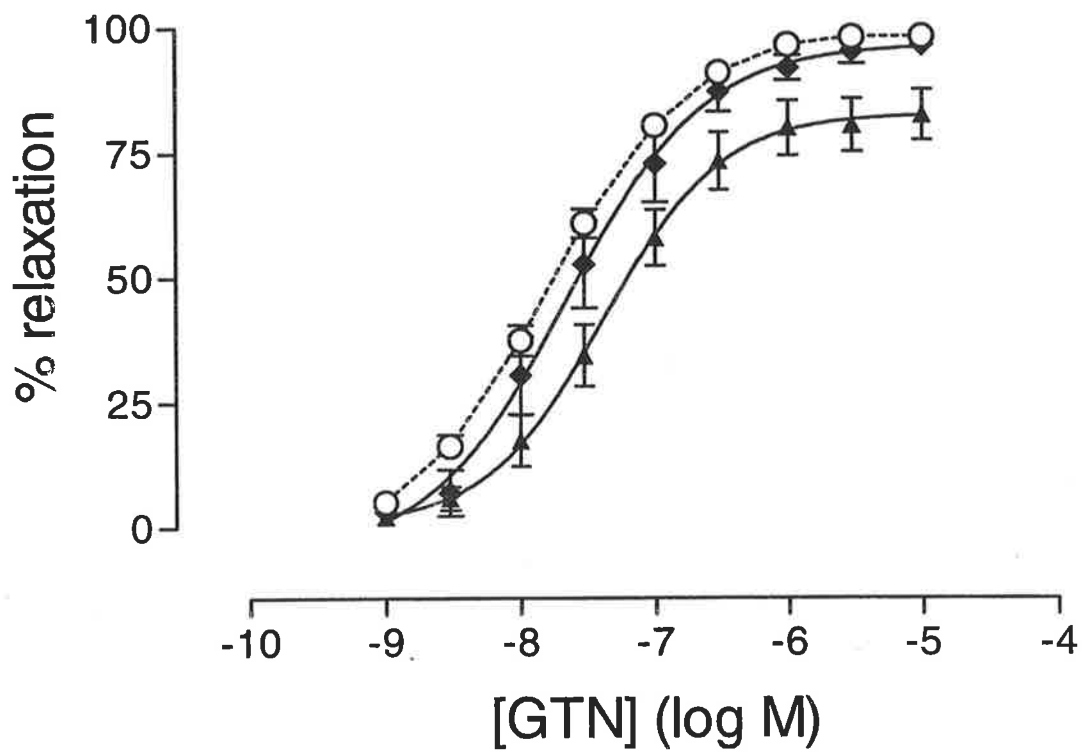


Figure 5.2
GTN responsiveness in IMA segments from patients receiving ISDN (n=7; closed diamonds) and ISMN (n=6; closed triangles). Responses in nitrate-free group (n=23; open circles, broken line) are shown for comparison.

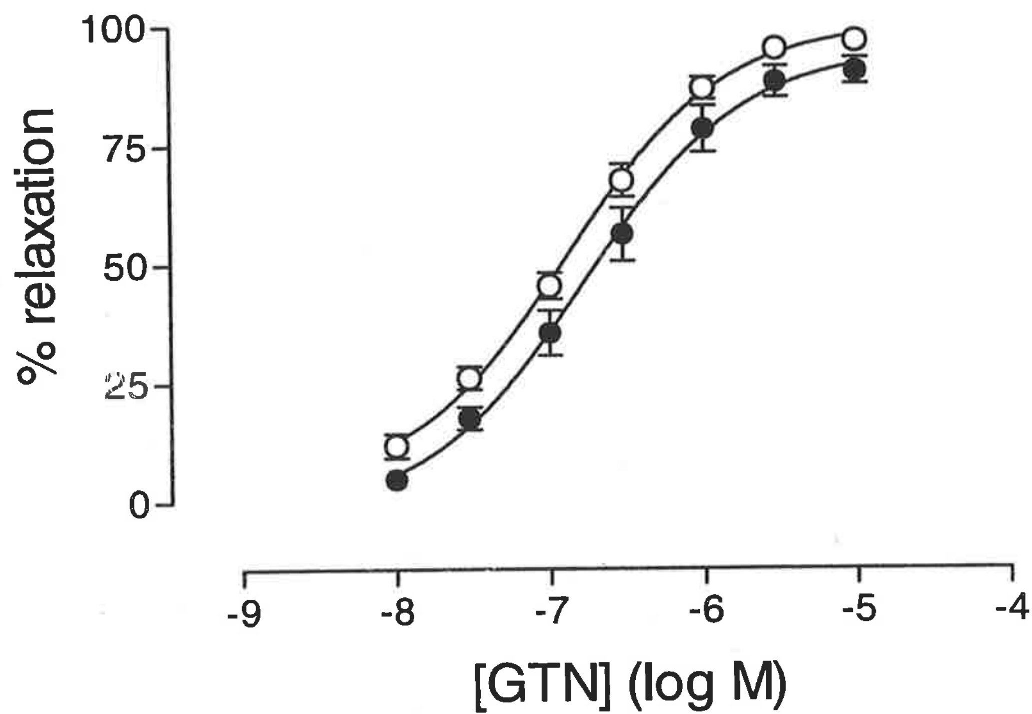


Figure 5.3
Effect of long-term oral nitrate therapy on responses of segments of SV to GTN. Open symbols indicate the nitrate-free group and closed symbols, the oral nitrates group. Maximum relaxation was significantly reduced in segments from the oral nitrates group (n=11) compared with the nitrate-free group (n=14).

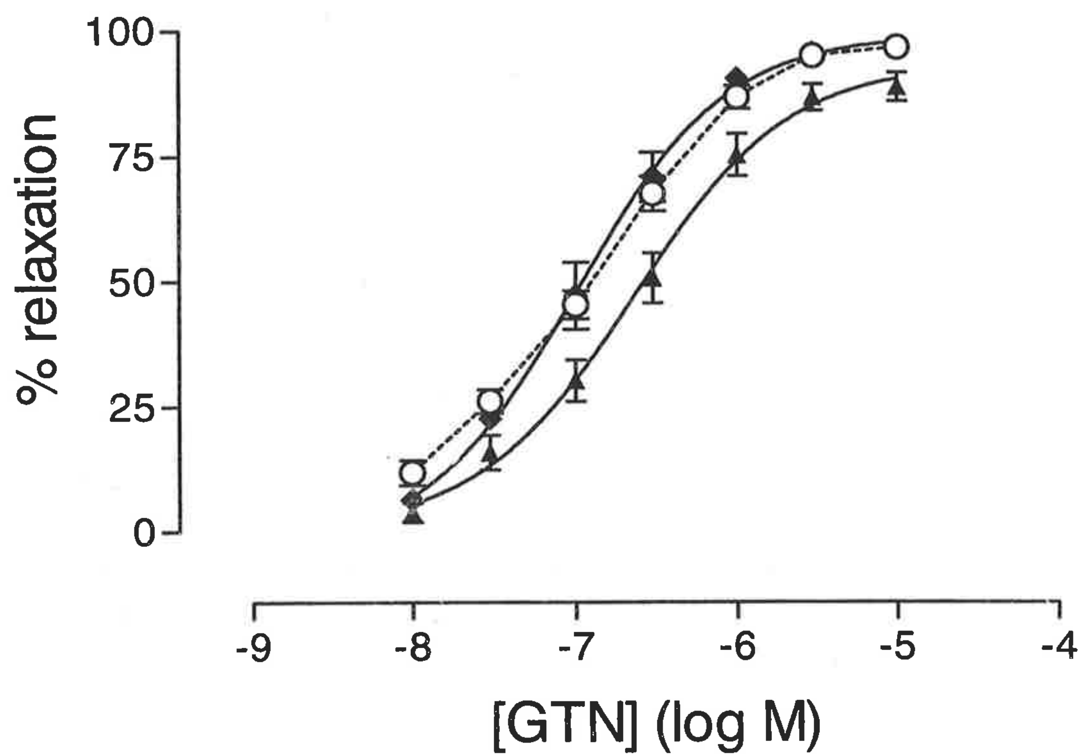


Figure 5.4
GTN responsiveness in SV segments from patients receiving ISDN (n=6; closed diamonds) and ISMN (n=5; closed triangles). Responses in nitrate-free group (n=14; open circles, broken line) are shown for comparison.

6. Nitrate Tolerance: Effect of Incremental Sulphydryl Bioavailability

6.1 Introduction

Since the "sulphydryl depletion" hypothesis was proposed by Needleman and coworkers(1973b), there have been a large number of investigations into the possibility that increasing sulphydryl bioavailability might increase nitrate effects and/or prevent nitrate tolerance (see also section 1.8.3.2). These investigations were further stimulated by other early studies(Axelsson, *et al.* 1982; Ignarro, *et al.* 1981), showing that modulation of sulphydryl groups in broken cell preparations markedly potentiated the activation of soluble guanylate cyclase by organic nitrates.

Subsequently, considerable evidence has emerged from both animal (Boesgaard, *et al.* 1993; Fung, *et al.* 1988; Hutter, *et al.* 1988; Munzel, *et al.* 1989; Munzel, *et al.* 1992) and human studies(Boesgaard, *et al.* 1994b; Creager, *et al.* 1997; Horowitz, *et al.* 1983; Levy, *et al.* 1988; Mehra, *et al.* 1994; Nishikawa, *et al.* 1998; Pizzulli, *et al.* 1997; Svendsen, *et al.* 1989; Vekshtein, *et al.* 1990; Winniford, *et al.* 1986) that sulphydryl agents (such as NAC and possibly methionine) can potentiate the systemic and coronary vasodilating effects of nitrates in vivo. In addition, NAC has been shown to potentiate the effects of GTN in inhibiting platelet aggregation(Loscalzo 1985). Importantly, it has been suggested that this nitrate-potentiating effect of sulphydryl agents occurs predominantly via an extracellular interaction in the plasma(Fung, *et al.* 1988) and/or in the small microvessels(Kurz, *et al.* 1991; Munzel, *et al.* 1992; Sellke, *et al.* 1991; Wheatley, *et al.* 1994), since increasing intracellular sulphydryls in isolated large vessels does not seem to provide a further increase in nitrate vasorelaxant effects (Boesgaard, *et al.* 1993; Chong, *et al.* 1991; Fung, *et al.* 1988; Gruetter, *et al.* 1986; Henry, *et al.* 1989b; Lawson, *et al.* 1996).

The results from studies examining whether sulphydryl agents can reverse or prevent nitrate tolerance induction in vivo have been far more conflicting. Several studies have demonstrated "reversal" of nitrate tolerance with NAC administration in vivo(Boesgaard, *et al.* 1991b; Ghio, *et al.* 1992; Levy, *et al.* 1991; May, *et al.* 1987;

Packer, *et al.* 1987; Vincent, *et al.* 1992). However, as discussed in section 1.8.3.2, these studies cannot distinguish between actual reversal of nitrate tolerance, and tolerance-independent potentiation of nitrate effects (see above). Hence, it is difficult to draw conclusions regarding the effect of NAC administration on nitrate tolerance from these studies. The fact that the majority of in vitro studies attempting to reverse nitrate tolerance with NAC in isolated large vessels have shown no effect(Abdollah, *et al.* 1987; Chong, *et al.* 1991; Fung, *et al.* 1988; Gruetter, *et al.* 1986; Henry, *et al.* 1989a; Lawson, *et al.* 1996), may suggest that the "reversal" of nitrate tolerance by NAC in the in vivo studies, may be due to tolerance-independent potentiation of nitrate effects, either extracellularly and/or in small microvessels.

To date, studies investigating whether co-administration of sulphydryl agents can prevent nitrate tolerance have produced conflicting results. While some studies have shown partial prevention of tolerance(Boesgaard, *et al.* 1991a; Boesgaard, *et al.* 1992; Tsuneyoshi, *et al.* 1989), others(Boesgaard, *et al.* 1994b; Dupuis, *et al.* 1990b) have shown no effect.

The issue of whether sulphydryl agents reverse or prevent nitrate tolerance is further complicated by the fact that all sulfhydryl agents are also anti-oxidants(Aruoma, *et al.* 1989), making them non-specific as regards the potential mechanism(s) by which tolerance may be attenuated.

The clinical significance of the nitrate-sulphydryl interaction also remains uncertain(Horowitz 1991). In the setting of unstable angina, there is some evidence that the combination may exert incremental beneficial effects with regard to reducing major coronary events, over and above those of nitrate therapy(Ardissino, *et al.* 1997; Horowitz, *et al.* 1988a). In addition, combined GTN-NAC therapy may reduce oxidative stress and preserve left ventricular function in patients with acute myocardial infarction(Arstall, *et al.* 1995), and may have a role in the treatment of pulmonary oedema(Beltrame, *et al.* 1998; Ghio, *et al.* 1994). However, theoretical concerns have been raised regarding the effects of nitrate-NAC combinations on small vessels and the potential for the coronary steal phenomenon(Kurz, *et al.* 1991).

Nonetheless, since the induction of tolerance with continuous therapy remains the major factor limiting the utility of organic nitrates, whether co-administration of sulphydryl agents can prevent this phenomenon to any extent remains a critically important issue. Thus the objectives of the experiments described in this section were to determine whether (1) co-administration of intravenous NAC prevents tolerance to GTN in humans, (2) intravenous NAC therapy potentiates the ex vivo responses of human vessels to GTN and (3) incubation with NAC in vitro potentiates GTN effects and/or reverses GTN tolerance induced in human vessels in vivo.

6.2 Experimental Protocol

Study participants were patients undergoing elective CABG selected using the criteria described in Chapter 2. Patients were randomized to one of the following arms, 24 hours before operation:-

(1) no nitrate therapy (control group),
(2) a continuous intravenous infusion of GTN at 10 µg/minute via non-adsorptive tubing (GTN-only group),
(3) continuous co-infusion of intravenous GTN at 10 µg/minute and intravenous NAC at 10 grams/24 hours (GTN+NAC group), OR
(4) a continuous intravenous infusion of NAC at 10 g/24 hours (NAC-only group).

In this series patients were randomized to the four arms in a 1:2:2:1 ratio, to ensure there were sufficient numbers in the GTN+NAC arm for analysis. Arms (1) and (2) received identical treatment to the two cohorts studied in Chapter 3.

All other nitrate therapy was held during the 24 hours prior to surgery, unless indicated for medical reasons, in which case the patient was withdrawn from the study. All other prophylactic anti-anginal agents were continued unchanged, as summarized in Table 6.1. Patients were withdrawn from the study if angina recurred in the 24 hour study period, if significant hypotension (systolic BP<90 mmHg) or a headache unresponsive to paracetamol occurred, or if surgery was postponed for other reasons. GTN and/or NAC infusions were continued unchanged throughout operation. During

the operation, discarded segments of distal left internal mammary artery (IMA) and/or proximal saphenous vein (SV) were collected as described in Chapter 2.

6.2.1 Vascular Reactivity Studies

Segments of IMA and SV obtained at operation were mounted in organ baths as described in Chapter 2. Following equilibration contractile responses to KCl solution and increasing concentrations of NA were determined as described in Chapter 2.

Following further washout cumulative CR curves to GTN were performed in each segment of IMA and SV. Time from harvest of vessels to assessment of responses to GTN was held constant at 3 hours 15 minutes. Following further washout all segments were then assessed for endothelium-dependent relaxation; segments without intact endothelium were not used for analysis.

Segments from further patients randomized to either the control group or GTN-only group were allocated to incubation with NAC 100 μM for 30 minutes before the relaxant responses to GTN were determined.

6.2.2 GTN Bioconversion Studies

When sufficient tissue was available, segments of SV from each patient were allocated to assess GTN bioconversion by measurement of GTN and dinitrate metabolites following incubation with GTN (1.0μM: 2 mins), as described in detail in Chapter 2. These studies were performed at approximately 3 hours post-harvest to coincide with the vascular reactivity studies.

Further segments of SV from a small number of additional nitrate-free patients (n = 4) were used to assess the effect of NAC on in vitro GTN bioconversion; segments from each patient were allocated to incubation with either NAC 100μM or Krebs solution for 30 minutes before assessing GTN bioconversion.

6.2.3 Data Analysis

Data are expressed as mean ± SEM unless otherwise indicated. The vascular relaxant responses to GTN are compared using the parameters log EC_{50} and E_{max}. Tissue GTN and dinitrate content are expressed in pmole/mg and the ratio of 1,2-GDN content to GTN content is expressed as log units. The primary comparison was the incremental effect of NAC co-infusion on these parameters, compared to those of the GTN-only group, assessed in each case by an unpaired t-test. Secondary comparisons were made between the control group and NAC-only group via unpaired t-test. The effect of in vitro NAC on GTN responses and GTN bioconversion in control vessels was analysed with a paired t-test.

6.3 Results

A total of 27 patients were randomized; the clinical characteristics of the patients studied are summarized in Table 6.1. Four patients were withdrawn from the study prior to surgery; the GTN infusion was interrupted in one patient in the GTN-only group, one patient in the control group received oral nitrates during the study period, and one patient in both GTN+NAC and NAC-only group had the operation postponed due to reasons unrelated to the study.

