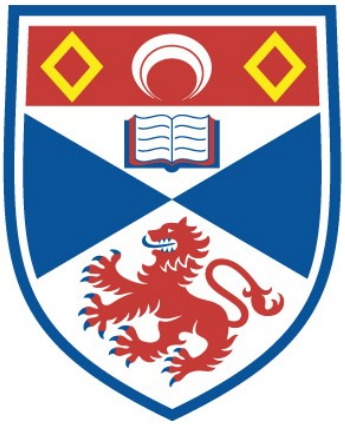
VASODILATOR MECHANISMS OF SOME NOVEL AND ESTABLISHED NITRIC OXIDE 'DONOR' DRUGS AND LASER LIGHT ON THE ISOLATED RAT TAIL ARTERY

Ian L. Megson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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VASODILATOR MECHANISMS OF SOME NOVEL AND ESTABLISHED NITRIC OXIDE 'DONOR' DRUGS AND LASER LIGHT ON THE ISOLATED RAT TAIL ARTERY

A Thesis

Submitted to the University of St. Andrews for the degree of Doctor of Philosophy.

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ABSTRACT

The mechanism of action of some conventional (sodium nitroprusside, NP and S-nitroso-N-acetyl penicillamine, SNAP) and novel (iron-sulphur cluster nitrosyls) nitric oxide (NO) 'donor' drugs has been studied.

Experiments were performed on segments of internally-perfused, rat isolated tail artery. Bolus injections (10μl) of SNAP and NP produce fully reversible (transient or T-type) vasodilator responses. Injections of Roussin's black salt (RBS), an iron-sulphur cluster containing 7 ligated nitrosyl groups, and a related cubane-like iron-sulphur cluster with 4 ligated nitrosyls (CUB), produce conventional T-type responses at doses below a critical threshold concentration (Dt). However, both compounds generate extraordinarily long-lasting (sustained or S-type) responses when the injected dose >Dt: these comprise an initial, rapid vasodilation, followed by partial recovery only. The resulting 'plateau' of reduced tone persists for several hours. Histochemical and X-ray microprobe analyses demonstrate that the plateau is due to slow release of NO from RBS or CUB taken up into the endothelium. RBS and CUB are also shown to be photosensitive: vasodilator responses to both compounds are potentiated by light.

A study has also been made of the mechanism of vascular smooth muscle (VSM) photorelaxation. The phenomenon is shown to be due to release of NO from a photodegradable molecular store, which probably exists in the form of nitrosothiol(s), located in VSM cells. The store can be depleted by exposure to laser light, and then regenerated in the dark. The repriming process is dependent on basal release of NO: it is prevented by known inhibitors of NO synthase and by haemoglobin. Prior treatment of vessels with ethacrynic acid, a thiol alkylating agent, also prevents repriming.

The therapeutic implications of these findings are discussed.

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INTRODUCTION

INTRODUCTION

The functional significance of the endothelium has only been fully appreciated in the last 20 years. This monolayer of cells, continuous throughout the vascular system, has emerged as a crucial element involved in the local control of blood pressure, as well as platelet adhesion and aggregation.

The original breakthrough came with the discovery that endothelial cells release prostacyclin, a prostaglandin synthesised from arachidonic acid with vasodilator and anti-aggregating properties (Moncada, Gryglewski, Bunting & Vane, 1976). It soon became clear that this was one of a number of vasoactive substances released by the endothelium. Of particular interest is so-called 'endothelium-derived relaxing factor' (EDRF). The free radical nitric oxide (NO) is now recognised as being responsible for the vasodilator actions of EDRF, as well as fulfilling a wide variety of biological roles outwith the vascular system.

The importance of a fully functional endothelium is highlighted by results that show that in some diseased states, notably hypertension (Winquist, Bunting, Baskin, & Wallace, 1984; Luscher & Vanhoutte, 1986) and atherosclerosis (Beetens, Coene, Verheyen, Zonnekeyn & Herman, 1986; Harrison, Armstrong, Frieman & Heistad, 1987; Forstermann, Mugge, Alheid, Haverich & Frolich, 1988), endothelium-dependent relaxations are attenuated. Damage to the endothelium is thought to be responsible for this attenuation. In the case of atherosclerosis, recent results have suggested that a defect in endothelial NO function precedes intimal thickening in a new model for the disease (Dusting, Arthur & Zembetis-Bellesis, 1992).

This study focuses on the discovery, actions and pharmacological uses of NO, currently the subject of widespread interest in a variety of biological fields.

1.1 DISCOVERY OF ENDOTHELIUM-DERIVED RELAXING FACTOR

In 1980, Furchgott & Zawadzki showed that vasodilation caused by acetylcholine was endothelium-dependent and that endothelial cells were responsible for the release of a labile relaxing factor. Their experiments showed that precontracted aortic rings which relax on exposure to acetylcholine when intact, fail to relax (or even contract) on exposure to the drug once the endothelium is removed by gentle rubbing of the intimal surface.

Acetylcholine emerged as one of many compounds capable of releasing EDRF. Others include adenine nucleotides, thrombin, substance P, the calcium ionophore A23187, bradykinin and histamine. In addition, local physical changes such as hypoxia, increased luminal flow and electrical stimulation are all capable of eliciting endothelium-dependent relaxations (Furchgott, 1984; Moncada, Palmer & Higgs, 1986).

1.2 CHEMICAL IDENTITY OF EDRF

Furchgott (1984) showed that an intact strip of aorta was capable of relaxing an endothelium-denuded strip of aorta when placed intimal surface to intimal surface and stimulated with acetylcholine. Other workers at about the same time (Rubanyi, Lorenz & Vanhoutte, 1985 Griffith, Edwards, Lewis, Newby & Henderson, 1984) used alternative 'donor-detector' models, where the effluent from an internally perfused length of aorta (donor) was made to superfuse endothelium-denuded aortic rings (detector). Stimulation of the donor vessel with ACh led to relaxation in the detector tissue. It was later shown that endothelial cells cultured on microcarriers packed in chromatography columns were capable of bradykinin-stimulated relaxation of detector tissues when superfused with the column effluent (Cocks, Campbell & Campbell, 1985; Gryglewski, Moncada & Palmer, 1986). Such bioassay techniques offered unique opportunities to study the chemical, physical and pharmacological properties of EDRF.

The first property to be investigated was the biological half-life of EDRF. This was estimated by monitoring the effect on relaxation of increasing the delay between the site of release and the detector tissue. Results published by Griffith *et al* (1984) & Cocks *et al* (1985) suggest a half-life of a few seconds in oxygenated physiological solution. It was also found that EDRF was released under basal conditions as well as when stimulated by acetylcholine (Griffith *et al*, 1984; Rubanyi *et al*, 1984; Martin, Villani, Jothianandan & Furchgott, 1985) and that it was inhibited by haemoglobin and methylene blue (Martin *et al*, 1985). By this time, Rapoport & Murad (1983) had already shown that the effect of EDRF is mediated by cyclic guanosine 3',5'-monophosphate (cGMP) within the target (vascular smooth muscle) cells.

Gryglewski, Palmer & Moncada (1986b), Moncada, Palmer & Gryglewski, (1986) and Rubanyi & Vanhoutte (1986) showed that the instability of EDRF was largely due to the presence of superoxide free radicals in solution (O_2^-). Addition of superoxide dismutase (SOD; the endogenous enzyme which catalyses the conversion of O_2^- to H_2O_2) or cytochrome c (which causes oxidation of O_2^- to O_2) to the superfusate prolonged the existence of EDRF in solution whilst Fe^{2+} or pyrogallol (O_2^- generators) reduced it. Haemoglobin and methylene blue (renowned inhibitors of EDRF) are now thought to act at least in part through generating O_2^- (Steele, Stockbridge, Maljkovic & Weir, 1990; Marczin, Ryan & Catravas, 1992) although the major component of the inhibitory effect of haemoglobin is still attributed to its ability to bind EDRF.

Armed with these observations regarding the chemical and pharmacological behaviour of EDRF, Furchgott (1988) and Ignarro (1988) simultaneously suggested that the free radical NO had properties so similar to those of EDRF that either the radical itself, or a closely related compound, was EDRF.