6.3.1 Vascular Reactivity Studies

Vasoconstrictor responses to either KCl solution or NA did not differ between segments from the four groups (not shown).

6.3.1.1 Effect of IV NAC on Relaxant Responses to GTN

Comparative log EC_{50} and E_{max} values for relaxant responses to GTN of each group are shown in Table 6.2. The responses of segments of IMA and SV from the control and GTN-only groups were similar to those seen in the previous tolerance induction study (see Chapter 3); the GTN-only group displayed a moderate degree of

tolerance ($P<0.05$ for control group vs GTN-only group log EC_{50} and E_{max} values in both IMA and SVsegments).

Compared to segments of IMA and SV from the GTN-only group, segments from the GTN+NAC group did not differ significantly in responsiveness to GTN (Table 6.2; Figure 6.1 and 6.2).

Compared to segments of IMA and SV from the control group, segments from the NAC-only group did not differ significantly in responsiveness to GTN (Table 6.2; Figure 6.3 and 6.4), although a trend towards reduced responsiveness was evident.

6.3.1.2 Effect of NAC in Vitro on Relaxant Responses to GTN

The effect of exposure to NAC 10μM on the relaxant responses of isolated segments of IMA and SV to GTN is shown in Table 6.3 and Figures 6.5 to 6.8. Incubation of segments from either the control group or the GTN-only group with NAC did not alter responsiveness to GTN.

6.3.2 GTN Bioconversion Studies

Due to limited availability of SV, the numbers of subjects in each group in which bioconversion of GTN was assessed were small. The comparative tissue content of 1,2-GDN was 0.14 ± 0.01 (control group; n=3), 0.11 ± 0.02 (GTN-only group; n=5), 0.11 ± 0.01 (GTN+NAC; n=7), and 0.12 ± 0.03 (NAC-only group; n=3). Because of small numbers in other groups, the only formal comparison undertaken was between GTN-only and GTN-NAC groups.

Tissue content of 1,2-GDN was similar in segments from both GTN-only group and GTN+NAC group (p=NS; Figure 6.9, Table 6.4). The ratio of 1,2-GDN/GTN was slightly higher in segments from the GTN+NAC group than the GTN-only group but this did not reach statistical significance (Table 6.4).

Because of small numbers in the control and GTN-only groups, a secondary comparison was made including the data from the cohort studied in Chapter 3, thus increasing the number of subjects in the control and GTN-only groups. Tissue 1,2-GDN content and 1,2-GDN/GTN ratio were significantly lower ($P<0.05$) in segments from the GTN-only group compared with the control segments, indicating impairment of bioconversion of GTN to 1,2-GDN as demonstrated in Chapter 3 (Table 6.4, Figure 6.9).

The 24 hour infusion of NAC alone appeared to have no effect on subsequent bioconversion of GTN to 1,2-GDN compared with that of the control group(Figure 6.10). Similarly, incubation of isolated segments with NAC 100μM for 30 minutes had no effect on the extent of bioconversion of GTN in segments from nitrate-free patients ($p = 0.8$).

6.4 Discussion

The major findings of these experiments are that (1) co-administration of continuously infused intravenous NAC at 10 g/24 hours does not prevent induction of tolerance to GTN in the human vessels studied to a significant extent, (2) intravenous NAC 10 g/24 hours does not result in potentiation of the ex vivo responses of these isolated human vessels to GTN and (3) incubation of isolated human vessels with NAC does not potentiate the response to GTN and has no effect on the sensitivity of tolerant vessels to GTN.

The main thrust of these experiments was to determine to what extent if any, incremental sulphydryl bioavailability alters the previously demonstrated attenuation of GTN vasodilator efficacy and GTN bioconversion in isolated large vessels from patients following induction of tolerance to GTN (see Chapter3). To date, the results of studies investigating whether co-administration of sulphydryl agents can prevent nitrate tolerance have been conflicting(Boesgaard, *et al.* 1991a; Boesgaard, *et al.* 1992; Boesgaard, *et al.* 1994b; Dupuis, *et al.* 1990b; Pizzulli, *et al.* 1997; Tsuneyoshi, *et al.* 1989).

Whether sulphydryl agents can reverse nitrate tolerance also remains controversial. Interpretation of the results of previous in vivo studies demonstrating reversal of the nitrate tolerance with NAC(Boesgaard, *et al.* 1991b; Ghio, *et al.* 1992; Levy, *et al.* 1991; May, *et al.* 1987; Packer, *et al.* 1987; Vincent, *et al.* 1992) is complicated by the known GTN-NAC interactions in plasma(Fung, *et al.* 1988) and in small microvessels(Kurz, *et al.* 1991; Munzel, *et al.* 1992; Sellke, *et al.* 1991; Wheatley, *et al.* 1994). Thus, the incremental vasodilation produced by NAC in tolerant subjects in these studies, although still possibly beneficial, may be produced entirely via mechanisms independent of intracellular bioconversion of GTN in large vessels, and therefore may be independent of true/cellular tolerance. The use of an isolated large vessel model in the current study permits investigation of the effect of NAC on cellular tolerance to GTN in a pure form.

Using this methodology, we could find little evidence of an effect of NAC, at the dose studied, on GTN tolerance in large vessels. In the presence of a moderate but significant degree of tolerance, the vascular relaxant responses of segments of IMA and SV to GTN were unaffected by either co-infusion of intravenous NAC or by incubation of the tolerant segments with NAC. Similarly, bioconversion of GTN by tolerant segments was not significantly affected by co-infusion of NAC. Overall, these results suggest that increasing sulphydryl bioavailability does not prevent the induction of cellular tolerance to GTN in vivo and does not reverse cellular tolerance in vitro.

As discussed above, a large number of in vivo studies(Boesgaard, *et al.* 1994b; Boesgaard, *et al.* 1993; Creager, *et al.* 1997; Fung, *et al.* 1988; Horowitz, *et al.* 1983; Hutter, *et al.* 1988; Levy, *et al.* 1988; Mehra, *et al.* 1994; Munzel, *et al.* 1989; Munzel, *et al.* 1992; Nishikawa, *et al.* 1998; Pizzulli, *et al.* 1997; Svendsen, *et al.* 1989; Vekshtein, *et al.* 1990; Winniford, *et al.* 1986) have demonstrated potentiation of nitrate effects by sulphydryl donors. However, the mechanism by which this occurs remains less than clear-cut. Boesgaard(1993) showed that increasing intracellular sulphydryls did not provide further increase in nitrate effects, whereas increasing extracellular sulphydryl availability resulted in significant nitrate potentiation. Supporting evidence for a plasma and/or small vessel nitrate-sulphydryl interaction is

provided by animal studies in isolated large vessels(Abdollah, *et al.* 1987; Chong, *et al.* 1991; Fung, *et al.* 1988; Gruetter, *et al.* 1986; Henry, *et al.* 1989a; Lawson, *et al.* 1996) the majority of which have shown that NAC does not affect responses to GTN. The results of the current study which demonstrated that neither infusion of NAC nor exposure to NAC in vitro affects responses to GTN in isolated human vessels, are consistent with these previous animal studies, and suggest that incremental intracellular nitrate bioconversion to NO has a minimal contribution to the potentiating effects of sulphydryl donors on nitrates in vivo.

Indeed, infusion of NAC alone appeared to result in a small non-significant degree of inhibition of GTN responses in IMA and SV. If larger numbers of experiments were to demonstrate that NAC alone (either in vitro or ex vivo) impairs responses to GTN, one possible explanation might reside in the possibility that the active moiety of GTN is not purely NO. One recently presented study(Kleschyov, *et al.* 2000), (not yet published in full) failed to identify any released NO from GTN. Other recent investigations point to the possibility that the release of a component of NO_2^- (as well as NO) from GTN might result in complex effects on vascular reactivity, given that sulphydryls such as cysteine have been shown both to potentiate NO and to inhibit NO_2^- in isolated aorta(Ellis, *et al.* 2000). NO_2^- release might theoretically occur via S-nitrosothiols generated from NAC(Wong, *et al.* 1998) and might account for NAC-induced nausea via inhibition of aldehyde dehydrogenase(Shoeman, *et al.* 2000). However, in order to investigate this possibility definitively, it would be necessary first to demonstrate conclusively a component of NO_2^- release from GTN, which is extremely difficult to do with currently available technology.

The study has several limitations. Critically, we cannot completely exclude a small incremental effect of co-administered NAC on bioconversion of GTN in tolerant vessels (ie. partial prevention of tolerance). Although absolute content of 1,2-GDN was similar in the segments from the GTN-only and GTN+NAC groups, the 1,2-GDN/GTN ratio was slightly greater in the GTN+NAC group. Since the numbers of patients studied were quite small, it is conceivable the study was underpowered to detect a small increment in GTN bioconversion conferred by NAC co-infusion. However, the results of the vascular reactivity studies seem clear-cut, suggesting any beneficial effect of this

NAC regimen on GTN bioconversion in the presence of tolerance is minimal. Secondly, only one GTN dosing regimen and one NAC regimen was studied; we cannot exclude the possibility of a beneficial effect of NAC in the presence of a greater degree of tolerance induction, or a higher NAC regimen. In relation to this, we did not attempt to measure changes in tissue sulphydryl content induced by NAC co-administration or incubation. Lastly, the results, while suggesting NAC may not affect cellular GTN tolerance, do not exclude a beneficial role for NAC in the treatment of myocardial ischaemia or heart failure. As discussed above, there is considerable evidence for a nitrate-potentiating effect of NAC in vivo, perhaps via an extracellular interaction; whether or not this effect results in incremental clinical benefits(Ardissino, *et al.* 1997; Arstall, *et al.* 1995; Horowitz, *et al.* 1988b) in these settings requires further investigation.

Table 6.1

Patient Characteristics: Vascular Reactivity and GTN Bioconversion.

	Control (n=5)	GTN-only (n=6)	GTN+NAC (n=8)	NAC-only (n=4)
Age (years)	59 ± 6	59 ± 10	64 ± 10	63 ± 14
Sex (M : F)	5:0	2:4	6:2	3:1
Conduit used (IMA:SV)	4:5	5:5	8:7	4:4
Prior nitrate therapy	2	4	5	2
Concomitant therapy:-				
ß-adrenoceptor antagonist	2	1	4	2
L-calcium channel blocker	2	5	4	2
ACE inhibitor	1	2	3	1
Statin	4	3	5	3
Coronary risk factors:-				
Hypercholesterolemia	4	3	6	3
Hypertension	1	4	3	1
Smoking	2	2	0	1
Diabetes	0	2	1	0

Values are number of patients or mean ± SD.

Table 6.2

Vascular reactivity of IMA and SV segments to GTN.

Group	No of Subjects	Log EC_{50} (M)	E_{max} (%)
IMA			
Control	4	-7.7 ± 0.1	99 ± 1
GTN-only	6	-7.1 ± 0.1	90 ± 3
GTN+NAC	8	-7.0 ± 0.1	93 ± 3
NAC-only	4	-7.5 ± 0.1	98 ± 2
SV			
Control	5	-6.8 ± 0.1	96 ± 3
GTN-only	5	-6.4 ± 0.1	83 ± 3
GTN+NAC	7	-6.3 ± 0.1	83 ± 3
NAC-only	4	-6.6 ± 0.2	91 ± 7

Values are mean ± SEM. Comparisons between the GTN-only and GTN+NAC groups were not significant. Comparisons between the control and NAC-only groups were not significant.

Table 6.3

Effect of incubation with NAC on GTN relaxant responses of IMA from control and tolerant patients.

Patient Group	Log EC_{50} (M)		E_{max} (%)	
	control segment	NAC segment	control segment	NAC segment
IMA				
Control	-7.7 ± 0.1	-7.6 ± 0.1	96 ± 1	96 ± 1
GTN	-7.2 ± 0.1	-7.2 ± 0.1	87 ± 4	89 ± 2
SV				
Control	-7.1 ± 0.1	-7.1 ± 0.1	96 ± 3	98 ± 2
GTN	-6.4 ± 0.1	-6.4 ± 0.1	86 ± 2	79 ± 4

Values are mean ± SEM. There were no significant differences between the control and NAC-incubated segments from either the control or GTN-only group.

Table 6.4

Tissue 1,2-GDN content and 1,2-GDN/GTN ratio.

Group	No of Subjects	1,2-GDN (pmole/mg)	1,2-GDN/GTN ratio
Control	11	0.16 ± 0.1	0.33
GTN-only	13	0.11 ± 0.1	0.23
GTN+NAC	7	0.11 ± 0.1	0.27

Values are mean ± SEM. Comparisons between the GTN-only and GTN+NAC groups were not significant.

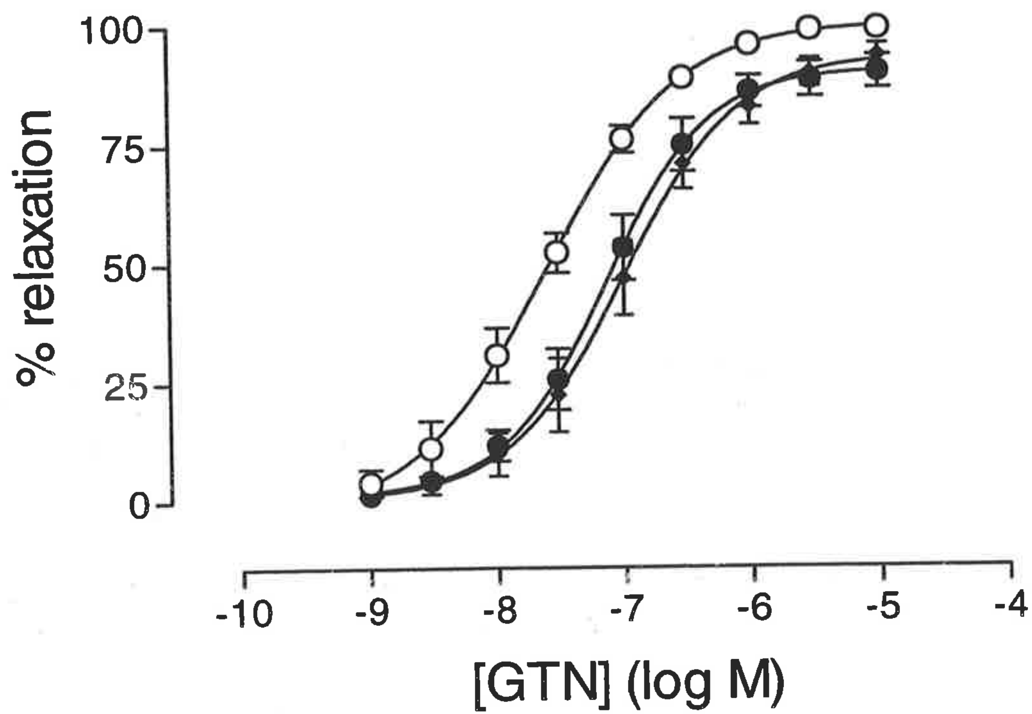


Figure 6.1
Effect of GTN infusion and GTN-NAC co-infusion on responses of IMA to GTN. Open circles indicate the control group (n=4), closed circles the GTN-only group (n=6) and close triangles the GTN+NAC group (n=8). (see Table 6.2)

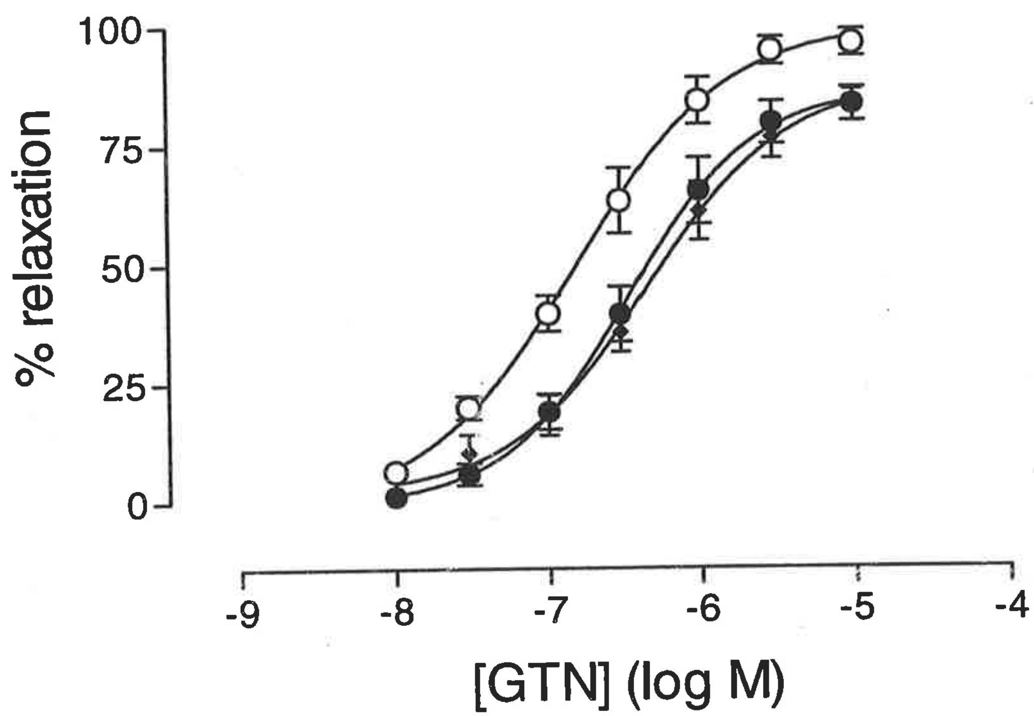


Figure 6.2
Effect of GTN infusion and GTN-NAC co-infusion on responses of SV to GTN. Open circles indicate the control group (n=5), closed circles the GTN-only group (n=5) and close triangles the GTN+NAC group (n=7). (see Table 6.2)