However, during the preceding year crucial evidence was obtained finally identifying EDRF as NO. Palmer *et al* (1987), using the reaction of NO with ozone to yield a

chemiluminescent product, showed that bradykinin was capable of generating enough NO from cultured endothelial cells to account for the physiological effects of EDRF. Comparative experiments using EDRF and NO in cascade systems showed them to have similar if not identical physical and chemical properties. NO was also shown to be susceptible to the same inhibitors as EDRF (Fe^{2+}, methylene blue, other redox reagents and haemoglobin) and its actions were enhanced by agents such as SOD and cytochrome c (Palmer *et al*, 1987). The actions of both EDRF and NO had by this time been shown to be mediated by the stimulation of soluble guanylate cyclase and the subsequent elevation of intracellular cGMP (Rapoport & Murad, 1983).

The evidence for EDRF being NO is compelling but not watertight. Controversy still reigns over the wide variation in estimated half-lives for EDRF (between 3 and 50 seconds, Griffith *et al*, 1984; Rubanyi *et al*, 1985; Gryglewski *et al*, 1986). It is thought that the discrepancy lies largely in differing experimental conditions, especially with regard to the relative O_2 and O_2^- concentrations, now known to be crucial in determining the half-life of EDRF. The reactions of NO with O_2 and O_2^- are considered in section 1.5.

Other controversial issues include the differential binding of EDRF and NO to anion exchange columns. EDRF has been reported to bind to anion exchange columns (Long, Shikano & Berkowitz, 1987) whereas NO (which is not anionic) does not. Other workers have shown that NO in physiological concentrations binds to a lesser extent than EDRF to anion exchange columns (Khan & Furchgott, 1987). It has been postulated that EDRF and NO undergo a chemical reaction with amines when passing down the column but it is also possible that the results indicate that EDRF is an NO-containing compound rather than the free radical itself.

Evidence from electron paramagnetic resonance (EPR) studies also raises a question mark concerning the exact identity of EDRF, since NO, but not EDRF, can form paramagnetic nitrosyl haem (Rubanyi, Greenberg & Wilcox, 1990). Despite these irregularities in the evidence that EDRF is NO, it is generally agreed that the actions of EDRF are *mediated* by NO.

1.3 SYNTHESIS OF EDRF

Palmer, Ashton & Moncada (1988a) were the first to identify L-arginine as the precursor to EDRF. Their experiment involved the removal of L-arginine from the medium of cultured endothelial cells. These cells were shown to have a reduced EDRF output when stimulated with bradykinin or A23187. The output could be restored to normal levels by introducing L- but not D-arginine. The results were confirmed by experiments using mass spectrometry, where ^{15}N-labelled L-arginine (at both the G- and U- nitrogen sites) was shown to release ^{15}NO on stimulation with bradykinin. The release was shown to depend upon an enzymatic reaction, since D-arginine was not a suitable substrate for the production of NO.

The enzyme involved, formerly known as the citrulline-forming enzyme, and now nitric oxide synthase (NOS), has been shown to be inhibited by an L-arginine analogue, N^G monomethyl - L- arginine (LNMMA) but not the D- enantiomer (Palmer *et al*, 1988). LNMMA has since been used extensively, both *in vitro* and *in vivo*, specifically for this purpose. *In vivo* studies on a wide variety of preparations, including the human brachial artery (Vallance, Collier & Moncada, 1989), have shown that LNMMA increases resting arterial tone, suggesting a 'basal release' of EDRF by the enzyme even when seemingly unstimulated. Palmer, Rees, Ashton & Moncada (1988b) refer to this phenomenon as the 'dilator tone' of the resting vessel. These results are supported by the existence of a basal cGMP concentration within unstimulated vascular smooth muscle cells. Removal of the vasodilator tone, using agents such as LNMMA, has been shown to supersensitise the tissue to NO delivered by endothelium-independent means, both *in vivo* and *in vitro* (Moncada, Rees, Schulz & Palmer, 1991). This might also explain the 'desensitisation' reported when using NO-donor drugs (Axelsson & Andersson, 1983; Waldman & Murad, 1987).

LNMMA is now known to be one of a family of inhibitory L-arginine analogues (figure 1.1). Others include N-iminoethyl-L-ornithine (LNIO); N^G-nitro-L-arginine methyl ester (LNAME); (Rees, Palmer, Schultz, Hodson & Moncada, 1990) and N^G-amino-L-arginine

(Fukuto *et al,* 1990). A variety of iodio compounds(figure 1.1; Stuehr, Fasehun, Kwon, Gross, Gonzalez, Levi & Nathan, 1991) have also been shown to inhibit the enzyme but via inhibition of the cofactor nicotinamide adenine dinucleotide phosphate (NADPH). The inhibition in this case is irreversible and less specific than with the competitive inhibitors mentioned above.

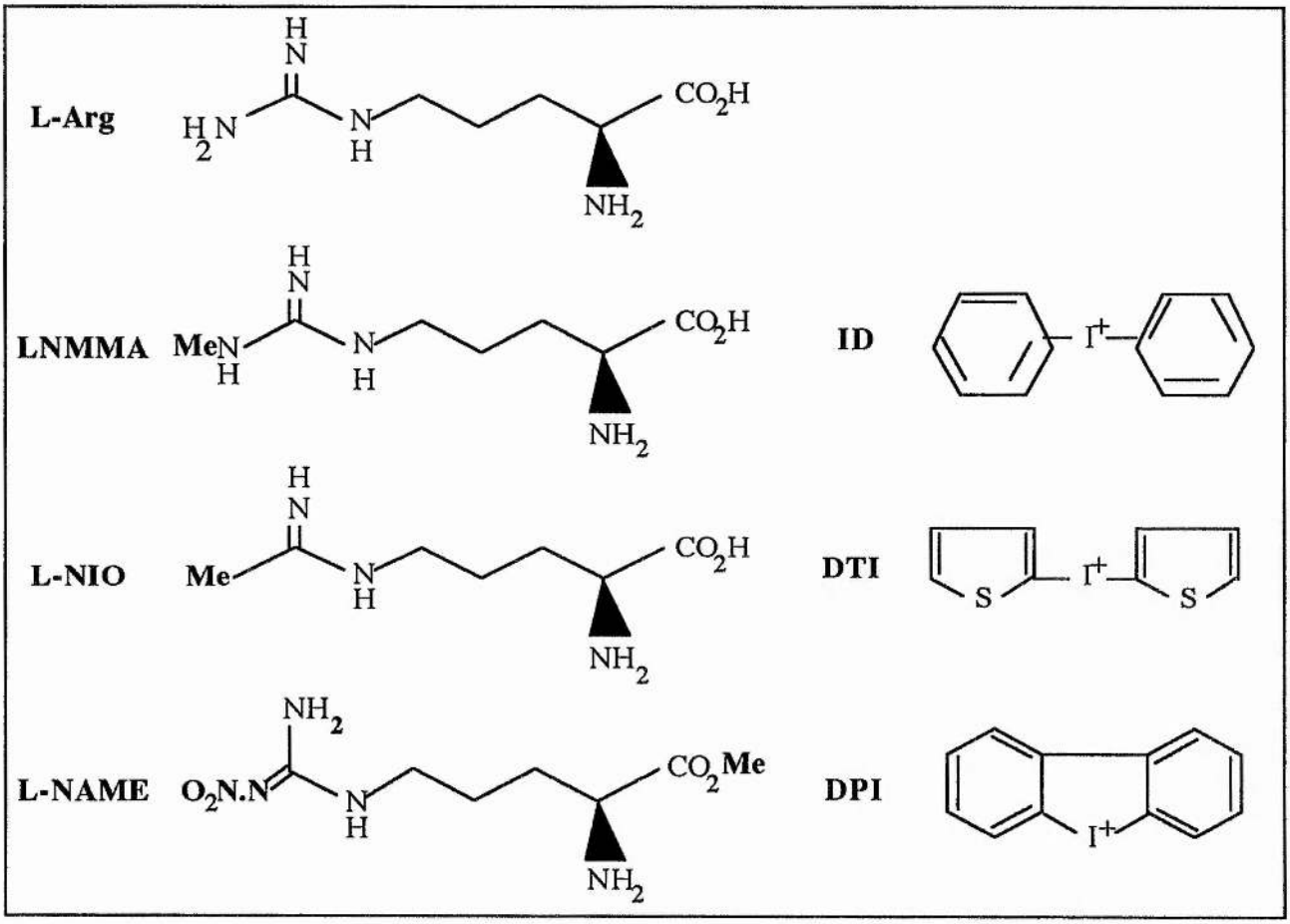


Figure 1.1. *Structural formulae of L-arginine and some inhibitors of NO synthase. LNMMA, LNIO and L-NAME are reversible, competitive inhibitors. Iodoniumdiphenyl (ID), di-2-thienylidonium (DTI) and diphenyleneiodonium (DPI) act irreversibly through inhibition of the cofactor NADPH.*

As intimated above, NO synthase is NADPH-dependent (Palmer & Moncada 1989). It can also be inhibited by calcium ion chelators indicating a dependence on calcium (Moncada & Palmer 1990). NO synthase has recently been shown to be stimulated by the calcium-calmodulin complex since it is inhibited by calmodulin-binding peptides (Busse & Mulsch 1990). Sequence analysis of cloned NOS has revealed a structural resemblance to P450

cytochrome c P450 reductase, with binding sites for NADPH, flavine adenine dinucleotide (FAD), flavine mononucleotide (FMN) (Bredt, Hwang, Glatt, Lowenstein, Reed & Snyder, 1991), tetrahedrobiopterin (BH_4) (Kwon, Nathan & Stuehr, 1989) as well as phosphorylation sites, (Bredt *et al*, 1991), highlighting the multi-factor regulation of the enzyme. Full classification of different isoforms so far identified is included in section 1.9.

NOS is now thought to synthesise NO from L-arginine via the intermediate N^G-hydroxy-L-arginine (Stuehr, Kwon, Nathan, Griffith, Feldman & Wiseman, 1991). Molecular oxygen is incorporated into both end products, citrulline and NO (Leone, Palmer, Knowles, Francis, Ashton & Moncada, 1992). The current hypothetical reaction mechanism for enzymatic NO production is shown in figure 1.2:

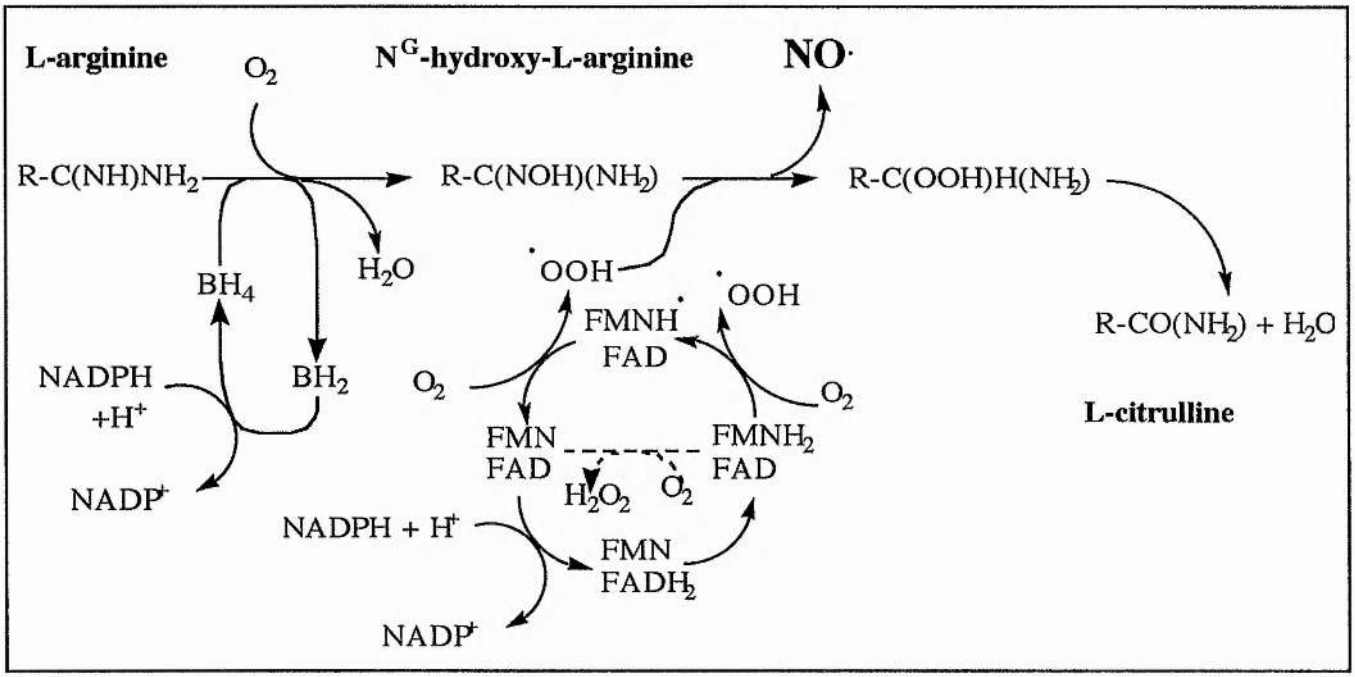


Figure 1.2. *Hypothetical reaction mechanism of enzymatic NO formation (after Stuehr, Cho, Kwon & Nathan, 1992)*

1.4 STIMULATION OF GUANYLATE CYCLASE BY NO

The target enzyme for NO (whether endothelium-derived or from an exogenous source) has been shown to be guanylate cyclase (Waldman & Murad, 1987). More specifically, it appears that its main site of action is on the cytoplasmic isoenzyme (soluble guanylate cyclase).

There is some controversy over the exact constitution of soluble guanylate cyclase but it is now thought to be a heterodimer with Mr 82,000 and 70,000 subunits (Waldman & Murad, 1987). Soluble guanylate cyclase purified from bovine lung contains a haem and a copper moiety. Highly purified enzyme extracts exhibit three absorption maxima at 433, 550 and 565nm which are shifted on exposure to either carbon monoxide or NO (Gerzer, Bohme, Hoffman & Schultz, 1981). This is consistent with the binding of these species to the haem moiety of the enzyme. The involvement of the haem group in the stimulation of the enzyme was clarified by the inhibitory effect of adding ferro (Fe^{2+})-metalloproteins (but not the ferric Fe^{3+} forms) such as haemoglobin or myoglobin, to highly purified extracts of the enzyme (section 1.6). Application of NO donor drugs to the purified enzyme in the presence of Fe^{2+} metalloproteins was no longer capable of activating cGMP production (Mittal, Arnold & Murad, 1978; Murad, Mittal, Arnold, Katsuki & Kimura, 1978). The likely explanation for these observations is that the Fe^{2+} metallo-proteins act as efficient scavengers for NO and prevent its binding to the target haem group. The presence or absence of ferro-haem compounds in the cellular environment, therefore, is a significant factor in determining the susceptibility of the enzyme to NO stimulation.

In light of these and other experiments, it is thought that the activation of soluble guanylate cyclase by NO involves at least one oxidation-reduction event (although O_2 and protein activators are not necessary for its effects; Murad *et al*, 1978). In fact, O_2 and other oxidising agents, such as methylene blue, H_2O_2, O_2^- and ferrocyanide, appear to inhibit the activity of the enzyme, whilst reducing agents (ascorbate, cysteine, glutathione and dithiothreitol) promote its activation by NO (Braughler, Mittal & Murad, 1979).

The inhibitory effect of oxidising agents may occur at different levels. NO itself may be converted to higher oxides of nitrogen (e.g. NO_2), whilst the haem group on the enzyme may also be oxidised to the Fe^{3+} form which is not capable of binding NO (sections 1.5 and 1.6).

NO stimulation results in striking alterations in the enzyme's properties. The inactivated enzyme displays a preference for Mn^{2+}-GTP (Km 5-50 μM) over Mg^{2+}-GTP (Km 20-150μM). However, once activated by NO, the enzyme can use GTP bound to either cofactor equally well, resulting in an increase in the Vmax. The Km for Mg^{2+}-GTP drops to about 40μM whilst that for Mn^{2+}-GTP remains unchanged. The proposed reason for the change is that a secondary binding site develops for Mg^{2+}-GTP on activation (Lewicki, Brandwein, Mittal, Arnold & Murad, 1982). Another curiosity concerning the state of the activated enzyme is its ability to form cAMP from Mg^{2+}-ATP. This is inhibited by the presence of GTP just as formation of cGMP is inhibited by the presence of ATP (Mittal & Murad, 1977).