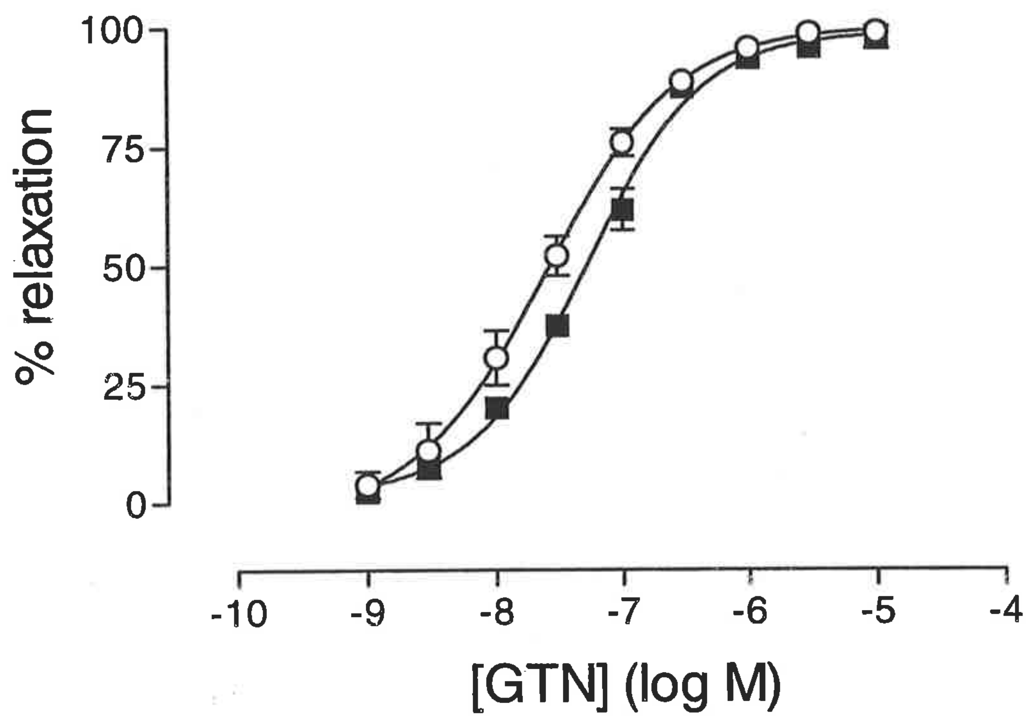


Figure 6.3
Effect of infusion of NAC only on responses of IMA to GTN. Open symbols indicate control group (n=4), closed symbols NAC-only group (n=4). The CR curves did not differ significantly.

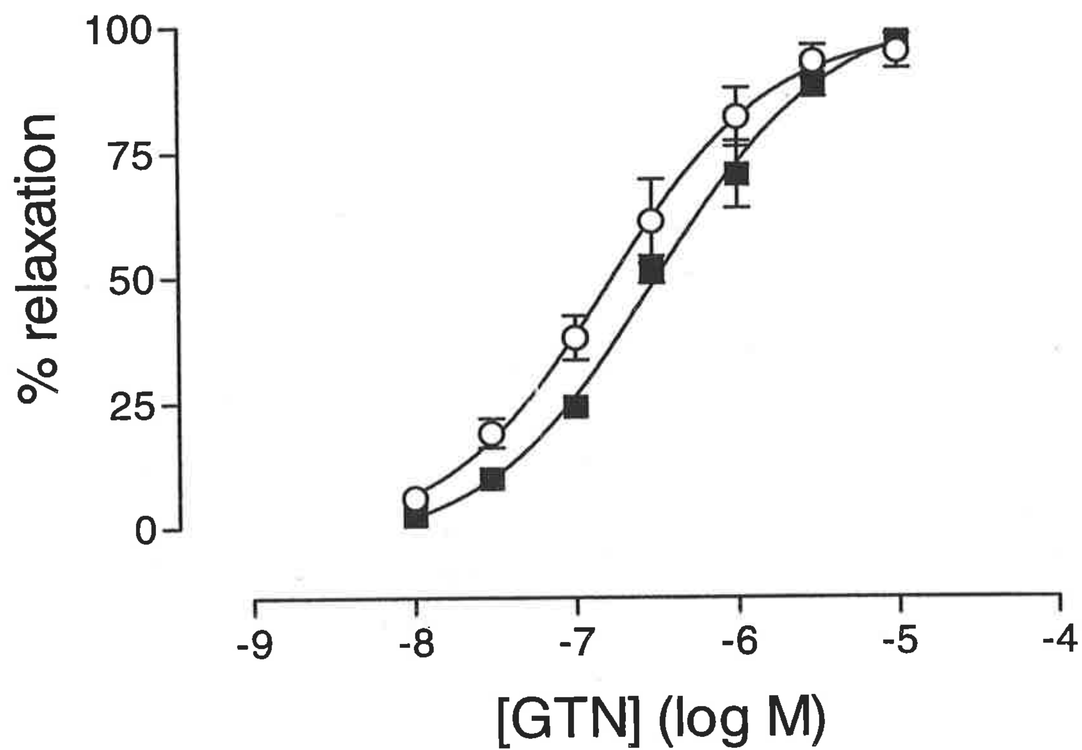


Figure 6.4
Effect of infusion of NAC only on responses of SV to GTN. Open symbols indicate control group (n=4), closed symbols NAC-only group (n=4). The CR curves did not differ significantly.

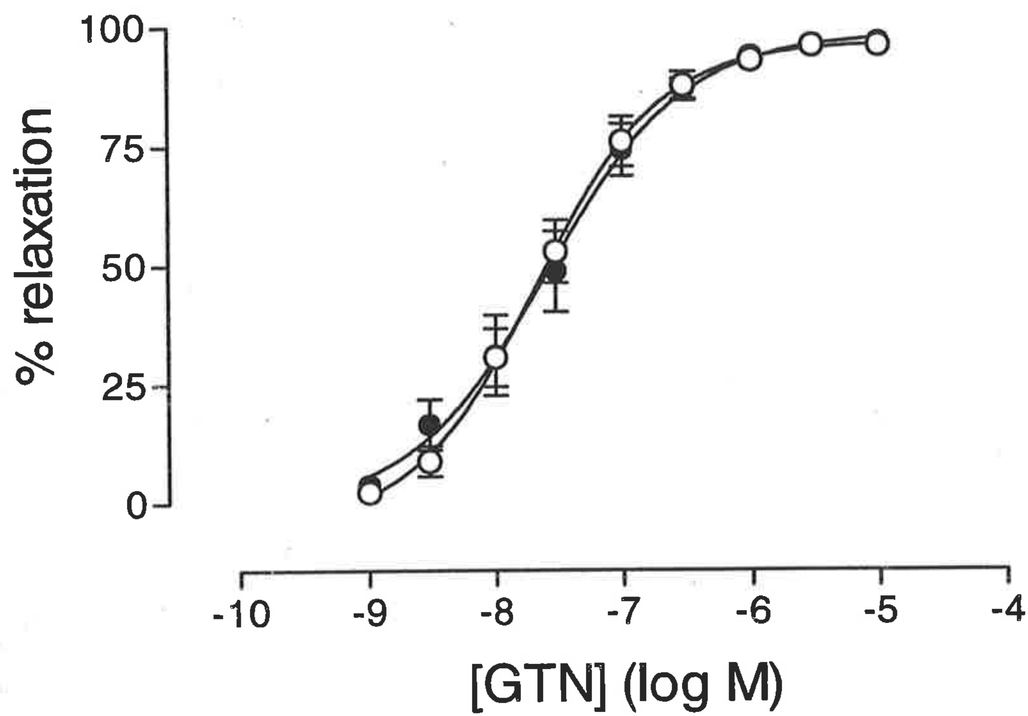


Figure 6.5
Effect of incubation with NAC (100 μM: 30 minutes) on the response to GTN of segments of IMA from nitrate-free patients (n=4).

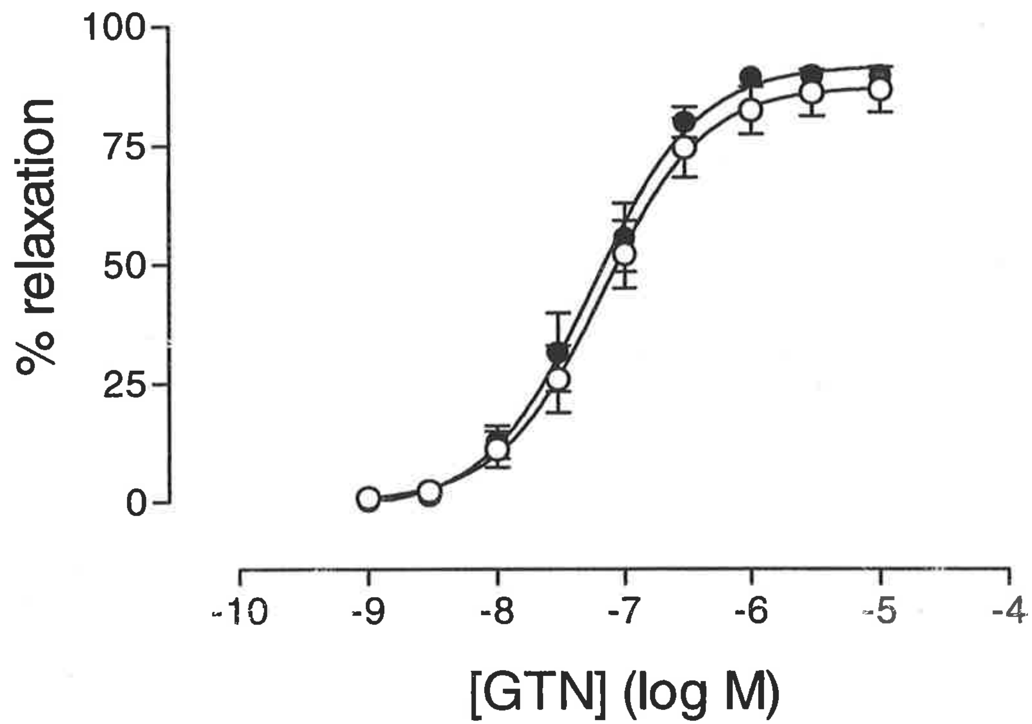


Figure 6.6
Effect of incubation with NAC (100 μM: 30 minutes) on the response to GTN of segments of IMA from patients in the GTN-only group (n=6).

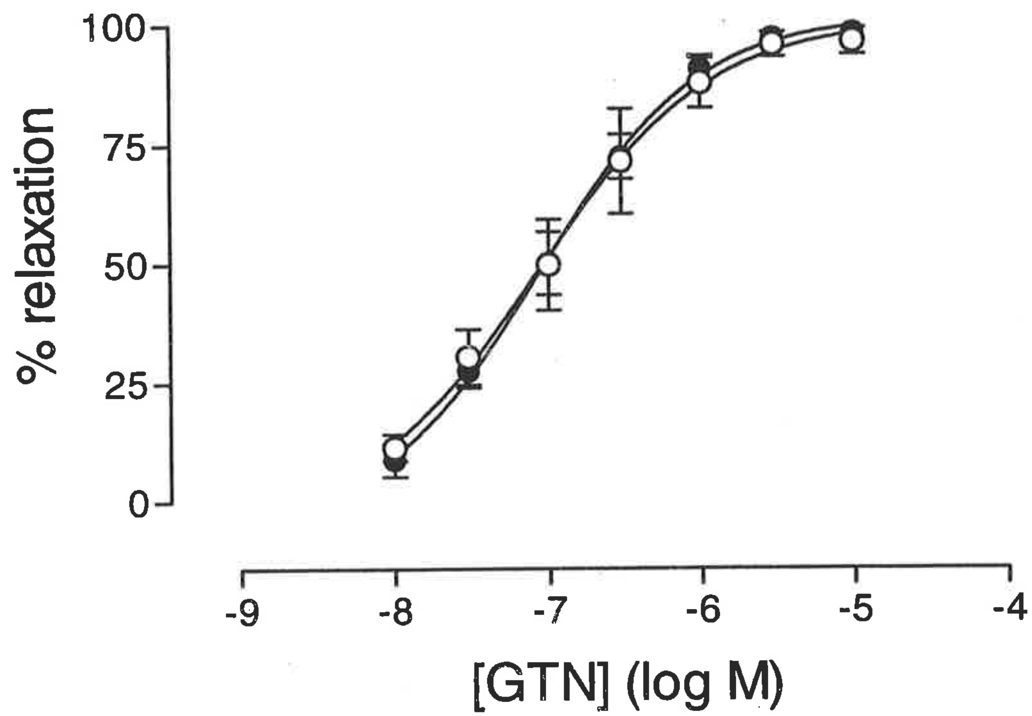


Figure 6.7
Effect of incubation with NAC (100 μM: 30 minutes) on the response to GTN of segments of SV from nitrate-free patients (n=5).

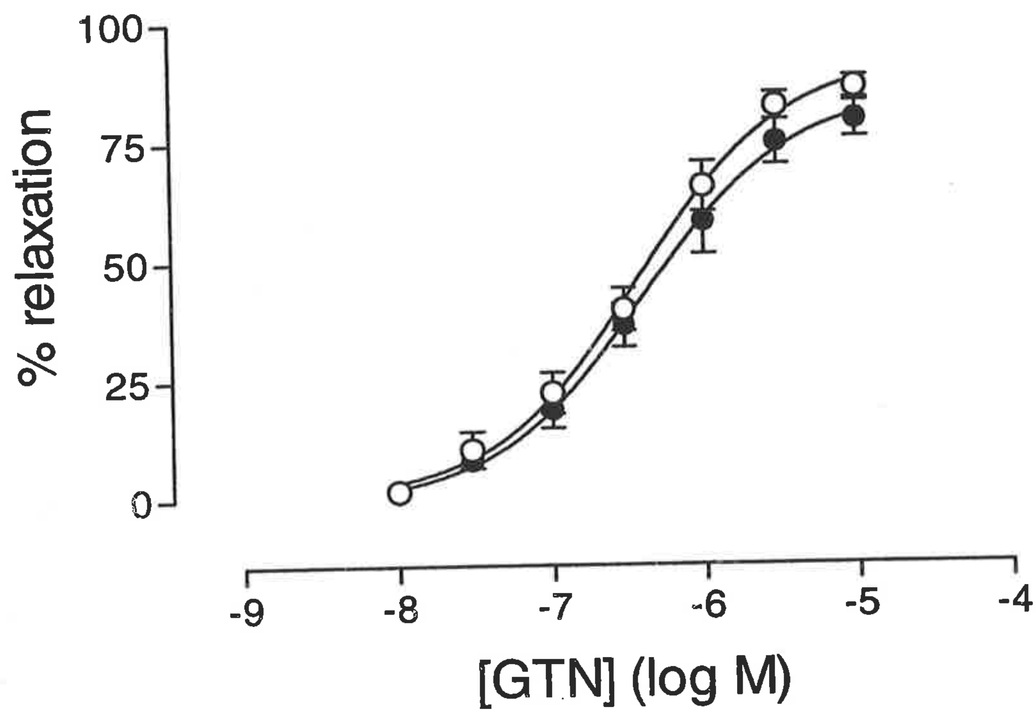


Figure 6.8
Effect of incubation with NAC (100 µM: 30 minutes) on the response to GTN of segments of SV from patients in the GTN-only group (n=5).