Studies on the activation of particulate guanylate cyclase by NO have been hampered by the effects of detergents in purification but it appears likely that this too is stimulated by NO in favourable (similar to soluble guanylate cyclase) redox conditions (Braughler, 1982; Katsuki, Arnold, Mittal & Murad, 1977).

1.5 INTRACELLULAR INTERACTIONS OF NO

The intracellular environment is host to a variety of free radical and molecular species which have the potential to react with and inactivate NO before it can stimulate guanylate cyclase activity.

A. FREE RADICALS

The most common free radicals present in cells are oxygen-derived byproducts of the respiratory chain; superoxide (O_2^-) and hydroxyl radicals ($OH^\cdot$; Freeman & Crapo, 1982). Other sources of these species are haem and other metal-containing proteins and both free and complexed metals (particularly Fe^{2+}; Freeman & Crapo, 1982). The relative concentrations of these, and of other potentially harmful radicals, greatly increase when cells are exposed to irradiation, pollutants, toxic chemicals or become diseased (Freeman & Crapo, 1982; McCord & Fridovich, 1972; Bielski & Gebieki, 1977; Mason & Chignell, 1982).

Normal levels of oxygen radicals are controlled by enzymes with rapid rate constants and low Km values, minimising their cytotoxic effects.

$$O_2^- + O_2^- + H^+ \xrightarrow{\text{superoxide dismutase (SOD)}} H_2O_2 + O_2 \qquad K = 2 \times 10^9\ M^{-1}sec^{-1}$$

$$2H_2O_2 \xrightarrow{\text{Peroxidase/Catalase}} 2H_2O + O_2 \qquad K = 4 \times 10^7\ M^{-1}sec^{-1}\ \text{(Peroxidase)}$$
$$K = 1 \times 10^7\ M^{-1}sec^{-1}\ \text{(Catalase)}$$

$OH^\cdot$, the extremely reactive and potentially most harmful radical, is controlled as a consequence of the activity of these two enzymes, since it arises as a result of the reaction between O_2^- and H_2O_2 (Waldman & Murad, 1987).

Despite the rapid activity of these enzymes, it is well documented that sufficient O_2^- exists in the intracellular environment to reduce NO stimulation of guanylate cyclase, since addition of exogenous SOD to the perfusate has been reported to double the half-life of EDRF (Rubanyi & Vanhoutte, 1986). The same workers showed that the effect is augmented by reducing the O_2 concentration from 95% to 10%, resulting in a five-fold increase in the half-life. The inference of these experiments is that O_2^- inactivates EDRF and that hyperoxia favours the inactivation. In addition, inactivation has been found to be potentiated by incorporating nitrate reductase, a flavoprotein which catalyses the formation of O_2^- from O_2 (Waldman & Murad, 1987). A more recent study, involving inhibition of endogenous SOD in bovine coronary arteries using diethyldithiocarbamate (DETCA), showed attenuation of endothelium-dependent and -independent nitrovasodilator relaxation (Omar, Cherry & Martelli, 1991). Exogenous O_2^- generators (Fe^{2+}, pyrogallol, cytochrome c and phenidone) severely attenuate bradykinin-induced (i.e. EDRF mediated) vascular relaxation.

Under physiological pH conditions, NO reacts with O_2^- to produce peroxynitrite ($ONOO^-$) which then decays following first order kinetics to NO_2 and ultimately NO_2^- (Saran, Michel & Bors, 1990).

Evidence is mounting to show that O_2^- generation is responsible, at least in part, for the actions of traditional inhibitors of NO activation of guanylate cyclase, including haemoglobin (Hb) and methylene blue (MB). The inhibition is thought to result from direct oxidation of the enzyme, as well as its reaction with NO. Hb is known to have a high affinity for NO and its inhibitory effect on NO stimulation of guanylate cyclase has been attributed solely to this fact. However, it is now thought that generation of free radicals during autoxidation of Hb is an important factor in mediating its inhibitory action in cerebrovascular smooth muscle cells (Steele, Stockbridge, Malkjovic & Weir, 1991). Similarly, it has been postulated that MB exerts its inhibitory effect on guanylate cyclase via the production of O_2^-, since its effects are abolished in the presence of exogenous SOD (Marczin, Ryan, Catravas, 1992; Wolin, Cherry, Rodenburg, Messina & Caley, 1990).

Results concerning the interactions of H_2O_2 with NO have been conflicting. Rubanyi & Vanhoutte (1986) found that catalase had no effect on the half-life of EDRF under their experimental conditions, whilst work by Marczin *et al*, (1992) showed that H_2O_2 has a profound effect on the production, transport or action of basally released EDRF, as reflected by smooth muscle cGMP levels. To complicate matters further, activation of guanylate cyclase by the addition of SOD has been shown to be inhibited by catalase, despite the fact that H_2O_2 alone is incapable of elevating intracellular cGMP (Mittal & Murad, 1977). These data imply that both O_2^- and H_2O_2 are required to activate guanylate cyclase via the formation of the short-lived radical, OH·.

In smooth muscle, therefore, the interaction between NO and oxygen free radicals ultimately results in attenuation of NO-induced vascular relaxation. In other tissues, particularly in diseased states, the relevance of the interaction lies in the inactivation of O_2^- by NO, resulting in protection against tissue injury (Rubanyi, Ho, Cantor, Lumma & Botelho, 1991). The cytoprotective effect may also be due to NO inhibition of NADPH oxidase, the enzyme largely responsible for O_2^- production in activated neutrophils (Clancy, Leszczynska-Piziak & Abramson, 1992).

B. MOLECULAR 'SCAVENGING' OF NO

NO is capable of reacting with a vast range of intracellular molecules as well as free radicals. First, NO reacts with molecular O_2. Originally it was thought that the reaction yielded both nitrite and nitrate (Feelisch, 1991), but in a recent review (Butler & Williams, 1993) nitrite was seen to be the only product in the knowledge that the intermediate steps in nitrite production occur at a faster rate than those for nitrate. Hence the reaction sequence is as follows:

1. $2NO + O_2 \longrightarrow NO_2$
2. $NO + NO_2 \longrightarrow N_2O_3$
3. $N_2O_3 + H_2O \longrightarrow 2NO_2^- + 2H^+$

The reaction shown in equation 1 is third order and as such is thought to be slow at physiological NO concentrations (Butler & Williams, 1993). *In vivo*, therefore, inactivation of NO is probably due more to its interaction with oxygen-derived free radicals than with oxygen itself.

It has recently been shown that albumin readily binds NO (Stamler, Jaraki, Osborne, Simon, Keaney, Vita Singel, Valeri & Loscalzo, 1992). This research demonstrates that NO binds to the single free cysteine (cys 34) of serum albumin to produce S-nitroso-serum albumin. The inference to be drawn from this is that NO can bind to any proteins containing accessible cysteines in their structure as well as to free thiols (Stamler *et al*, 1992).

Porphyrins also bind NO avidly (Antonini & Brunori, 1971). The intracellular importance of this is limited since Hb is unable to enter cells. Nevertheless, various other intracellular ferroporphyrins, including catalase, myoglobin and cytochrome c, bind NO, forming stable nitrosylhaem complexes (Kimura, Mittal & Murad, 1975; Murad, Arnold, Mittal & Braughler, 1979; Mittal & Murad, 1979; Murad *et al*, 1978 - Section 1.6).

Guanylate cyclase in vascular smooth muscle is therefore in direct competition with a wide range of free radical and molecular species for NO binding. Interactions with free radicals serve both to protect the tissue from damage and, in conjunction with binding to proteins and molecules other than guanylate cyclase, to attenuate the vasodilator effect of endogenous or exogenous NO.

Evidently, the activation of guanylate cyclase by NO is a complex process which can be modulated at a variety of levels, primarily by the redox conditions in the immediate environment of both the NO and the haem group of the enzyme.

1.6 ROLE OF PORPHYRINS IN REGULATION OF GUANYLATE CYCLASE

The fact that ferro-haemoglobin and other haemoproteins are capable of inhibiting the stimulation of guanylate cyclase by NO, whilst the actions of hydroxylamine are dependent on the presence of other haemoproteins (catalase or cytochrome c), suggests a role for porphyrins in regulating guanylate cyclase activity. The role of catalase in the hydroxylamine scenario has been shown to be due to the catalytic release of NO from hydroxylamine in the presence of H_2O_2. There seemed however, to be a necessity for the formation of a catalase-NO intermediate to facilitate enzyme activation (Craven, De Rubertis & Pratt, 1979). This suggests that the role of the porphyrin is more as an NO acceptor than as an enzyme catalysing the release of NO. The nitrosyl-haem complex is now recognised as an important regulator of guanylate cyclase activity.

Further evidence supporting this hypothesis arose from early attempts to purify soluble guanylate cyclase. The process repeatedly resulted in a haem-free protein which could not be stimulated by NO or nitrovasodilators. Partial activation was restored following exposure of the enzyme to haematin, haemoglobin, met-haemoglobin or catalase. The effect could be potentiated by incorporating a reducing agent, to maintain the haem iron in the ferrous (Fe^{2+}) state. Preformed NO-haemoglobin maximally activated the partially purified enzyme (Craven & De Rubertis, 1978, Craven *et al,* 1979).

As purification techniques improved, dissociation of the haem moiety from the enzyme became less problematic. Confirmation of the essential role played by haem in NO activation came when haem-containing guanylate cyclase was chromatographically separated from haem-free enzyme and it was shown that only haem-containing enzyme could be stimulated by NO (Gerzer, Bohme, Hoffman & Schultz, 1981; Gerzer, Hoffman, Bohme, Krassimira, Spies & Schultz, 1981; Gerzer, Hoffman & Schultz, 1981). A clue to the mechanism by which the NO-haem complex causes activation arose from experiments where haem-free guanylate cyclase was reconstituted using protoporphyrin IX, the demetallated precursor of ferroprotoporphyrin (Gerzer, Radney & Garbers, 1982). This was shown to cause activation of the enzyme, with changes in Km and Vmax similar to

those obtained with nitrosyl-haem activation (Waldman, Rapoport & Murad, 1987). Activation of guanylate cyclase, therefore, is thought to be related to the absence of Fe^{2+} from the porphyrin ring (e.g. protoporphyrin IX - induced activation) or to withdrawal of the Fe^{2+} from the plane of the porphyrin ring (NO - induced activation; figure 1.3).

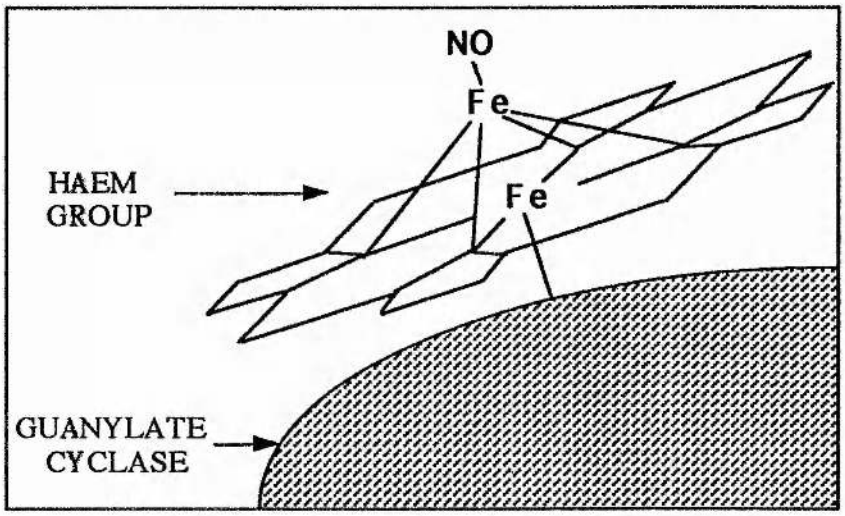


***Figure 1.3** Proposed mechanism for NO- induced stimulation of guanylate cyclase.*

The mechanism is undoubtedly more complex than illustrated in figure 1.3, with a possible role for an activating factor, as yet unidentified. It has also been proposed that protoporphyrin IX acts through an alternative mechanism (Ohlstein, Wood & Ignarro, 1982).

Regulation of particulate guanylate cyclase by protoporphyrin IX (6-10-fold activation) and a variety of other porphyrin structures has also been demonstrated by Lacombe & Eberenz-L'Homme (1983). Haemin was found to activate the particulate isoenzyme from some tissues (rat lung) but not others. These results suggest that porphyrin regulation of activity is not identical for the two isoforms of the enzyme. The soluble form is activated by low concentrations of haemin whilst high concentrations inhibit its activity. The particulate form, however, requires high haemin concentrations for activation (Waldman, Sinacore, Lewicki, Chang & Murad, 1984).

1.7 ROLE OF THIOLS IN REGULATION OF GUANYLATE CYCLASE

It has become clear that porphyrins are not the only regulatory factor involved in the stimulation of guanylate cyclase. As already discussed, regulators of guanylate cyclase activity generally exert their effect by altering the redox state of the enzyme. Since sulphydryl groups can undergo oxidation-reduction reactions, and are known to regulate protein structure and function, it is reasonable to suppose that they may also be involved in the regulation of guanylate cyclase.

Even in early studies, alkylating agents which covalently modify free thiol groups (e.g. ethacrynic acid) were shown to inhibit basal and nitro-activated cGMP production (Katsuki *et al*, 1977). Similarly, disulphide bridge formation, on addition of cystine or cystamine, also inhibited both basal and nitro-activated cGMP production (Kimura, Mittal & Murad, 1975;Waldman, Lewicki, Chang & Murad, 1983). The link was confirmed when radiolabelled cystamine was shown to be incorporated into the enzyme in a manner consistent with the time course of inhibition of the enzyme. This effect was reversed using the reducing agent dithiothreitol, which breaks disulphide bridges (Brandwein, Lewicki & Murad, 1981).

Oxidation of the enzyme, induced by preincubation of crude enzyme preparations in an oxygen-rich atmosphere, initially causes stimulation of the enzyme but ultimately leads to inhibition of its activity (Lewicki *et al*, 1982). Reduction by dithiothreitol potentiates activation of the enzyme (Bohme, Jung & Mechler, 1974). The apparent anomaly in these results has been explained in terms of the initial oxidation of sulphydryl groups leading to activation, developing into an, as yet unexplained, 'overoxidation', which causes irreversible loss of both basal and nitro-activated cGMP production. The inhibition of nitro-activated cGMP production could be due to direct inhibition of NO by O_2 (or the more potent oxygen radicals), as well as by oxidation of the recipient haem group of the enzyme (Waldman & Murad, 1987).

Since these findings were published, it has been suggested that different sulphydryl groups on the enzyme are responsible for (a) the regulation of basal cGMP production; and (b) for regulation of nitro-activated cGMP production (Braughler, 1983). At least one of these sulphydryl groups is located at or near the catalytic site of the enzyme, since preincubation of the enzyme with either the activating agent or with Mg^{2+}-GTP protects the enzyme against inactivation (Ignarro, Kadowitz & Baricos, 1981). Again, care must be taken when interpreting results involving the effects of oxidising and reducing agents on nitro-stimulation of the enzyme because such treatments will invariably affect the viability of both the NO donor and NO itself *en route* to the active site of the enzyme.

Ignarro, Lipton, Edwards, Baricos, Hyman, Kadowitz & Greutter (1981) showed that partially purified soluble guanylate cyclase requires added free thiol groups to undergo activation by a variety of NO-donor compounds. In addition, nitrosocysteine and other nitrosothiols are capable of elevating cGMP levels, as well as causing a brief arterial relaxation in anaesthetised cats. Ignarro *et al* (1988) argue that nitrosothiols more closely mimic the actions of NO-donor drugs than NO itself, and have suitable half-lives in physiological conditions to account for the actions of EDRF.

In summary, therefore, the endogenous vasodilator, EDRF, is NO generated during the NO synthase catalysed conversion of L-arginine to L-citrulline. Binding of EDRF to the haem moiety of vascular smooth muscle guanylate cyclase stimulates cGMP production from GTP leading to relaxation (see section 1.8).