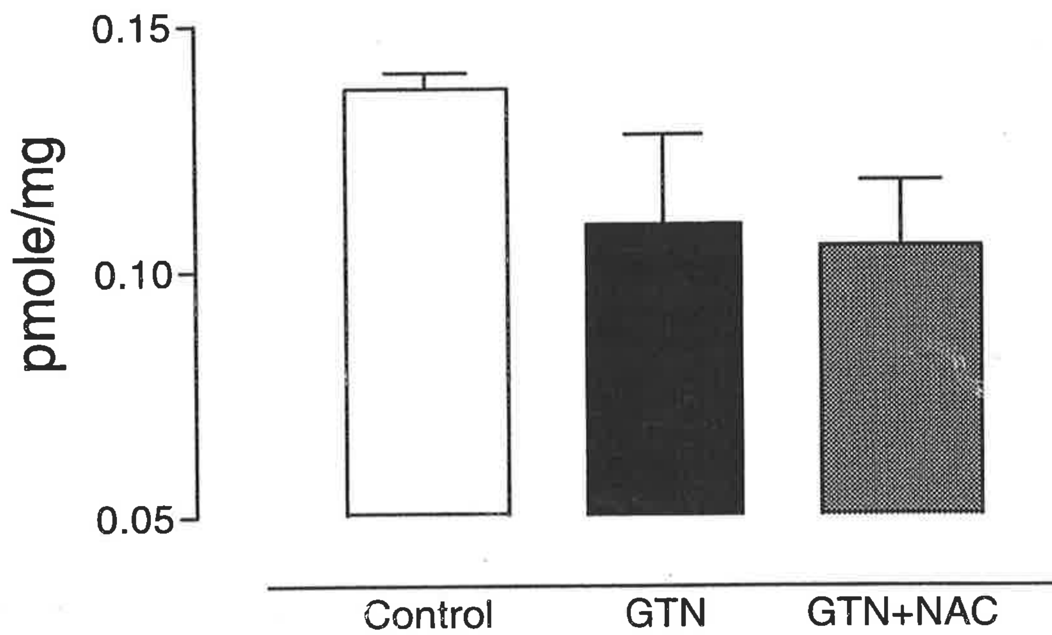


Figure 6.9
SV content of 1,2 GDN after incubation of segments with 1.0 μM GTN. 1,2-GDN content was similar in GTN-only (n=5) and GTN+NAC (n=7) groups. Control group 1,2-GDN content (n=4) is shown for comparison only.

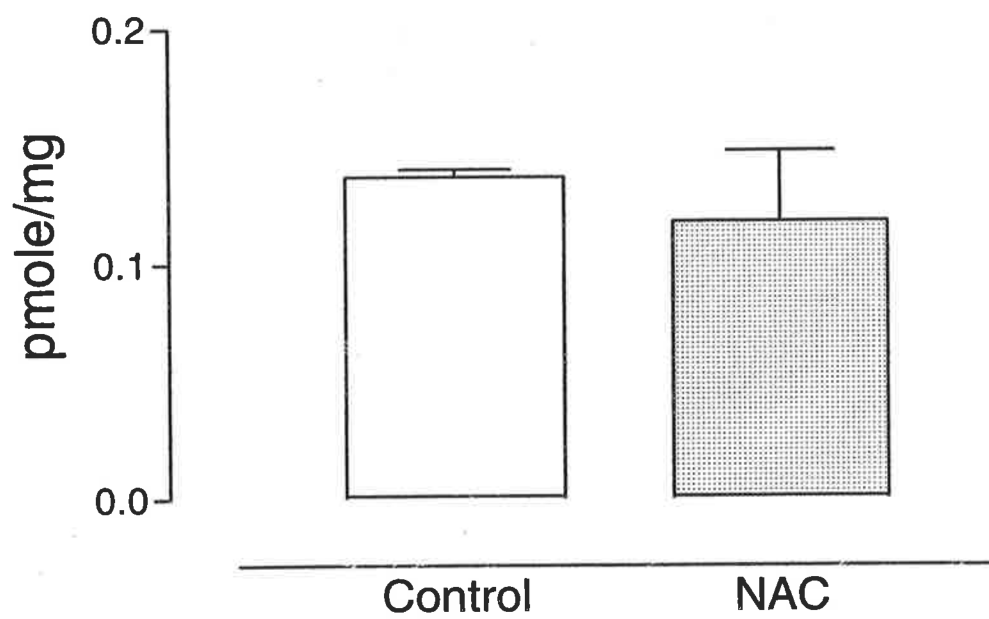


Figure 6.10
SV content of 1,2-GDN after incubation of segments from control group (n=3) and NAC-only group (n=3) with GTN 1 μM. 1,2-GDN content did not differ significantly between groups.

7. Nitrate Resistance

7.1 Introduction

The aim of the experiments described in this chapter is to examine the determinants of impaired de novo responsiveness to GTN, or GTN resistance, in isolated segments of large human arteries and veins. Historically, this term was first used to describe a phenomenon observed in the setting of severe congestive heart failure. Several workers(Armstrong 1987; Armstrong, *et al.* 1980; Elkayam, *et al.* 1987; Elkayam, *et al.* 1985; Kulick, *et al.* 1988; Packer, *et al.* 1986; Roth, *et al.* 1987; Varriale, *et al.* 1991) observed that 25-50% of patients with severe heart failure were unable to achieve a significant reduction in pulmonary artery pressure despite infusion of nitrates at high doses. This appeared to be associated with the presence of elevated right atrial pressure and severe peripheral oedema(Armstrong, *et al.* 1980; Kulick, *et al.* 1988; Packer, *et al.* 1986; Varriale, *et al.* 1991). It was suggested that these might be markers of an impaired ability to respond to vasodilators, due to a combination of abnormal mechanical forces(high tissue and extravascular pressure) and elevated vasoconstrictor forces(Abrams 1991). However, there is increasing evidence that de novo impaired responsiveness to vasodilators, including nitrates, can occur in the absence of severe heart failure.

As discussed in Chapter 1 (sections 1.7 and 1.9), a large body of data has accumulated underlying the importance of the function of the endothelium and EDRF in the regulation of vascular tone, as well as platelet and monocyte function(Celermajer 1997; Rubanyi 1993). Considerable research has been generated by the hypothesis that dysfunction of the endothelial is a precursor to atherosclerosis. Since it was first demonstrated that atherosclerosis in human coronary arteries is associated with endothelial dysfunction(Ludmer, *et al.* 1986), (manifested by a vasoconstrictor response to acetylcholine), further studies(Cox, *et al.* 1989; Forstermann, *et al.* 1988; McLenachan, *et al.* 1990; Nabel, *et al.* 1990; Zeiher, *et al.* 1993) have confirmed this observation. Others have linked endothelial dysfunction to the risk factors for coronary artery disease, including hypertension(Panza, *et al.* 1990), hypercholesterolaemia(Celermajer, *et al.* 1992; Creager, *et al.* 1990; Sorensen, *et al.*

1994; Verbeuren, *et al.* 1986; Zeiher, *et al.* 1993), diabetes mellitus(Clarkson, *et al.* 1996; McVeigh, *et al.* 1992; Watts, *et al.* 1996) and cigarette smoking(Celermajer, *et al.* 1994; Celermajer, *et al.* 1992; Zeiher, *et al.* 1995), providing support for the hypothesis that dysfunction of the endothelium is a precursor to atherosclerosis.

Endothelial dysfunction has classically been defined as an impairment of the vasodilator response to agents causing the release of NO from the endothelium, in conjunction with an intact vasodilator response to exogenous sources of NO such as GTN(Celermajer 1997). Proposed mechanisms include a failure of the signal transduction pathway for NO release or increased inactivation of NO(Cai, *et al.* 2000), perhaps by NAD(P)H generated O_2^-(Guzik, *et al.* 2000).

However, in recent years there have been an increasing number of reports of reduced responses to endothelium-independent vasodilators from workers examining the function of large vessels in the presence of specific disease states or risk factors. Abnormal endothelium-independent responses have been observed in patients with atherosclerosis(Liao, *et al.* 1991) coronary artery disease(Celermajer, *et al.* 1992; Forstermann, *et al.* 1988; Nishikawa, *et al.* 1998), and heart failure(Carville, *et al.* 1998; Katz, *et al.* 1994). This phenomenon has also been observed in subjects with risk factors for coronary artery disease, including hypercholesterolaemia(Creager, *et al.* 1990; Duffy, *et al.* 1999; Sorensen, *et al.* 1994), hypertriglyceridaemia(Lundman, *et al.* 1997), cigarette smoking(Celermajer, *et al.* 1992; Zeiher, *et al.* 1995), and diabetes(Adams, *et al.* 1998; Clarkson, *et al.* 1996; McVeigh, *et al.* 1994; McVeigh, *et al.* 1992; Watts, *et al.* 1996; Williams, *et al.* 1996). In addition, it appears that responsiveness to endothelium-independent NO donors may vary depending on race(Cardillo, *et al.* 1999), and level of fitness(Haskell, *et al.* 1993). Interestingly, there is some evidence suggesting that this phenomenon may occur in large arteries but not veins(Huvers, *et al.* 1997). Lastly, reduced responsiveness to exogenous NO donors has been demonstrated at the platelet level, in association with stable angina pectoris(Chirkov, *et al.* 1999), and in obese diabetics(Giugliano, *et al.* 1995).

Support for these observations in humans has been provided by studies in animal models of hypercholesterolaemia(Cooke, *et al.* 1991; Miller, *et al.* 1998;

Verbeuren, *et al.* 1986), diabetes mellitus(Bucala, *et al.* 1991) and hypertension(Bauersachs, *et al.* 1998; Laursen, *et al.* 1997; Rajagopalan, *et al.* 1996) which have demonstrated reduced vascular responsiveness to NO donors, and also a NO-independent stimulator of guanylate cyclase(Mulsch, *et al.* 1997).

These animal studies have also provided some of the first information regarding a possible mechanism of this "resistance" to exogenous vasodilators. Several studies have linked the phenomenon to increased O_2^- generation(Laursen, *et al.* 1997; Rajagopalan, *et al.* 1996) (Kojda, *et al.* 1998a; Mulsch, *et al.* 1997), the source of which appears to be NAD(P)H oxidase(Rajagopalan, *et al.* 1996). Of relevance in this regard, increased O_2^- generation has been demonstrated in genetic animal models of hypertension(Kerr, *et al.* 1999), and has also been shown to be induced in intact animals(Rajagopalan, *et al.* 1996) and isolated human vessels(Berry, *et al.* 2000) by angiotensin II. In the latter study, the source of the incremental O_2^- generation appeared to be both NAD(P)H oxidase and xanthine oxidase. Further supporting a role for increased O_2^- generation in resistance to NO donors, oxidized low-density lipoprotein has been shown to inhibit vascular smooth muscle relaxation and cGMP response to nitrovasodilators in vitro(Galle, *et al.* 1992).

Others studies have associated resistance with reduced soluble guanylate cyclase expression(Bauersachs, *et al.* 1998) or activity(Weisbrod, *et al.* 1997). Studies in human platelets(Chirkov, *et al.* 1999) suggest that resistance to the effects of exogenous NO donors is related to both increased O_2^- generation as well as impairment of soluble guanylate cyclase activity.

It is important to note that there have been many studies investigating endothelial dysfunction in which an impaired response to endothelium-independent vasodilators has not been seen (see (Celermajer 1997) for review). There are several possible explanations for this. Firstly, many studies use a single large dose when assessing responses to the endothelium-independent vasodilator, eliciting a near-maximal response, and are therefore unable to assess potential differences in sensitivity to these agents. Secondly, as mentioned above, there is evidence that endothelium-independent vasodilator responses may be heterogenous with regard to different

vascular beds(Huvers, *et al.* 1997), and therefore the presence of an impaired response may depend on the vessels studied. Lastly, in the studies outlined above, the degree of impairment of endothelium-independent responses has tended to be less than that of the endothelium-dependent responses. Hence, small changes in responsiveness to endothelium-independent agents may not have been detected or recognized.

Overall however, the data outlined above provide evidence supporting the concept that "dysfunction", is not limited to the endothelium, but also affects the underlying vascular smooth muscle. Clearly any heterogeneity in response to exogenous vaosdilators could have considerable clinical importance. Underscoring this fact, a recent study(Schachinger, *et al.* 2000) observed that an impaired vasodilator response to intracoronary GTN, was associated with a significantly higher risk of cardiovascular events over a 7 year period.

The availability of control data from the experiments in previous chapters made it possible to evaluate the determinants of de novo vascular hyporesponsiveness to GTN in patients with stable angina pectoris using the ex vivo isolated large vessel model.

7.2 Experimental Protocol

Study participants were patients undergoing elective CABG selected using the criteria described in Chapter 2. Patients exposed to nitrate therapy were also excluded from the study; hence all subjects were nitrate-free. During the operation, discarded segments of distal left internal mammary artery (IMA) and/or proximal saphenous vein (SV) were collected.

7.2.1 Vascular Reactivity Studies

Segments of IMA and SV were mounted in organ baths as described in Chapter 2. Following equilibration contractile responses to KCl solution were elicited to assess viability and cumulative CR curves for NA were performed, to determine pre-constriction concentration as outlined in Chapter 2.

Following further washout cumulative CR curves to GTN were performed in each segment of IMA and SV. Time from harvest of vessels to assessment of responses to GTN was held constant at 3 hours 15 minutes. Following further washout all segments were then assessed for endothelium-dependent relaxation; segments without intact endothelium were not used for analysis.

7.2.2 Vascular O_2^- Generation

Segments of IMA were obtained from another cohort of nitrate-free patients for determination of O_2^- generation via lucigenin-enhanced chemiluminescence as described in Chapter 2. These data were used to evaluate the determinants of vascular O_2^- generation in patients with stable angina pectoris.

7.2.3 Data Analysis

The CR curves of IMA and SV segments to GTN were analysed to determine the parameters log EC_{50} and E_{max}. Vascular O_2^- generation via lucigenin-enhanced chemiluminescence is expressed as counts/min/mg. The effect of the clinical characteristics of the patients on these parameters was assessed by multivariate analysis. Dependent variables were as follows: a past history of hypercholesterolaemia (defined as total cholesterol $\geq$ 5.5 mmol/L), hypertension, smoking (within previous 6 months) or diabetes mellitus, concomitant therapy with beta-adrenoceptor blockers, L-type calcium channel antagonists, or ACE-inhibitors; statin therapy was not included since this variable has a large degree of co-dependence with another variable (hypercholesterolaemia). The cumulative effect of the total number of risk factors for coronary artery disease on vascular reactivity and O_2^- generation was assessed by one-way analysis of variance (Kruskal-Wallis test).

7.3 Results

7.3.1 Determinants of Vascular Responsiveness to GTN

Vasodilator responses to GTN were studied in segments of IMA and SV obtained from 28 and 29 patients, respectively.

IMA Segments

The data for GTN relaxant responses in IMA segments are shown in Figure 7.1 (log EC_{50} data) and Figure 7.2 (E_{max} data). The results of the multivariate analysis are shown in Table 7.1.

Analysis of the log EC_{50} values revealed that the presence of each of the individual risk factors for coronary artery disease was associated with reduced responsiveness to GTN (Figure 7.1). By multivariate analysis (Table 7.1), the presence of a history of hypercholesterolaemia, smoking or diabetes mellitus was associated with significant hyporesponsiveness to GTN ($p<0.05$). A trend was also evident for age >60. In contrast, concomitant medical therapy did not appear to affect responsiveness to GTN.

With regard to the parameter E_{max}, trends towards reduced values were seen in patients with a history of hypercholesterolaemia and age >60 (Figure 7.2; Table 7.1), but these did not reach statistical significance.

Combining the risk factors for coronary artery disease (Figure 7.3) revealed a significant correlation ($p<0.01$) between the total number of risk factors and hyporesponsiveness to GTN, as assessed by log EC_{50} values. A similar trend was evident for the parameter E_{max} (Figure 7.4) but this did not reach statistical significance ($p=0.06$).

Lastly, a strong correlation was observed in IMA segments between sensitivity to GTN and sensitivity to A23187, as assessed by the respective log EC_{50} values (Figure 7.5; $p<0.001$).

SV Responses

The data for GTN relaxant responses in SV segments are shown in Figure 7.6 (log EC_{50} data) and Figure 7.7 (E_{max} data). The results of the multivariate analysis are shown in Table 7.2.

In contrast to the IMA responses, none of the individual clinical characteristics appeared to affect responsiveness to GTN, as assessed by either the log EC_{50} or E_{max} parameters.

Similarly, combining the risk factors for coronary artery disease revealed no correlation between the total number of risk factors and responsiveness to GTN (Figure 7.8 and 7.9).

7.3.2 Vascular O_2^- Generation

Vascular O_2^- generation was studied in segments of IMA from 23 patients. The data are illustrated in Figure 7.10. The results of the multivariate analysis are shown in Table 7.3.

The presence of the individual risk factors for coronary artery disease tended to be associated with higher O_2^- generation (Figure 7.10). By multivariate analysis, this reached significance in the case of prior hypercholesterolaemia and diabetes mellitus (Table 7.3).

Combining the risk factors for coronary artery disease (Figure 7.11) revealed a significant correlation ($p<0.01$) between the total number of risk factors and O_2^- generation.

7.4 Discussion

The main findings of this study are that (1) de novo vascular hyporesponsiveness (resistance) to GTN occurs in the human IMA but not the SV. (2) In the IMA, resistance to GTN is associated with the presence of increasing total number of risk factors for coronary artery disease and specifically with prior

hypercholesterolaemia, smoking and diabetes mellitus. (3) Resistance to GTN correlates with impairment of response to A23187, a conventional measure of endothelial function. (4) Increased vascular O_2^- generation is associated with the presence of increasing total number of risk factors for coronary artery disease and specifically with prior hypercholesterolaemia and diabetes mellitus.