The free radical O_2^-, a by product of respiration, is an important inhibitor of NO. However, NO may also bind to ferroporphyrin-containing compounds or thiols (both free and protein-associated), all of which will prevent its stimulating guanylate cyclase.

Regulation of guanylate cyclase is strongly dependent on the immediate redox environment. The oxidation state of the haem-iron as well as the protein-bound thiol groups determine the affinity of NO for the enzyme.

The vascular L-arginine:NO pathway is summarised in figure 1.4 below:

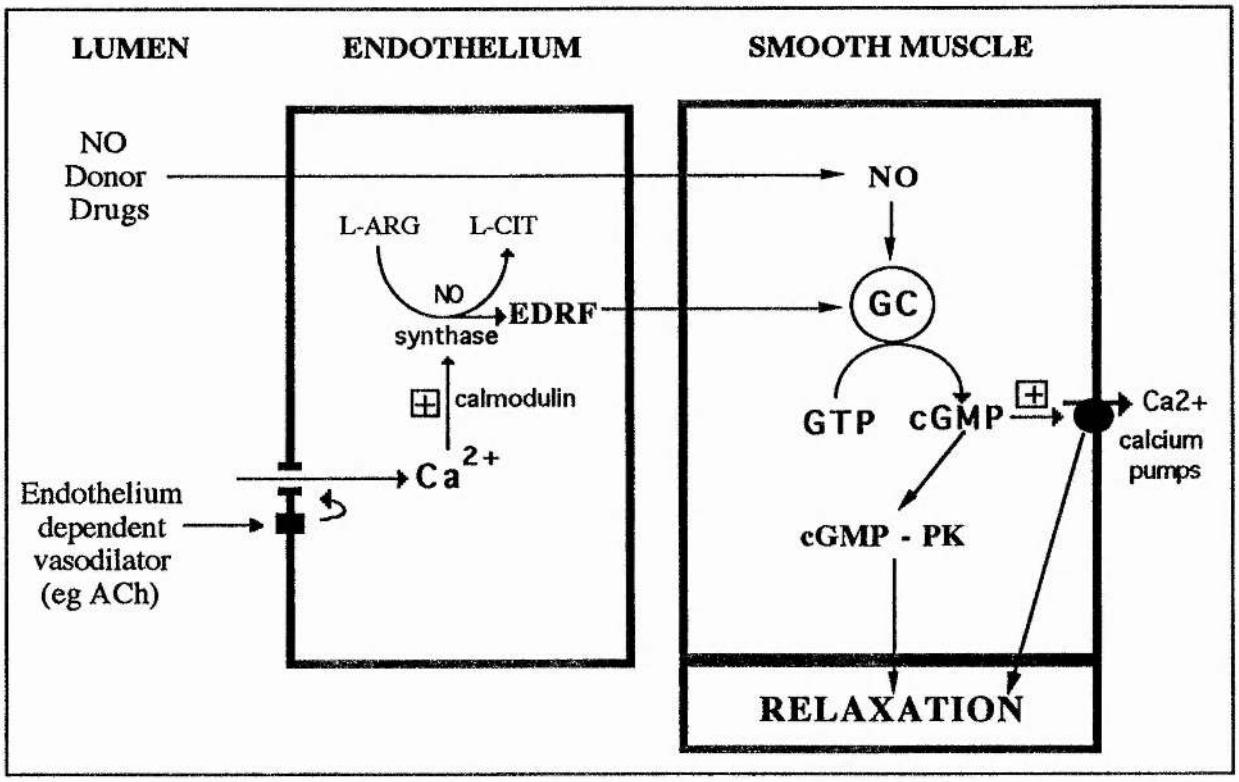


***Figure 1.4**: Diagram summarising the synthesis and actions of NO in vascular tissue.*

1.8 MECHANISMS OF ACTION OF cGMP IN VASCULAR SMOOTH MUSCLE.

It is now widely accepted that cGMP is the intracellular mediator involved in NO-stimulated relaxation of vascular smooth muscle. Since the discovery of a specific cGMP-dependent protein kinase in lobster tail muscle (Kuo & Greengard, 1970), it has been considered likely that this mediates the actions of cGMP, in much the same way that cAMP-dependent protein kinases regulate the actions of cAMP (Lohmann & Walter, 1984). However, it has since been shown that cGMP is also capable of regulating cyclic nucleotide

phosphodiesterases (Walter, 1984) and plasmalemmal Ca^{2+}-pump activity in vascular smooth muscle (Popescu, Panoui, Hinescu & Nuto, 1985). In order to understand how an elevation in intracellular cGMP causes vasodilation, it is necessary to outline the process involved in vasoconstriction.

A. Contraction of vascular smooth muscle.

Cytosolic Ca^{2+} is the second messenger for activation of vascular (and other) smooth muscle cells (Haslam, 1987; Rasmussen, Takuwa & Park, 1987). Intracellular Ca^{2+} can be elevated either directly by voltage- or receptor -dependent stimulation of calcium channels in the membrane (Exton, 1988), or by the release of intracellular stores via receptor-mediated stimulation of phospholipase C (e.g. α_1 adrenoreceptors). Phospholipase C is the constituent enzyme of the inositolphosphate pathway that hydrolyses phosphatidylinositol 4,5-bisphosphate to inositoltrisphosphate. This is a second messanger that stimulates Ca^{2+} release from intracellular stores. The byproduct of the hydrolysis, 1,2 diacylglycerol (DAG), is a potent activator of phosphokinase C.

The rise in intracellular Ca^{2+} triggers a cascade of events which ultimately leads to contraction of smooth muscle. The first step involves the activation of calmodulin. Ca^{2+}/calmodulin-dependent myosin light chain kinase activity is then stimulated to phosphorylate myosin light chains, a prerequisite for smooth muscle contraction (Walter, 1989).

The role of phosphokinase C, activated by DAG, is not so clearly defined as that of Ca^{2+}. It is known that phosphokinase C phosphorylates myosin light chains but it is thought that this occurs at different sites from those phosphorylated by myosin light chain kinases (Haslam, 1987). The site of phosphokinase C phosphorylation is thought to be on the structural and regulatory components of filamin-actin-desmin fibrillar domain of myosin light chains. It is proposed that phosphorylation at these sites produces sustained smooth muscle tone, whilst Ca^{2+}/calmodulin-stimulated phosphorylation is responsible for the initial phase of contraction (Rasmussen *et al*, 1987).

B. Role of cGMP in relaxation of vascular smooth muscle.

Essentially, relaxation of smooth muscle involves the removal of intracellular calcium and dephosphorylation of myosin light chains. As yet, there is no evidence to suggest that cGMP is capable of myosin light chain dephosphorylation directly (as is the case with cAMP; Lamb *et al*, 1988). The major contribution made by cGMP with regard to vascular smooth muscle relaxation has therefore been attributed to its ability to down-regulate free intracellular Ca^{2+}.

Most, but not all, of the actions of cGMP are mediated by cGMP-dependent kinases. Following the discovery of cGMP-dependent protein kinase in lobster tail muscle (Kuo *et al*, 1970), similar findings have come to light in a wide variety of tissues (Hofmann & Sold, 1972; Gill, Holdy, Walton & Kanstein, 1976). Protein kinase purified from bovine lung has now been characterised and is recognised as a Mr 150,000 enzyme composed of two identical subunits. All rat cell lines so far tested using radio immunoassay techniques have been found to have cGMP-dependent kinases, although highest concentrations exist in the cerebellum, heart, lung and aorta (Walter, 1981). The enzyme was primarily thought to be soluble but recent evidence, from studies of vascular smooth muscle and platelets (Waldman, Bauer, Gobel, Hofmann, Jacobs & Walter, 1986), suggests that in some cases particulate protein kinase may be as prevalent.