Several previous studies have demonstrated hyporesponsiveness of large arteries to GTN in humans in vivo, in the presence of various disease states or risk factors for coronary artery disease. Impaired responses to GTN have been seen in the brachial artery in the presence of coronary artery disease(Celermajer, *et al.* 1992), Type-1 diabetes mellitus(Adams, *et al.* 1998; Clarkson, *et al.* 1996), hypercholesterolaemia(Sorensen, *et al.* 1994), hypertriglyceridaemia(Lundman, *et al.* 1997) or smoking(Celermajer, *et al.* 1992), in the forearm venous capacitance vessels in the presence of congestive heart failure(Katz, *et al.* 1994), in the femoral artery in the presence of Type-2 diabetes mellitus(Huvers, *et al.* 1997) and in the epicardial coronary arteries in the presence of coronary artery disease(Forstermann, *et al.* 1988; Nishikawa, *et al.* 1998) or smoking(Zeiher, *et al.* 1995). Similarly, impaired responsiveness to another exogenous NO donor, SNP, has been demonstrated in the femoral artery in the presence of peripheral vascular disease(Liao, *et al.* 1991) and in the forearm venous capacitance vessels in the presence of congestive heart failure(Carville, *et al.* 1998), Type-2 diabetes mellitus(Watts, *et al.* 1996; Williams, *et al.* 1996) or hypercholesterolaemia(Creager, *et al.* 1990; Duffy, *et al.* 1999). Lastly, there is evidence of heterogeneous vasodilator responses to NO donors even in normal individuals(Cardillo, *et al.* 1999; Haskell, *et al.* 1993). Ultradistance marathon runners were found to have enhanced vasodilating responsiveness in their coronary arteries to GTN as compared with inactive individuals(Haskell, *et al.* 1993). Another study suggested that black individuals have reduced forearm vasodilator responses to SNP as compared with whites(Cardillo, *et al.* 1999).

However, the above studies demonstrating reduced responsiveness (resistance) of large arteries in humans to NO donors have been performed in vivo. Hence, it is difficult to determine the relative contribution of altered vasoconstrictor forces/tone and/or impaired activity of the NO donor at the level of the vascular smooth muscle

cell. The current study is the first to demonstrate an association between impaired responsiveness to GTN and the presence of coronary artery disease risk factors, in isolated segments of human artery; it therefore provides evidence that the presence of these risk factors can impair the vasodilator effects of GTN at the level of the arterial smooth muscle cell.

A strong association was found between the total number of risk factors and hyporesponsiveness of IMA segments to GTN, as assessed by log EC_{50} values. By multivariate analysis, hypercholesterolaemia, smoking and diabetes mellitus were associated with significant hyporesponsiveness. However, trends were evident for the other age >60 and hypertension. Thus it is conceivable that with a greater number of subjects, correlations between hyporesponsiveness to GTN and these variables may have emerged. Importantly, the presence of risk factors for coronary artery disease, either individually or cumulatively, did not appear to affect responsiveness of the SV segments to GTN. Heterogeneity in responses of large arteries and veins to GTN has been seen previously in the femoral artery and vein in the presence of diabetes mellitus(Huvers, *et al.* 1997). The possible mechanism and clinical implications of this heterogeneity are discussed below.

The results of the current study are supported by several studies using animal models of specific risk factors for coronary artery disease. Isolated segments of aortae from hypercholesterolaemic rabbits have been demonstrated to have impaired vasodilator responses to GTN(Verbeuren, *et al.* 1986), SNP(Miller, *et al.* 1998) and NO itself(Weisbrod, *et al.* 1997). Similarly, isolated vessels from rats with chronic diabetes mellitus display impaired responses to GTN(Bucala, *et al.* 1991). Although the presence of hypertension was associated with only a trend towards reduced responses to GTN in the IMA in the current study, previous investigations in spontaneously hypertensive rats(Bauersachs, *et al.* 1998; Kojda, *et al.* 1998a) or rats with angiotensin II induced hypertension(Rajagopalan, *et al.* 1996) have demonstrated a significant reduction in vasodilator responses to NO donors.

Importantly, the impaired responsiveness to GTN seen in the IMA in association with an increasing number of risk factors appears to be manifested largely as an

increase in the parameter log EC_{50} rather than a reduction in the maximum relaxation(E_{max}). Furthermore, the presence of the individual risk factors did not significantly affect the E_{max} to GTN. This finding has important implications for previous studies examining vascular endothelial function which utilized a single large dose of GTN to assess endothelium-independent vasodilation. It is conceivable that such studies could completely miss a significant reduction in sensitivity to GTN. Thus, any "dysfunction" observed with the endothelium-dependent vasodilator may be erroneously attributed entirely to the endothelium, when in fact it may be partly due to abnormal response of the vascular smooth muscle to NO.

The mechanism(s) underlying the GTN resistance observed in the human IMA in this study remains uncertain. The strong correlation between responses of IMA to GTN and A23187, an endothelium-dependent vasodilator and conventional measure of endothelial function, suggests that the two phenomena, GTN resistance and "endothelial dysfunction" may share a common mechanism. This raises the possibility that GTN resistance is due to increased vascular O_2^- generation, since there is increasing evidence that oxidant stress plays a pivotal role in endothelial dysfunction(Cai, *et al.* 2000). This concept is supported by the results from the patients in which IMA O_2^- generation was examined. As with vascular reactivity to GTN, there was a strong correlation between increasing total number of risk factors for coronary artery disease and IMA O_2^- generation. In addition the presence of the individual risk factors tended to be associated with higher O_2^- generation; by multivariate analysis, this reached significance in the case of prior hypercholesterolaemia and diabetes mellitus. This finding is consistent with previous studies examining the determinants of vascular O_2^- production in human vessels, which have found similar correlations with prior hypercholesterolaemia(Guzik, *et al.* 2000; Huraux, *et al.* 1999) and diabetes mellitus(Guzik, *et al.* 2000) The role of oxidant stress in resistance to NO donors is also supported by previous studies in platelets from obese diabetics(Giugliano, *et al.* 1995) and patients with stable angina pectoris(Chirkov, *et al.* 1999) and in animal models of hypercholesterolaemia(Galle, *et al.* 1992; Miller, *et al.* 1998) and hypertension(Bauersachs, *et al.* 1998; Rajagopalan, *et al.* 1996) which have also found elevated O_2^- levels in association with resistance to NO donors.

However, a number of issues regarding the potential mechanism(s) of GTN resistance in blood vessels remain unclear. Firstly, the presence of elevated O_2^- generation does not necessarily imply a causal role. Indeed there is evidence of reduced expression and activity of soluble guanylate cyclase in association with resistance to NO/NO donors(Bauersachs, *et al.* 1998; Chirkov, *et al.* 1999; Galle, *et al.* 1992; Weisbrod, *et al.* 1997), which may be independent of O_2^- generation.

Secondly, the mechanism(s) by which increased O_2^- generation might impair responses to NO/NO donors is uncertain. While increased NO inactivation by O_2^- has been postulated to play a role in "endothelial dysfunction"(Cai, *et al.* 2000), the experiments described in Chapter 4.1 suggest that the relaxant response to GTN in human IMA is relatively unaffected by acute elevations in O_2^- generation. In addition, normalization of endothelial O_2^- generation in aortae from hypercholesterolaemic rabbits via transduction of SOD(Miller, *et al.* 1998) or in aortae of spontaneously hypertensive rats via pretreatment with SOD(Bauersachs, *et al.* 1998), does not appear to normalize the vasodilator response to SNP. The lack of effect of acute modification of oxidant stress on GTN and SNP responses suggests that resistance to NO/NO donors is not due to increased clearance of NO by O_2^-, but rather that it is caused by (redox-sensitive) modification of one or more components of the soluble guanylate cyclase-relaxation cascade (including possibly soluble guanylate cyclase itself) either via exposure to elevated O_2^- levels or by accumulation of O_2^- generated compounds, such as peroxynitrite or oxidized low-density lipoprotein. This also raises the possibility of hysteresis between the presence of elevated O_2^- levels and the development of impaired responses. This might provide an explanation for the discrepancy seen between the effect of smoking on GTN responses and O_2^- levels, as many of the smokers in the study may have been abstinent for several months.

Thirdly, the study did not investigate the source(s) of the incremental O_2^- generation. Possible sources include NAD(P)H-oxidase, xanthine oxidase and "dysfunctional" endothelial NO synthase. In this regard, a recent study(Guzik, *et al.* 2000) found that NAD(P)H-oxidase is the predominant source of O_2^- generation in segments of human saphenous vein, and that increased NAD(P)H-oxidase activity is associated with reduced endothelial NO bioavailability, as well as with the specific risk

factors hypercholesterolaemia and diabetes mellitus. These associations are consistent with those found in the current study, suggesting the source of observed incremental IMA O_2^- generation may be NAD(P)H-oxidase. Other workers(Rajagopalan, *et al.* 1996; Wang, *et al.* 1998) have demonstrated that the major source of elevated vascular O_2^- production in angiotensin II-mediated hypertension in rats is also NAD(P)H-oxidase. On the other hand a recent study(Berry, *et al.* 2000) found that both NAD(P)H-oxidase and xanthine oxidase contribute equally to basal O_2^- generation in segments of human IMA and SV. However, in the same study, exposure to angiotensin II induced further elevation of O_2^- levels via a largely NAD(P)H-oxidase-dependent mechanism. Lastly, while there is evidence that endothelial NO synthase can become uncoupled resulting in incremental O_2^- generation, animal studies have suggested the excess O_2^- production in models of hypercholesterolaemia(Miller, *et al.* 1998) and hypertension(Rajagopalan, *et al.* 1996) is not localized to the endothelium, but occurs throughout the vascular wall. Overall, therefore current evidence suggests the major source of the incremental O_2^- generation seen in this study is likely to be NAD(P)H-oxidase.

Interestingly, the study by Berry et al(Berry, *et al.* 2000) also demonstrated that in comparison with segments of IMA, O_2^- generation was significantly lower in segments of SV and was not increased by exposure to angiotensin II. These findings may provide an explanation for the absence of GTN resistance in large veins found in this study and that of Huvers et al(Huvers, *et al.* 1997).

A final issue with regard to potential mechanisms of GTN resistance is the extent to which resistance is specific for NO donors or NO. There is some evidence suggesting that vasodilator responses to agents which activate adenylate cyclase to generate adenosine 3',5'-cyclic monophosphate (cAMP), may also be impaired in certain clinical situations(Cardillo, *et al.* 1999; Galle, *et al.* 1992). Galle et al(Galle, *et al.* 1992) found that oxidized low density lipoproteins inhibited the responses of human IMA to both forskolin, an activator of adenylate cyclase, and SNP, which activates guanylate cyclase. Similarly, a more recent study(Cardillo, *et al.* 1999), demonstrated that vasodilator responses to both isoproterenol (mediated via adenylate cyclase) and SNP were attenuated in black subjects as compared with whites. These findings suggest

that "resistance" is not restricted to activators of soluble guanylate cyclase, but may affect all cyclic nucleotide-mediated vasodilators.

This study has several important clinical implications. Firstly, it suggests that the potential therapeutic benefit of organic nitrates may be adversely affected by the clinical characteristics of a patient. Hence patients with risk factors for coronary artery disease may have sub-optimal de novo responses to nitrates, in addition to the potential for the development of tolerance with continuous therapy. Similarly, the finding of a correlation between responses to GTN and responses to A23187, a conventional measure of endothelial function implies that individuals with "endothelial dysfunction" of the coronary arteries, in whom the greatest response to supplemental exogenous NO is desired(Bassenge 1994), may in fact have impaired responses.

Secondly, the study also raises the possibility that modulation of patient risk factors may improve the clinical efficacy of nitrates in the management of myocardial ischaemia. Thus, reduction of the number of uncontrolled risk factors might improve the responsiveness of the large epicardial coronary arteries to acute or chronic nitrate therapy, with the potential for greater relief of symptoms. Conversely, the findings in the SV segments suggest the effect on venodilator response to nitrates would be minimal and hence modulation of risk factors may have little effect on nitrate efficacy in the setting of congestive heart failure.

Lastly, there is evidence that GTN resistance in the coronary arteries may have adverse prognostic implications with regard to future cardiovascular events(Schachinger, *et al.* 2000). Hence investigating the determinants and mechanism of GTN resistance is of critical importance, since it may provide further insight into the relationship between the individual risk factors for coronary artery disease and acute coronary events.

Clearly, more research is required in the area of nitrate resistance. Future studies should address issues such as (1) the precise mechanism and relationship to the mechanism of "endothelial dysfunction" (2) the source of the incremental arterial O_2^- production (3) the apparent arterial-venous heterogeneity and (4) whether improved risk

factor control results in improved nitrate efficacy. These priorities are addressed in greater detail in Chapter 8.

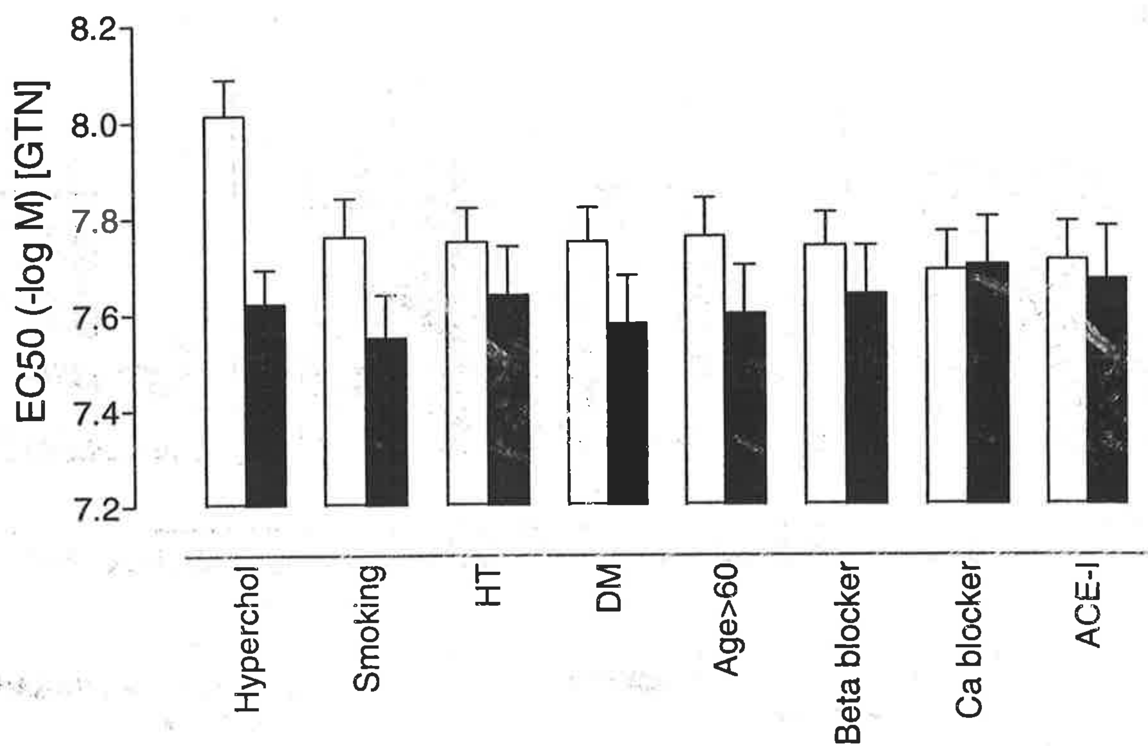


Figure 7.1
Relationship between clinical characteristics and vascular responses of segments of IMA (n=28) to GTN as assessed by log EC_{50}. Open bars indicate the absence of the clinical variable, closed bars the presence. The presence of hypercholesterolaemia, smoking or diabetes mellitus was associated with significant hyporesponsiveness to GTN by multivariate analysis (see Table 7.1; $p<0.05$ for each variable).

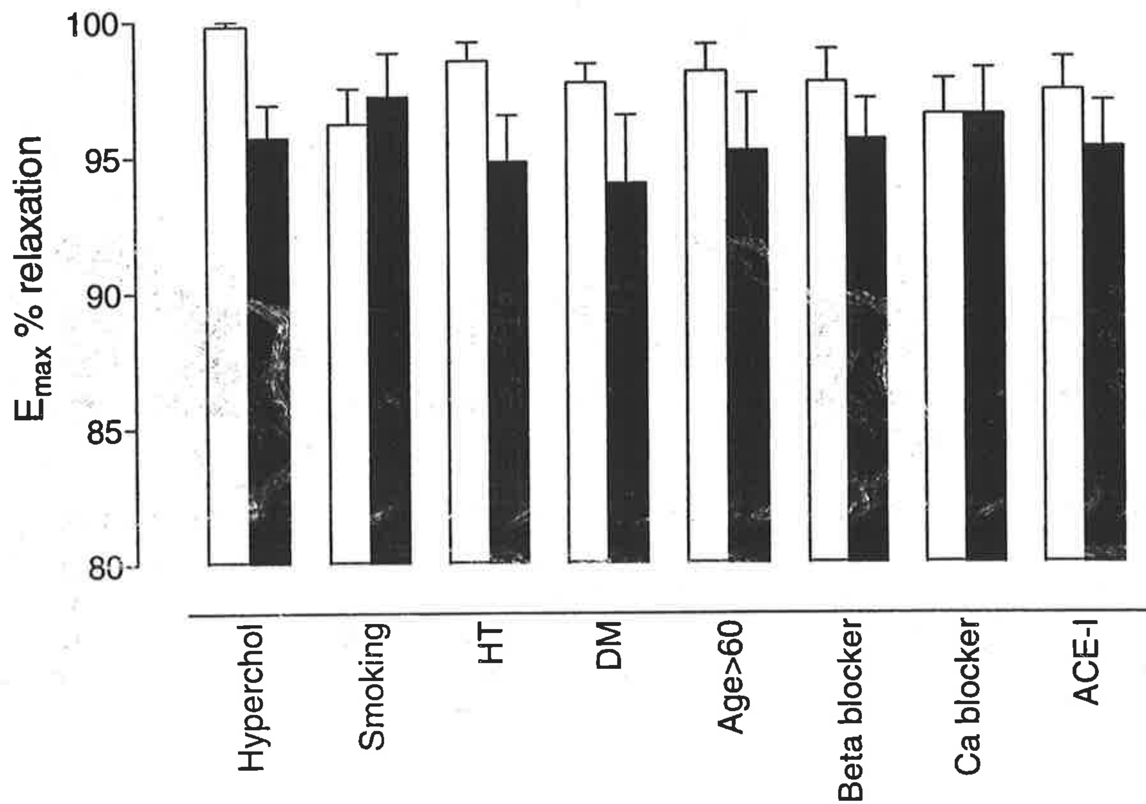


Figure 7.2
Relationship between clinical characteristics and vascular responses of segments of IMA (n=28) to GTN as assessed by E_{max}. Open bars indicate the absence of the clinical variable, closed bars the presence. E_{max} was not significantly affected by the individual although trends were evident (see Table 7.1).

Table 7.1

Determinants of relaxant responses to IMA to GTN as assessed by log EC_{50} and E_{max} values: Multivariate analyses.

Variable	Number of subjects with variable	log EC_{50}		E_{max}	
		reg coeff	p value	reg coeff	p value
Hypercholesterolaemia	23	0.47	0.013	-0.37	0.1
Smoking	9	0.60	0.012	-0.10	0.7
Hypertension	15	0.26	0.16	-0.31	0.3
Diabetes mellitus	9	0.46	0.04	-0.20	0.5
Age >60	15	0.38	0.06	-0.42	0.06
Beta-adrenoceptor blocker	16	0.22	0.2	-0.17	0.4
Calcium antagonist	8	-0.22	0.3	0.17	0.5
ACE-inhibitor	12	-0.11	0.6	0.03	0.9

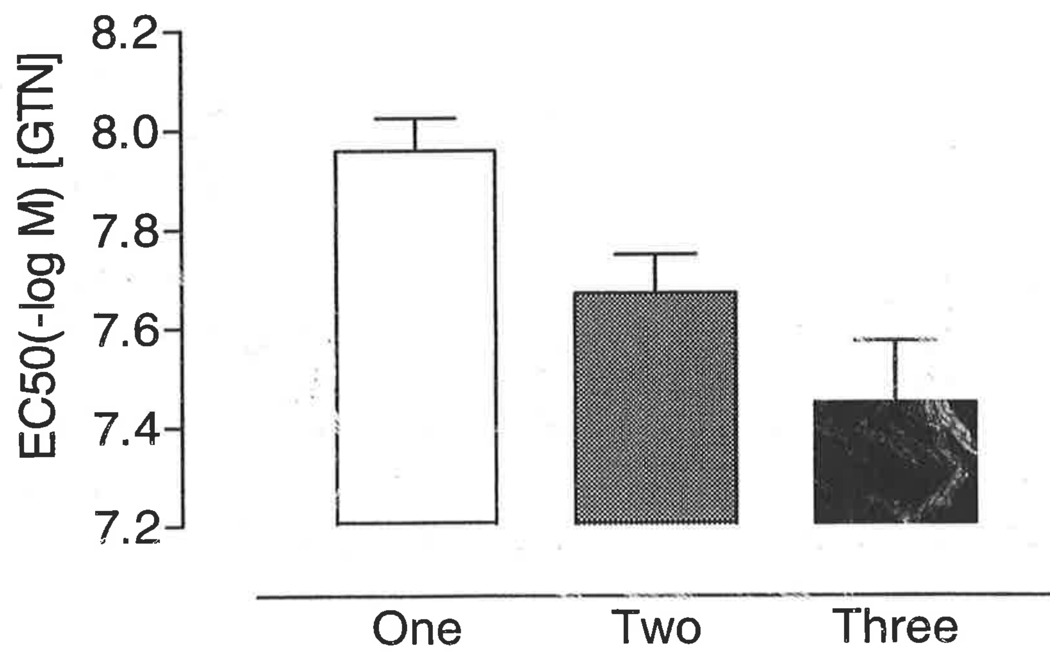


Figure 7.3
Effect of total number of risk factors for coronary artery disease on responses of segments of IMA (n=28) to GTN as assessed by EC_{50}. $p < 0.01$ one-way ANOVA.

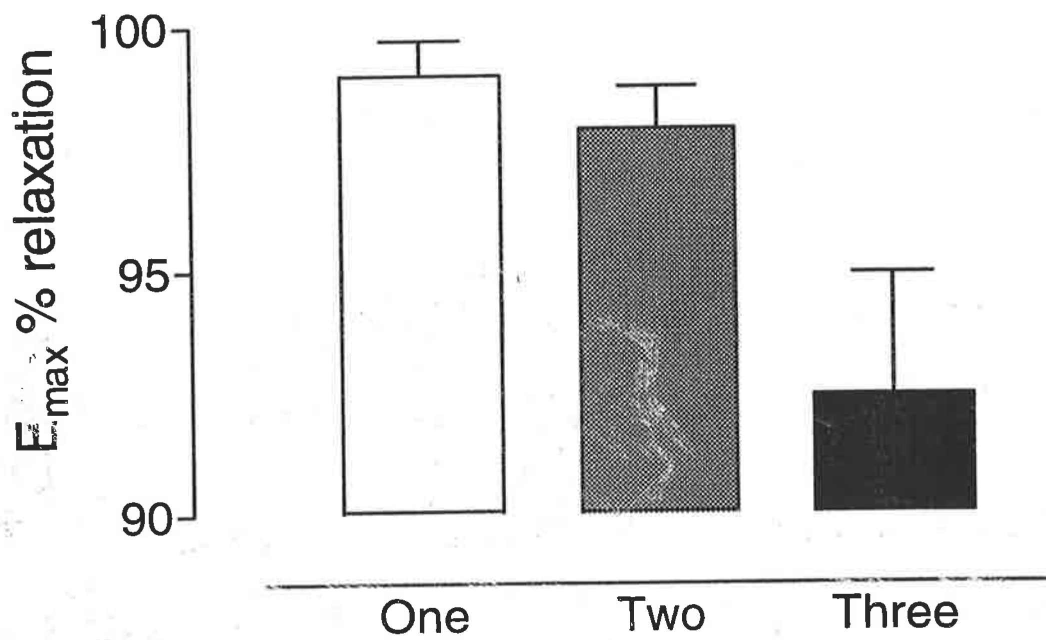


Figure 7.4
Effect of total number of risk factors for coronary artery disease on responses of segments of IMA to GTN as assessed by E_{max}. p =0.06 one-way ANOVA.

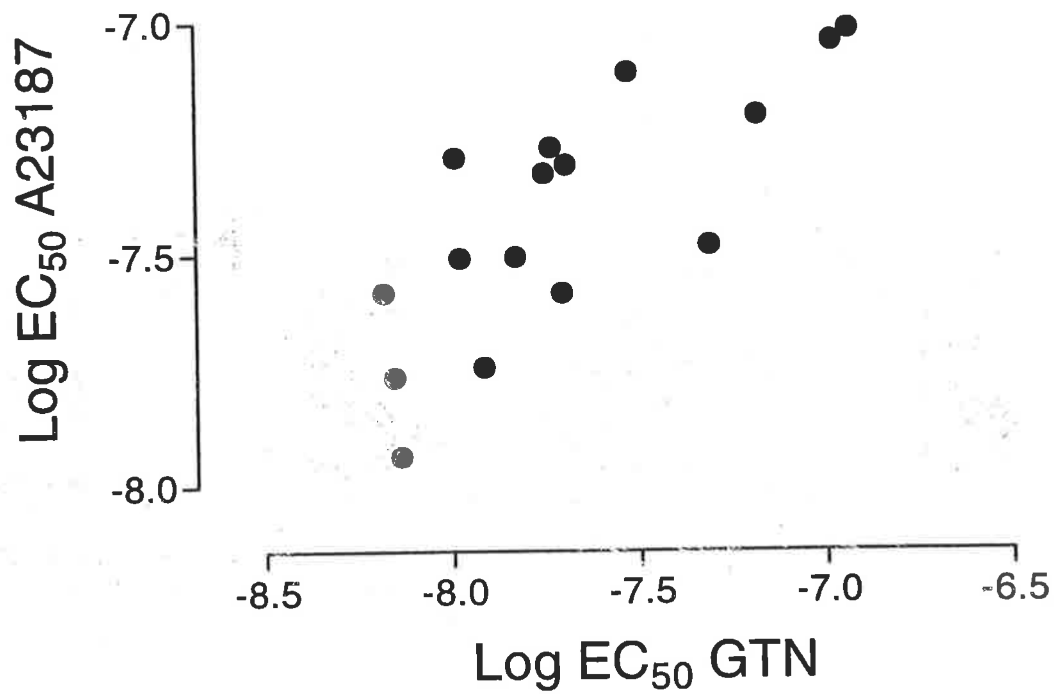


Figure 7.5
Correlation between sensitivity of segments of IMA to GTN and sensitivity to A23187 as assessed by respective log EC_{50} values (r^2 0.60; $p<0.001$).

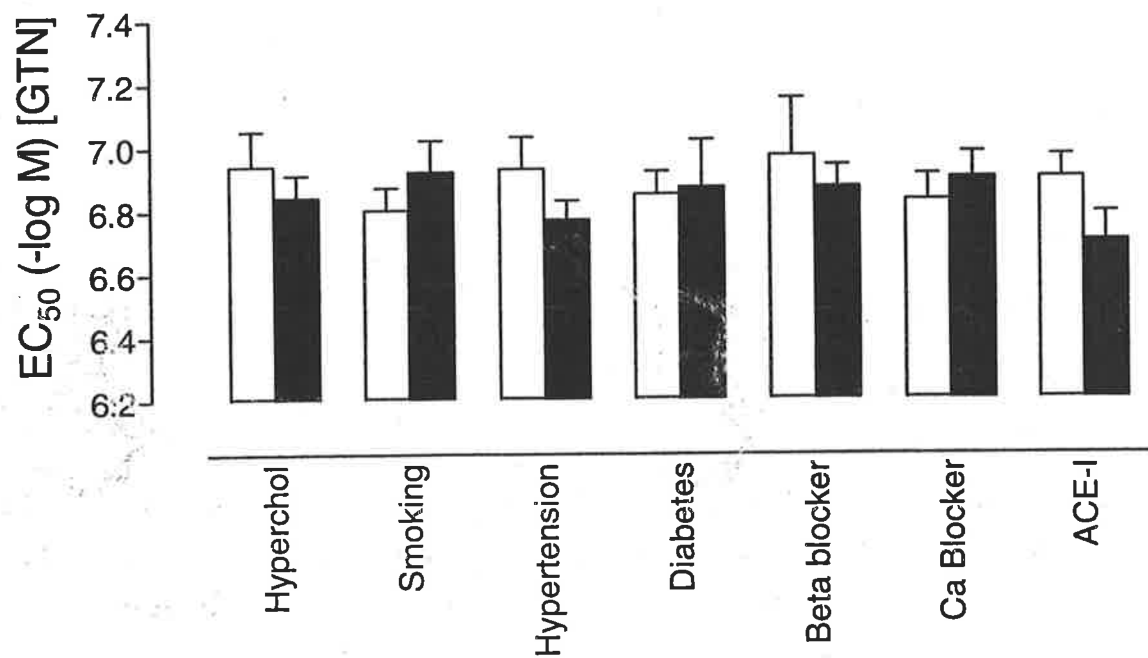


Figure 7.6
Relationship between clinical characteristics and vascular responses of segments of SV (n=29) to GTN as assessed by log EC_{50}. Open bars indicate the absence of the clinical variable, closed bars the presence. See Table 7.2 for multivariate analysis.

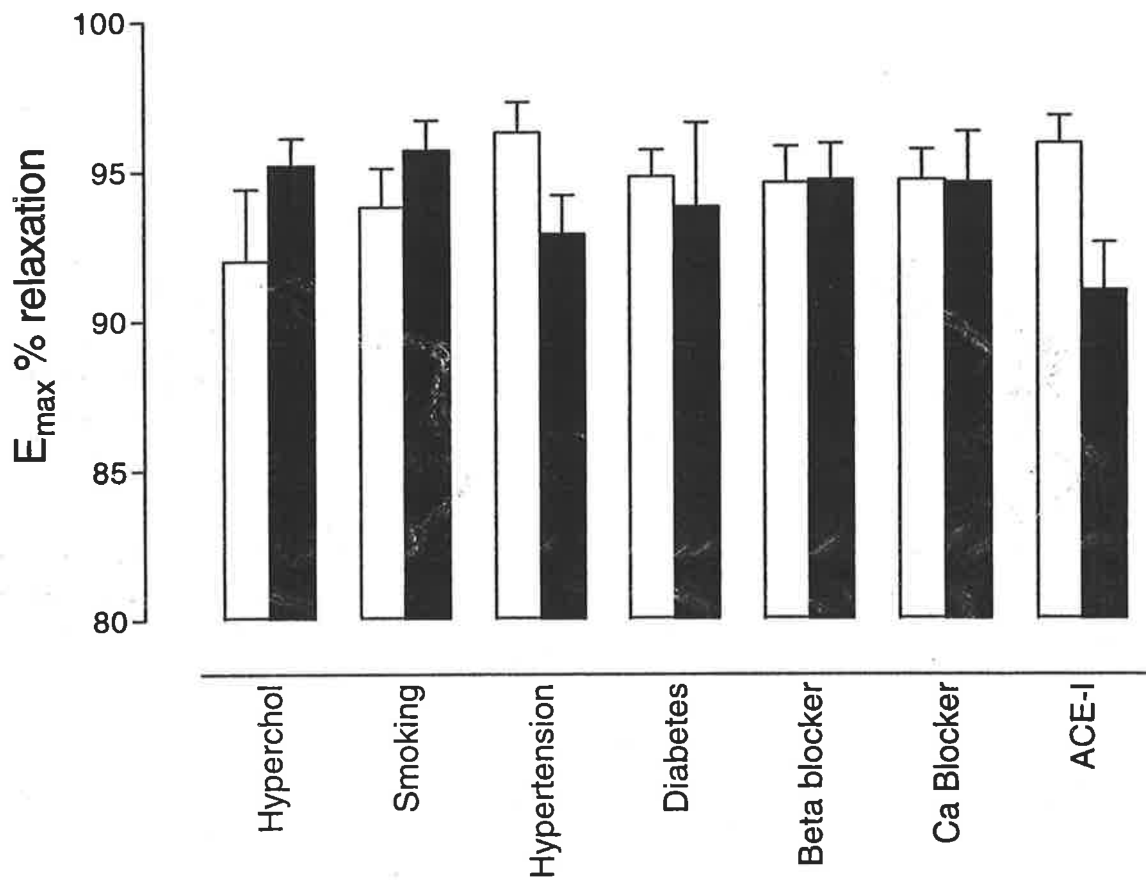


Figure 7.7
Relationship between clinical characteristics and vascular responses of segments of SV (n=29) to GTN as assessed by E_{max}. Open bars indicate the absence of the clinical variable, closed bars the presence. See Table 7.2 for multivariate analysis.

Table 7.2

Determinants of relaxant responses to SV to GTN as assessed by log EC_{50} and E_{max} values: Multivariate analyses.