The principle function of protein kinases is to phosphorylate specific proteins. The pattern of phosphorylation, and the resulting response of the tissue, is dependent on whether the protein kinase is cGMP- or cAMP-dependent and on the tissue concerned. Attention will now focus on the situation in vascular smooth muscle.

cGMP is thought to regulate intracellular Ca^{2+} in four ways. Firstly, the processes responsible for elevating cytosolic Ca^{2+} are inhibited by both cAMP and cGMP in smooth muscle cells (Jones, Byland, Forte, 1984; Morgan & Morgan, 1984). It has been demonstrated that cGMP-dependent kinases mediate the inhibitory effects of 8-bromo-

cGMP on vasopressin- and depolarisation-induced elevation of cytosolic Ca^{2+} in vascular smooth muscle (Cornwell & Lincoln, 1989). As yet, it is unclear how cGMP-dependent protein kinases are able to lower the intracellular calcium concentration but it is likely to involve one or more of the following mechanisms:

1. Regulation of phospholipid-metabolising enzymes.

Evidence exists to support the view that both cAMP & cGMP-elevating vasodilators inhibit agonist-induced activation of the phosphatidylinositol cycle in platelets (Haslam, Salama, Fox, Lynam & Davidson, 1980; Waldman & Walter, 1989). c-GMP-elevating vasodilators have been shown to have this effect on vascular smooth muscle cells (Rapoport, 1986). The resultant reduction in inositoltrisphosphate leads to a decrease in intracellular Ca^{2+}, whilst the reduction in DAG leads to a decrease in stimulation of phosphokinase C. The overall effect of both processes is to reduce protein phosphorylation involved in platelet or muscle activation (Waldman & Walter, 1989; Rapoport, 1986).

2. Regulation of pathways responsible for Ca^{2+} removal.

cGMP is now known to activate plasmalemmal Ca^{2+}-ATPase in intact vascular smooth muscle cells (Kobayashi, Kanaide & Nakamura, 1985; Furukawa, Tawada & Shigekawa, 1987; Lincoln, 1989). Ca^{2+}-ATPase is responsible for removal of Ca^{2+} from the cytoplasm, a process which has been shown to be activated by partially purified cGMP-dependent protein kinase (Furukawa & Nakamura, 1987). The precise mechanism is not fully understood. It is also unclear whether cGMP-dependent kinases affect Ca^{2+} uptake into smooth muscle sarcoplasmic reticulum (Lincoln, 1989).

Despite uncertainties about the mechanism, the reduction of intracellular Ca^{2+} concentration is clearly the main role of cGMP and cGMP-dependent kinases in vascular smooth muscle cells. However, other processes are also thought to play a part.

3. Regulation of cGMP - dependent cAMP phosphodiestererases

It has been known for some time that cGMP stimulates the hydrolysis of cAMP in some tissues (Beavo, Hardman, Sutherland, 1971). The picture, however, is now known to be far more complex. Evidently, cAMP phosphodiesterases fall into numerous sub-classes, only some of which are stimulated by cGMP (Beavo, 1988). Whether a particular cell contains cGMP-inhibited or -stimulated cAMP phosphodiesterase seems to be determined by the cell type. As a result, elevation of cGMP levels in certain tissues leads to a subsequent decrease in cAMP, whilst the reverse is true in other tissues.

In the cardiovascular system, this differentiation is clearly illustrated. Acetylcholine is known to exert its negative inotropic effects by stimulating cGMP production (Goldberg & Haddox, 1977). It is now believed that the associated drop in cAMP levels (potentiating the negative inotropic effect) in cardiac tissue is due to stimulation of cGMP-dependent cAMP phosphodiesterases (Flitney & Singh, 1981; Hartzell & Fischmeister, 1986). In vascular smooth muscle, there is circumstantial evidence that elevation of intracellular cGMP leads to inhibition of cGMP-dependent cAMP phosphodiesterase, elevating intracellular cAMP and potentiating vasodilation. The evidence is that cAMP- and cGMP-elevating vasodilators act synergistically, both in vascular smooth muscle and platelets (Walter, Niederding & Waldman, 1988) and also from experiments that show such inhibition in platelets (Macphee, Harrison & Beavo, 1986).

4. Regulation of voltage-dependent Ca^{2+} channels

Recently, NO derived from a variety of sources has been shown to hyperpolarise arterial smooth muscle (Tare, Parkington, Coleman, Neild & Dusting, 1991). Whether this is cGMP dependent or not is as yet unknown, but hyperpolarisation would be likely to induce a reduction in voltage-dependent Ca^{2+}-channel opening, leading to a reduction in intracellular Ca^{2+}.

In vascular smooth muscle, therefore, cGMP is capable of causing vasodilation by a variety of mechanisms summarised in figure 1.5:

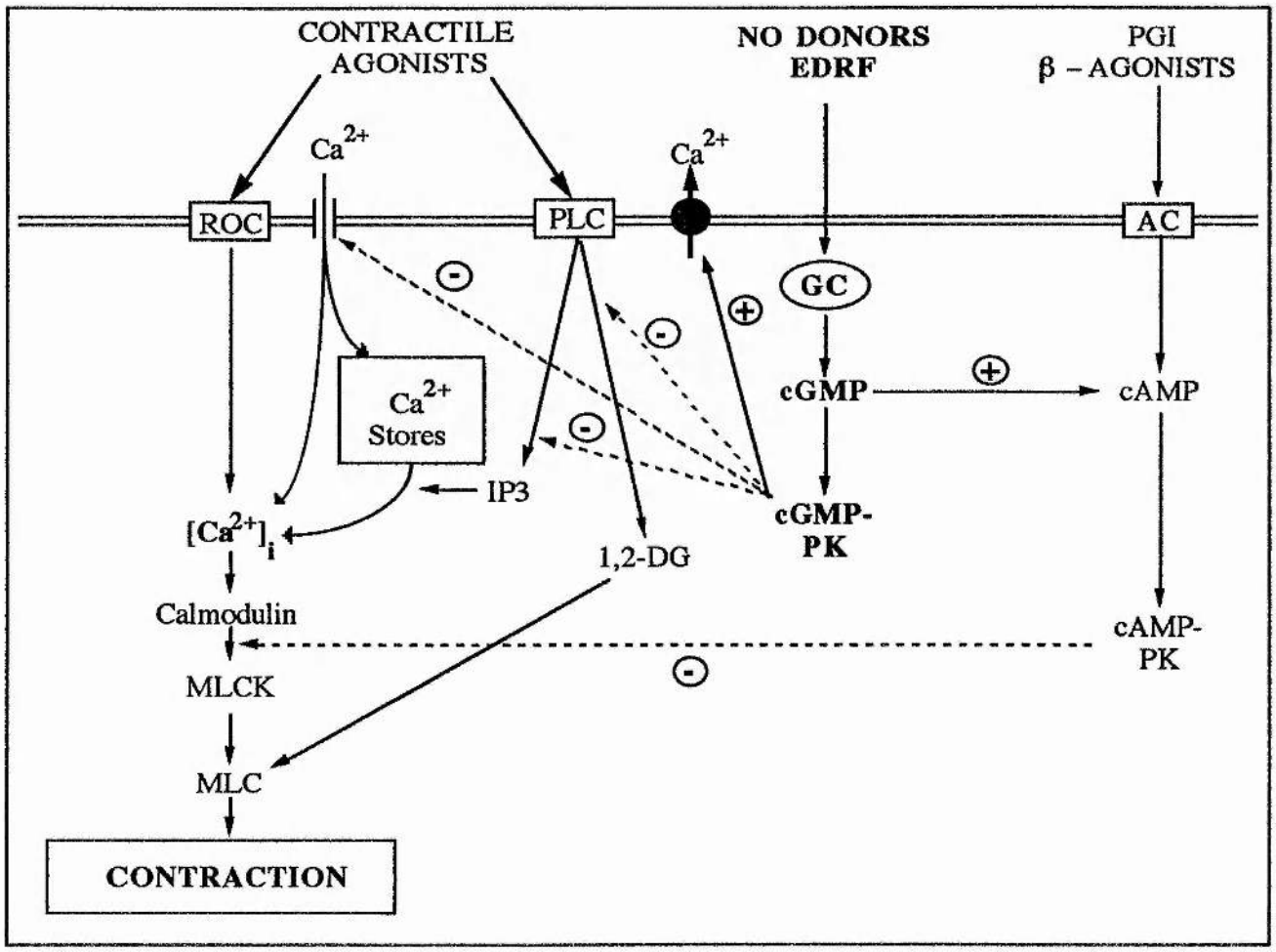


Figure 1.5 *Mechanisms of action of cGMP. Abbreviations: PLC-phospholipase C; ROC-receptor operated channel; GC/AC- Guanylate / adenylate cyclase: cGMP/AMP-PK - cGMP/AMP-dependent protein kinase; IP3 - inositol trisphosphate; 1,2-DG - 1,2 diacyl glycerol; MLC- myosin light chain; MLCK - myosin light chain kinase.*