Variable	**Number of subjects with variable**	**log EC_{50}**		**E_{max}**	
		reg coeff	p value	reg coeff	p value
Hypercholesterolaemia	24	0.17	0.4	0.31	0.1
Smoking	13	-0.18	0.5	0.21	0.3
Hypertension	14	0.38	0.1	-0.39	0.07
Diabetes mellitus	4	-0.35	0.2	0.25	0.3
Beta-adrenoceptor blocker	15	-0.12	0.6	-0.06	0.7
Calcium antagonist	11	-0.37	0.1	0.17	0.4
ACE-inhibitor	8	0.22	0.4	-0.32	0.2

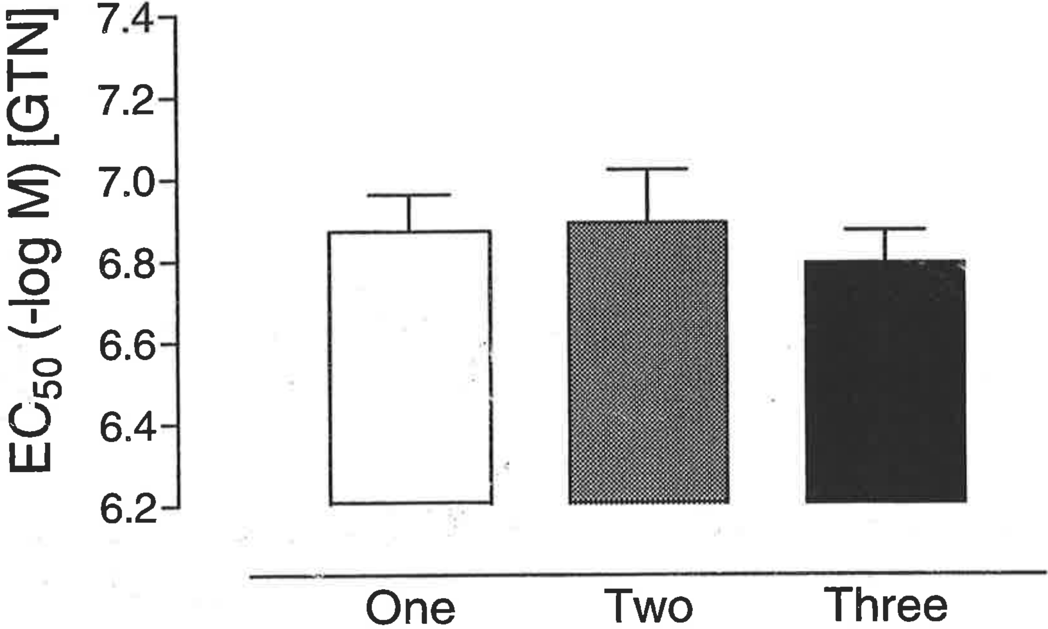


Figure 7.8
Effect of total number of risk factors for coronary artery disease on responses of segments of SV (n=29) to GTN as assessed by EC_{50}. P >0.05.

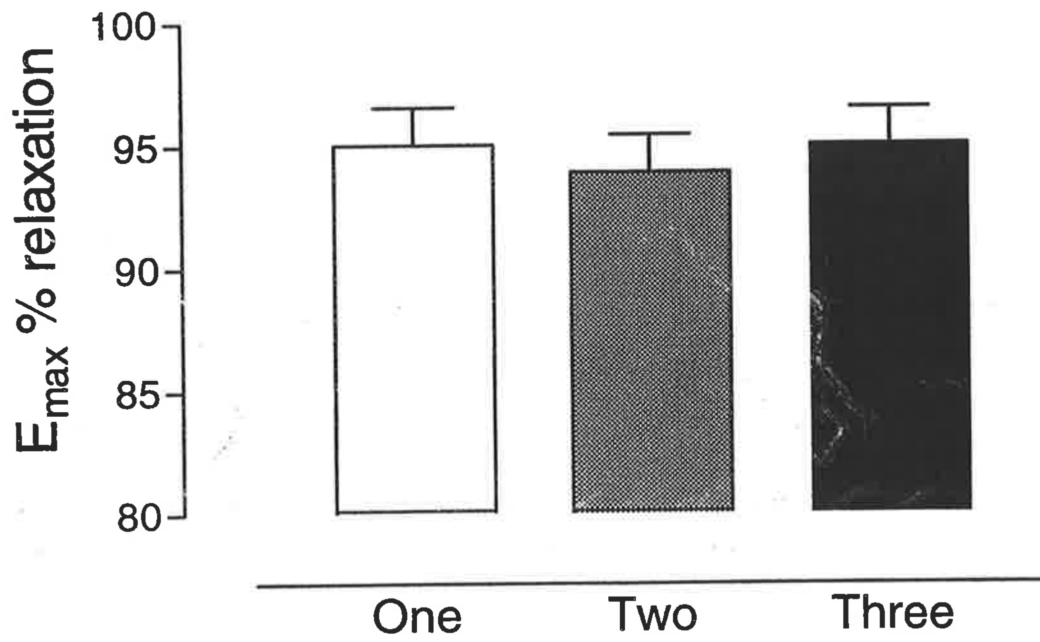


Figure 7.9
Effect of total number of risk factors for coronary artery disease on responses of segments of SV (n=29) to GTN as assessed by E_{max}. $P > 0.05$.

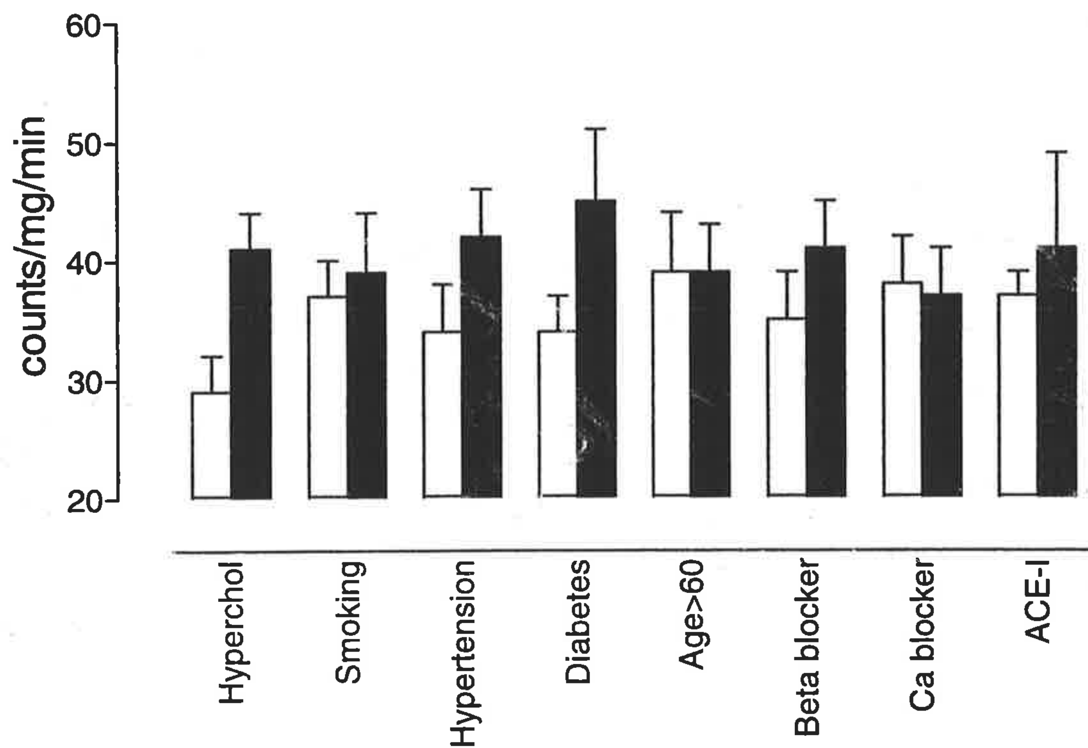


Figure 7.10
Relationship between clinical characteristics and lucigenin-enhanced chemiluminescence in segments of IMA (n=23). Open bars indicate the absence of the clinical variable, closed bars the presence. The presence of hypercholesterolaemia was associated with significantly greater O_2^- generation by multivariate analysis (see Table 7.3; $p<0.05$).

Table 7.3

Determinants of IMA O_2^- generation assessed via lucigenin-enhanced chemiluminescence: Multivariate analysis.

Variable	Number of subjects with variable	reg coeff	p value
Hypercholesterolaemia	17	0.40	0.048
Smoking	6	0.31	0.16
Hypertension	11	0.08	0.73
Diabetes mellitus	7	0.39	0.053
Age >60	13	0.03	0.9
Beta-adrenoceptor blocker	10	0.14	0.5
Calcium antagonist	10	0.13	0.6
ACE-inhibitor	6	0.12	0.6

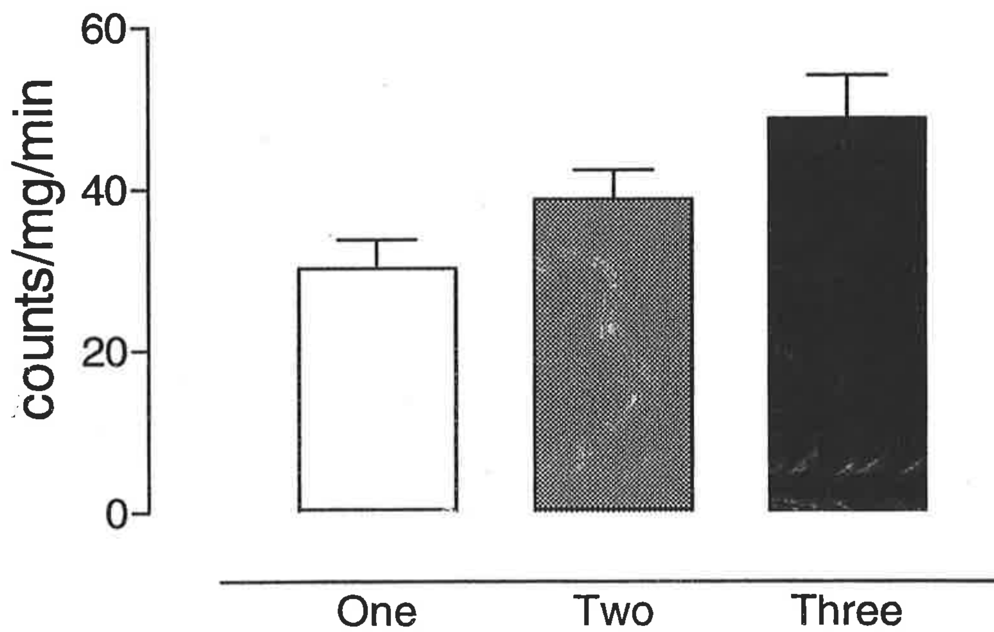


Figure 7.11
Effect of total number of risk factors on lucigenin-enhanced chemiluminescence in segments of IMA (n=23). $p < 0.05$ one-way ANOVA.

8. General Discussion and Future Directions

A major aim of the experiments described in this thesis was to examine the characteristics of nitrate action in arteries and veins from patients undergoing coronary bypass surgery to provide insight into the mechanism of nitrate tolerance and the determinants of de novo nitrate responsiveness in humans. With regard to mechanism of nitrate tolerance almost all previous studies have been confined to animal vessels. As outlined in the introduction (Section 1.8), most of these have used nitrate doses or concentrations much higher than those used in humans and have yielded conflicting information. These factors make it imperative to study human vessels under conditions of normal therapeutic nitrate administration. Hence, the first question is what have the present studies achieved?

In Chapter 3, the potential of the IMA and SV as ex vivo models of tolerance was indicated by the finding that following the relatively modest regimen of 10 μg/minute of intravenous GTN for 24 hours, both vessels were tolerant to GTN when tested 3 hours after their isolation. Further studies revealed a high degree of specificity of tolerance for the nitrate, as indicated by the failure to observe reduced sensitivity to a non-nitrate NO donor (SNP) and to a generator of NO from the endothelium (A23187) in the tolerant vessels. This nitrate- (as distinct from NO-) specificity of tolerance was reinforced by the demonstration in Chapter 4.4 that vasodilator responses to GTN and SNP in human vessels were equally sensitive to blockade by ODQ, providing strong evidence that both are mediated by guanylate cyclase activation. The situation appears somewhat different with A23187, because inhibition by ODQ was incomplete, implying that a component of the vasodilator responses to this agent was independent of NO. However, the inhibition by ODQ was substantial enough to suggest that NO was still the major component, so the absence of cross-tolerance to this agent is suggestive of a lack of cross-tolerance to endogenous NO. The above characteristics of tolerance pointed to a mechanism involving inhibition of GTN bioconversion. Additional evidence in favour of this mechanism was obtained in Chapter 3 by the finding of a lower concentration of the major GTN metabolite, 1,2-GDN in the tolerant veins following brief exposure to GTN. The significance of this finding is underlined by

animal studies showing that 1,2-GDN formation is a sensitive metabolic indicator of GTN-induced relaxation.

The above experiments emphasize the importance of impairment of bioconversion as a mechanism of tolerance in the human vessel. However, an alternative hypothesis, that increased generation of O_2^- is responsible for tolerance, has gained much support in recent years, largely from animal studies. As shown in Chapter 3, the tolerant human vessel (IMA) displayed increased generation of O_2^-. This observation is apparently at variance with the evidence for the impaired bioconversion hypothesis and hence prompted the experiments in Chapter 4, designed to test whether elevated O_2^- generation could account for reduced GTN sensitivity. These experiments, carried out in non-tolerant vessels, argued against a role for the excess O_2^- in so far as 1) inhibition of SOD, which produced a 3-fold increase in O_2^- generation, did not affect GTN sensitivity and 2) DPI, a flavoprotein inhibitor which has been shown to inhibit O_2^- generation in animal models, had little effect on GTN sensitivity, although the concentration studied (1 μM) was considerably below that used in the animal tolerance studies. Nevertheless, these findings, although preliminary, did not detract from the impaired bioconversion concept, unless it is postulated that prolonged exposure to excess O_2^- in vivo results in impairment of the enzyme system responsible for bioconversion. Indeed, this possibility is not too remote when account is taken of the early evidence for a role of sulphydryls in the bioconversion of GTN and the potential for loss of thiols via interaction with O_2^-.

The role of sulphydryls in nitrate tolerance has been the subject of intense debate for many years. There is considerable evidence that the effects of GTN are potentiated in vivo by co-administration of the sulphydryl agent, NAC. However, the situation with regard to the prevention or reversal of tolerance remains uncertain. The studies in Chapter 6 demonstrated that NAC, added to the isolated tolerant vessel, was without effect on sensitivity to GTN. Furthermore, vessels from patients infused with NAC, either alone or together with GTN, displayed no change in sensitivity to GTN. Similarly, changes in bioconversion of GTN to 1,2-GDN following brief exposure to GTN were minimal. Although the numbers of subjects was small, these experiments

provided no indication in the ex vivo model of an important interaction between sulphydryls and GTN.

The final experiments related to ex vivo tolerance explored the extent to which vessels from patients receiving oral nitrate therapy (ISDN or ISMN), exhibited evidence of cross-tolerance to GTN. The problem of the appropriate dosing regimen to minimize tolerance has been the subject of a number of clinical studies and it was hoped that the study of ex vivo vessels would provide further insight into this problem. The results (Chapter 5) reinforced the bulk of the clinical studies by the finding of significant cross-tolerance to GTN, and suggested that this was more apparent after ISMN than ISDN.

The last major study in this thesis took advantage of the accumulated data in no-tolerant vessels to explore the phenomenon of nitrate resistance. This concept is not universally accepted, although an increasing number of studies in human subjects and ex vivo animal models are providing support for its existence. The results in Chapter 7 demonstrated that subsensitivity to GTN in IMA is associated with prior hypercholesterolaemia, smoking, or diabetes mellitus, as well as increasing total number of risk factors for coronary artery disease. This was evident predominantly as a change in sensitivity rather than a reduced maximum response, and was not evident in the SV. Further experiments demonstrated an association between arterial O_2^- generation and hypercholesterolaemia, suggesting the phenomenon might be related to increased redox stress. The reason for the arterial-venous heterogeneity and the source of the O_2^- remain to be evaluated.

In summary, the various studies reported in the thesis have focused on ex vivo studies of human arteries and have provided mechanistic information concerning nitrate tolerance, as well as demonstration of the existence of nitrate resistance.

8.1 Major limitations and future directions

The ultimate objective of any investigation of the pharmacology of the organic nitrates should be to advance the therapeutic efficacy of this group of agents. As outlined in Chapter 1, it is clear that organic nitrate efficacy in the vasculature is potentially limited by three factors, resistance (de novo hyporesponsiveness), tolerance

and pseudo-tolerance. The results of the current experiments can be combined with those of previous investigations to provide a conceptual schematic (Figure 8.1) for the predominant mechanisms of these anomalies. It is clear that all three can exert incremental effects in impairing vasodilator responsiveness to organic nitrates.

The experiments in the current thesis have addressed the occurrence of nitrate resistance within the IMA primarily from a phenomenological, rather than a mechanistic, point of view. This is largely inevitable in the model studied, since it is impossible to obtain IMA from "normal" subjects and hence properly controlled studies cannot be performed. Thus full controlled studies of nitrate resistance using ex vivo approaches (to eliminate effects of circulating vasoactive agents), will remain limited to platelets(Chirkov, *et al.* 1999) and possibly small subcutaneous arteries. On the other hand it would be possible to use IMA from nitrate-free individuals with coronary disease for intervention studies aimed at minimization of nitrate resistance. Such studies might be performed using the population identified in the current study to have the greatest resistance (ie. those with multiple coronary risk factors). Potential interventions include lipid-lowering, or agents which inhibit NAD(P)H oxidase, such as ACE-inhibitors. Diabetics, not closely studied in this thesis, would be an important population for detailed study: it would seem worthwhile specifically to evaluate the possible benefit of improved diabetic control on nitrate/NO responsiveness. It would also be important to extend the resistance studies in two ways:-

(a) Biochemical: To evaluate whether changes in nitrate responsiveness are paralleled by changes in expression or activity of eNOS or agents modulating its activity (such as tetrahydrobiopterin and/or ADMA) or by changes in vascular production of O_2^- and the various enzymes catalysing its formation (notable NAD(P)H oxidase).
(b) Physiological correlations: Parallel studies should be performed in intact vascular beds, especially the coronary arteries, but perhaps also the forearm vessels, and in platelets, in order to determine whether the heterogeneity of resistance between IMA and SV in the current experiments is indicative of wide variability between vessels and tissues.

Although of lesser priority, it would also be potentially important to identify whether nitrate/NO resistance occurs in other groups of individuals not specifically

studies in the current work, but in whom "endothelial dysfunction" has been reported, such as those with renal dysfunction.

The major advance resulting from the experiments described in this thesis, as indicated earlier, has been the demonstration that nitrate tolerance results primarily from impairment of nitrate bioconversion. This finding is of considerable clinical importance. One implication is that it is unlikely that purely redox-based therapies (such as vitamin E or C) will attenuate tolerance development unless bioconversion is affected by redox state, although such therapies might conceivable ameliorate nitrate resistance.

What further steps need to be taken before the findings concerning tolerance induction can be translated into advances in nitrate therapeutics? Clearly more focus on nitrate bioconversion would be useful. As outlined in Chapter 1, previous studies have identified three major categories of enzymes that may release NO from nitrate within blood vessels. Of these, the sulphydryl-dependent microsomal enzyme characterized by Fung and coworkers seems of greatest importance, though cyctochrome-P450-dependent mechanisms cannot be ignored. Further experiments may rely heavily on changes in activity of these enzymes, provided they can be studied with greater precision. It is clearly of importance to determine the precise role of sulphydryl availability in nitrate bioconversion, especially in the coronary vessels and hence determine whether bioconversion is likely to be altered by changes in redox state.

Such studies should also help to resolve the complicated issue of therapeutic mechanisms of efficacy of sulphydryl agents, such as NAC. Although identified as one of the most effective thiols for potentiating stimulation of soluble guanylate cyclase by GTN in broken cell preparations, clinical efficacy of NAC in myocardial ischaemia simply be ascribed to limitation of nitrate tolerance. The possibility cannot be excluded that part of the effect is due to reversal of nitrate resistance via a redox based mechanism. However, it is still possible that an ex vivo examination of vascular pharmacology is not the right approach to study NAC effects. Perhaps an ideal approach would be to examine its interaction with GTN in vivo with controlled studies

using quantitative coronary arteriography and/or applanation tonometry to examine changes in vascular tone.

The approach of examination of nitrate bioconversion is also promising for evaluation of purported "tolerance-immune nitrates" such as penta-erythritol tetranitrate and possibly nicorandil. It would be possible not only to examine changes in bioconversion, but also to evaluate the possibility that while relatively free of tolerance themselves, they induce cross-tolerance to GTN(Henry, *et al.* 1990).

Finally, it is appropriate to add a cautionary note concerning <u>pseudo-tolerance</u>. This problem, which provides the basis for the occurrence of nitrate "rebound" and the "zero-hour" phenomenon, has not been addressed in the experiments in this thesis. Experiments in pseudo-tolerance represent a potential ethical problem in human studies, and probably the only acceptable methodology would involve comparison of "nitrate-free" periods with baseline data in blinded studies. A purely ex vivo approach, while permitting study of the changes in reactivity to vasoconstrictor agents, would miss changes in circulating vaso-active materials. Hence, such studies might best be performed in whole animal models of chronic nitrate therapy. Pseudo-tolerance, while the most difficult component of the factors limiting nitrate efficacy to study, may conceivably have a profound effect on clinical outcomes, and hence may prove on careful epidemiological studies to be of great interest.

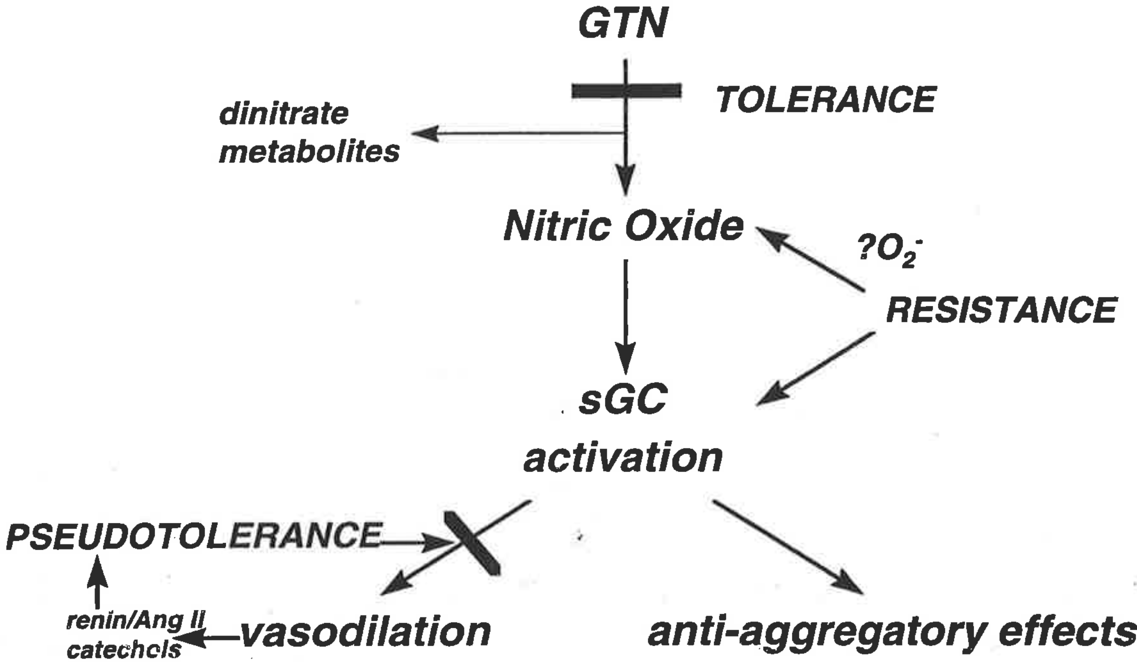


Figure 8.1
Factors potentially limiting organic nitrate efficacy.

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Nitroglycerin Tolerance in Human Vessels

Evidence for Impaired Nitroglycerin Bioconversion

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Addenda and Corrigenda

Chapter 1

Page 8: As stated, diphenyleneiodonium (DPI) inhibits conversion of GTN to 1,2-GDN in rat aorta. DPI is also known to inhibit NO synthase (NOS) mediated NO production in the endothelium and NADPH mediated superoxide production in smooth muscle cells. Hence, DPI may alter responsiveness to GTN via mechanisms other than impairment of bioconversion.

Page 18: NO has been demonstrated to have a number of additional effects on the myocardium and vasculature not mentioned in Section 1.5. Since the active moiety of the organic nitrates is probably NO these effects may therefore be of relevance. These NO effects include inhibition of L-type calcium channels, anti-proliferative and anti-hypertrophic effects, as well as either proapoptotic or anti-apoptotic effects depending on the concentration of NO or whether the NO is generated by iNOS or eNOS. NO is also postulated to play a role in ischaemic preconditioning. The anti-ischaemic implications of the recently demonstrated effects of NO on the efficiency of the electron transfer chain (Vallance et al 2001), have not been evaluated fully.

Chapter 2

Page 56: Cumulative CR curves to GTN were found to be monophasic, in contrast to a biphasic response found in animal arterial segments (Henry et al 1989c; Malta 1989; de la Lande 1990b).

Page 57: In some experiments the endothelium was denuded by abrasion. The efficacy of this removal was tested functionally via relaxant response to A23187. The vessels were not viewed microscopically. Hence, there may not have been complete anatomical abolition of the endothelium.

Page 58: Vascular superoxide production was normalized to wet weight. While these measurements could have been normalized to dry, rather than wet weight, it is unlikely that the use of wet weight would have introduced any bias in the study.

Chapter 2: Additional Figures

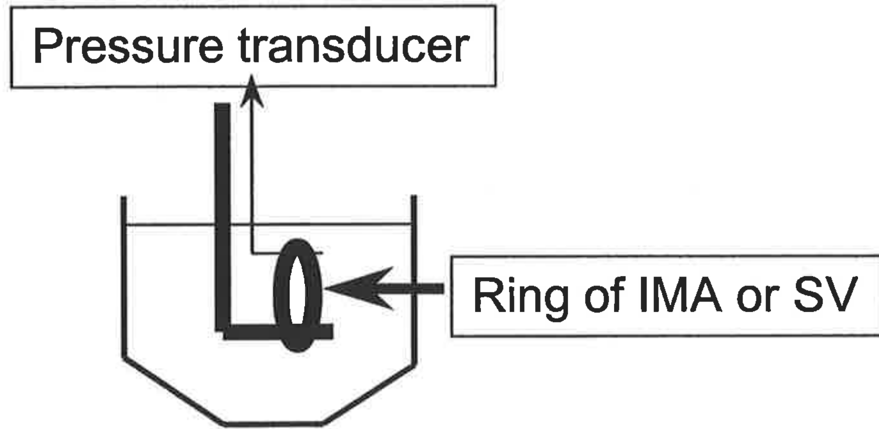

Figure 2.1. Segment of vessel mounted on pressure transducer in organ bath.

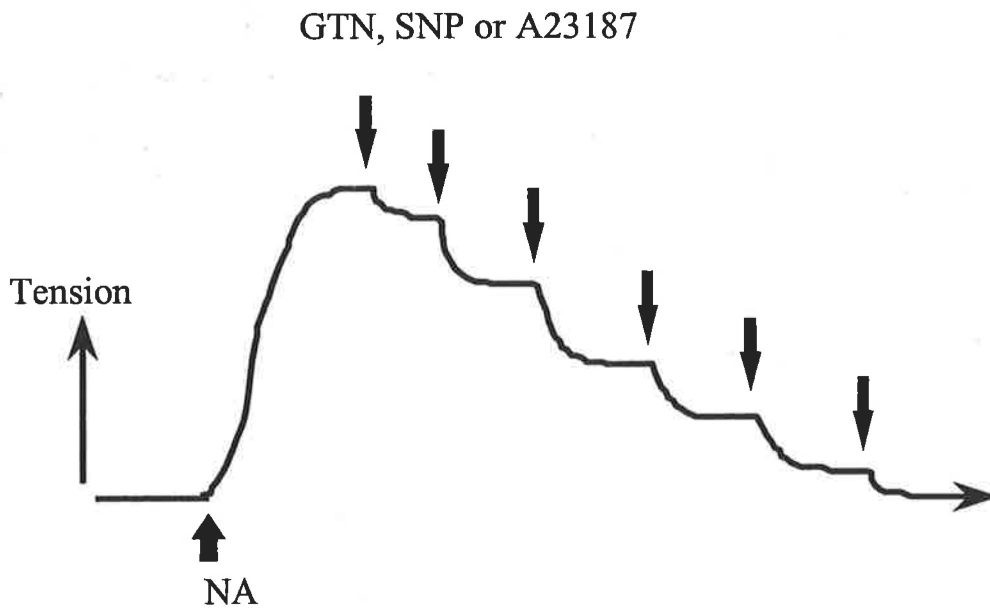

Figure 2.2. Representation of a cumulative concentration response curve to a vasodilator following preconstriction with noradrenalin.

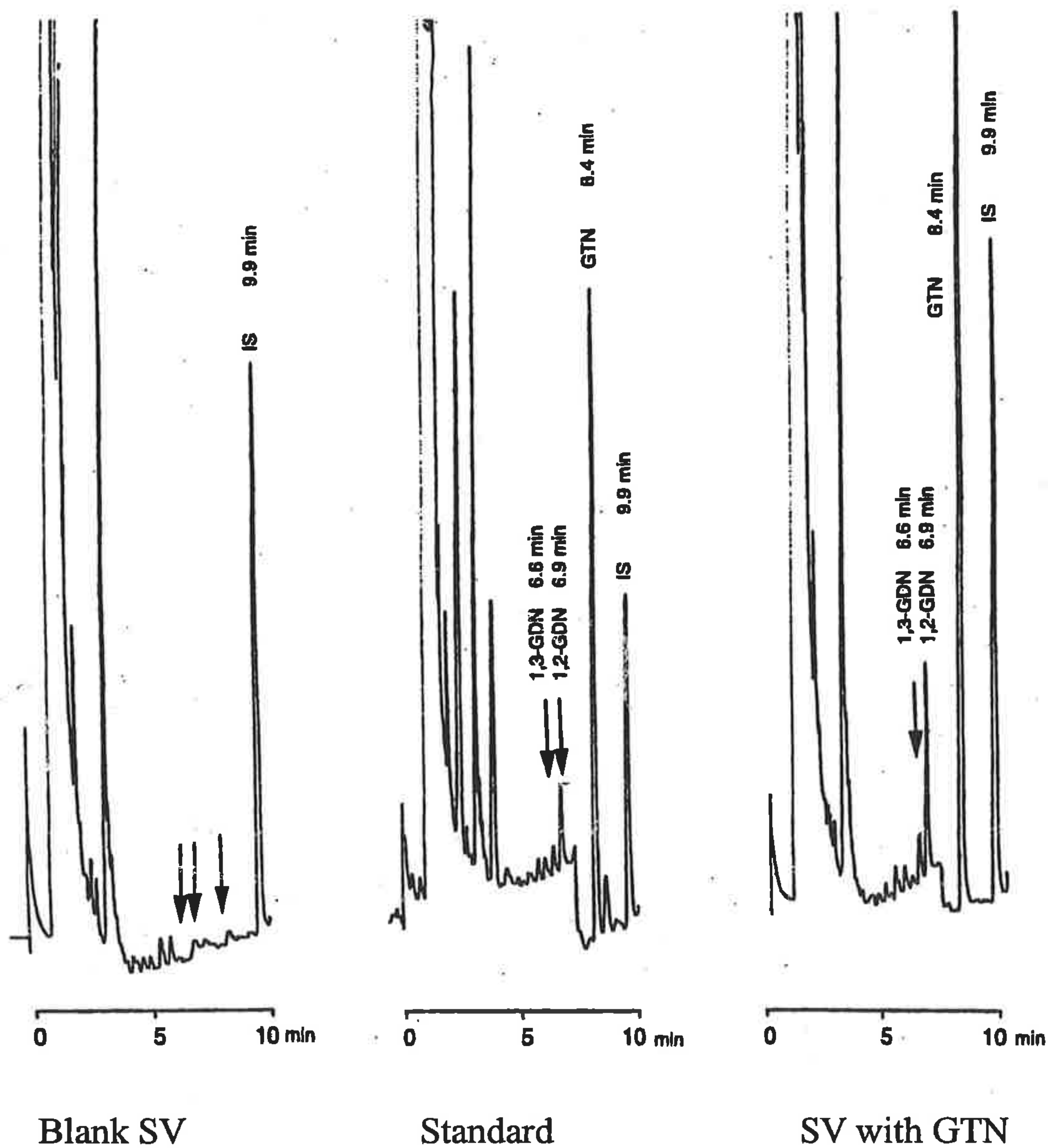

Figure 2.3. Typical GC chromatograms of blank SV incubated with no GTN, a standard (containing 0.25 pmoles 1,3-GDN, 0.5 pmoles 1,2-GDN and 4.8 pmoles GTN) and SV incubated with 1 microM GTN.

Chapter 3

All CR curves were compared using EC_{50} and E_{max} values. An alternative statistical test would have been two-way repeated measures ANOVA, although this would not have changed the results.

Chapter 4

Page 107: DPI inhibited the IMA relaxant response to lower concentrations of GTN, when these data points were compared using paired t-test. Analysis of the entire curve by two-way repeated measures ANOVA revealed a significant inhibitory effect of DPI on GTN responses ($p<0.05$).

The vehicle used in the ODQ and DETCA experiments was distilled water. Vehicle only controls were not performed.

Chapter 6

In figures 6.5 to 6.8, open symbols represent control segments and closed symbols represent NAC-treated vessels.

Typographical Errors

Page 4	Line 11	Insert "the" after "via"
Page 4	Line 12	Insert "the formation of" after "by"
Page 40	Line 4	Replace "demontrate" with "demonstrate"
Page 44	Line 12	Insert "in the" after "role"
Page 47	Line 8	Replace "of" with "have"
Page 56	Line 3	Delete"of"

1. Vallance P, Chan N. Endothelial function and nitric oxide: Clinical relevance. Heart 2001; 85: 342-50.