1.9 OTHER PHYSIOLOGICAL FUNCTIONS OF NITRIC OXIDE

A. Inhibition of platelet aggregation.

The discovery of a role for NO in platelet function occured in 1981, when it was found that NO and other nitrovasodilators inhibited ADP-induced platelet aggregation by a cGMP-dependent mechanism (Mellion, Ignarro, Ohlestein, Pontecorvo, Hyman & Kadowitz, 1981). It was not until 1986 that a link was identified between the actions of NO on platelet aggregation and those of EDRF on vascular smooth muscle (Azuma, Ishikawa & Sekikazi, 1986). It was shown by these and other workers that EDRF itself was capable of inhibiting platelet aggregation *in vitro* (Furlong, Henderson, Lewis & Smith , 1987; Radomski, Palmer & Moncada, 1987) and *in vivo* (Hogan, Lewis & Henderson, 1988). Radomski *et al* showed that NO and prostacyclin were both capable of enhancing the disaggregating effects of the other on platelets (Radomski, Palmer, Moncada, 1987b), although the effects of prostacyclin are mediated by cAMP rather than cGMP (Radomski, Palmer, Moncada, 1987c), and that both have cytoprotective properties (Radomski, Palmer & Moncada, 1988). The cytoprotective effects of NO are now thought to be due to its reacting with the potentially harmful oxygen free radicals (O_2^- in particular) (Rubanyi, Ho, Cantor, Lumma, & Botelho, 1991). At the same time, it was reported that NO inhibited platelet adhesion to collagen, endothelial cell matrix and cultured endothelial cell monolayers by a cGMP-dependent mechanism (Radomski, Palmer & Moncada, 1987c,d). This, however, is in contrast to prostacyclin, which only has a weak, cAMP-mediated inhibitory effect on platelet adhesion (Higgs, Moncada, Vane, Caen, Michel & Tobelom, 1978; Radomski *et al*, 1987c) and does not synergise with NO in this case.

It has been shown that nitrovasodilators which release NO spontaneously (sodium nitroprusside, organic nitrates and 3,morpholino-sydnonomine) are capable of inhibiting platelet aggregation by a cGMP-dependent mechanism. However, some organic nitrates (e.g. GTN) which can elicit vasodilation in vascular smooth muscle are not able to inhibit platelet aggregation (Gerzer, Karrenbrock, Siess, & Heim, 1988). The inference is that

platelets lack the necessary mechanism for uptake of organic nitrates and/or their biodegradation, leading to release of NO.

It is now known that NO is generated in platelets and that it acts as a negative feedback system to modulate aggregation (Radomski, Palmer & Moncada, 1990 a,b). Generation of platelet-derived NO is NADPH-dependent, inhibited by LNMMA and dependent on free intracellular Ca^{2+}. Addition of L-arginine to the medium does not alter basal NO production, but enhances the increase in NO production when aggregation is initiated using either collagen, ADP or arachidonic acid. The inference from this is that nitric oxide synthase exists in platelets and is stimulated to enhance NO synthesis from L-arginine once aggregation is initiated (due to an increase in intracellular Ca^{2+} : Ware, Johnson, Smith & Salzman, 1986). The increase in intracellular NO has been shown to be accompanied by an increase in cGMP but not cAMP. The mechanism by which cGMP inhibits aggregation is not fully understood, but it may involve sequestration of free intraplatelet Ca^{2+} (Busse, Luckhoff & Bassenge, 1987).

EDRF, therefore, may play an important antithrombotic role *in vivo* by inhibiting both platelet aggregation and adhesion to vessel walls, particularly as its actions are enhanced by the synergistic effect of prostacyclin.

B. In the central and peripheral nervous systems

In 1987, the central nervous system (CNS) transmitter, glutamate, was shown to elevate cGMP levels in the post synaptic neurone and give rise to an unstable intracellular factor (Garthwaite & Garthwaite, 1987). This factor was rapidly identified as having properties identical to EDRF or NO (Garthwaite, Charles & Chess-Williams, 1988) and was found to require Ca^{2+} for its production (Knowles, Palacios, Palmer & Moncada, 1988). Earlier work had established that NO is capable of stimulating guanylate cyclase (Miki, Kawebe & Kuriyama, 1977) and that a small molecule whose actions were inhibited by Hb is produced in rat forebrain (Deguchi, 1977). Furthermore, in 1982, the endogenous activator of soluble

guanylate cyclase in neuroblastoma cells was identified as L-arginine (Deguchi & Yoshioka, 1982). This led to an extensive study of both CNS and peripheral NS in the search for NO synthase and further evidence for NO as a neurotransmitter.

Stimulation of N-methyl-D-aspartate (NMDA) receptors has been shown to activate synthesis of NO postsynaptically. It seems that NO does not act on the generator cell (perhaps because high intracellular Ca^{2+} inhibits guanylate cyclase activity) but diffuses out and acts on one or more neighbouring structures, including presynaptic terminals and astrocytes. NO synthesis is blocked by LNMMA and enhanced by L-arginine.

NO is also formed presynaptically, in climbing fibres of the cerebellum. The synthesis is stimulated by the influx of Ca^{2+} into the fibres following action potentials (Shibuki & Okada, 1991 ; Southam & Garthwaite, 1991). The apparent effect of NO generated in these fibres is the long-term depression of responses in Purkinje fibres, since LNMMA blocks the depression.

NO synthase in the brain has since been characterised and shown to be similar to both the endothelial and platelet enzyme in that it is inhibited by LNMMA, LNIO and LNA, requires NADPH as a cofactor (Schmidt, Wilke, Evers, & Bohme, 1989) and is dependent on calcium-calmodulin activation (Bredt & Snyder, 1990). It has been shown to be most prevalent in rat cerebellum, although it is also found in the hypothalamus, midbrain, striatum, hippocampus and medulla oblongata (Forstermann, Gorsky, Pollock, Schmidt, Heller & Murad, 1990).

NO has also been implicated in the pathophysiology of seizures and brain damage in rats (Beckman, Chen & Conger, 1992; Bagetta, Mollace, Iannone & Nistico, 1992).

In the CNS, therefore, NO performs a variety of functions (although not as a classic neurotransmitter or neuromodulator), some of which are positively harmful to the brain tissue.

In the peripheral nervous system, NOS has been discovered in a wide variety of tissues fulfilling many different functions. NO is now commonly termed a 'non-adrenergic, non-cholinergic' (NANC) neurotransmitter found, for example, in the vagal nerves supplying gastrointestinal smooth muscle. In the presence of both adrenergic and cholinergic inhibitors, vagal stimulation results in relaxation, which can be inhibited by LNMMA, LNAME or LNA. NO has also been shown to be the NANC neurotransmitter involved in nocioception (Durte, Faccioli & Ferreira, 1992), relaxation of the lower oesophageal sphincter, gastrointestinal smooth muscles (Toda, Baba & Okamura, 1990; Thornbury, Ward, Dalziel, Carl, Westfall & Sanders,1992), bovine retractor penis (Liu, Gillespie, Gibson & Martin, 1991; Martin, Gibson & Gillespie,1992), the smooth muscle sphincter of the urinary bladder (Thornbury, Hollywood & McHale, 1992), urethral smooth muscle (Persson, Garcia-Pascaul, Forman, Tottrup & Andersson, 1992) and corpus cavernosum (Goessl, Knispel & Beckmamn, 1992). In each case, competitive inhibitors of NO synthase block nerve-induced relaxations.

C. In activated macrophages.

In 1987, it was demonstrated that activated macrophages required L- (but not D-) arginine to exert their cytotoxic effects on tumour target cells (Hibbs, Taintor, & Vavrin, 1987). It was also shown that L-citrulline and NO_2^- were formed in the process. LNMMA competitively inhibits the synthesis of both of these compounds and also the expression of cytotoxicity (Hibbs *et al*, 1987). With the recent identification of the L-arginine:NO synthetic pathway in endothelial cells, platelets and in the nervous system, NO was swiftly recognised as the most likely active intermediate in activated macrophages (Marletta, Yoon, Iyenger, Leaf & Wishnok, 1988 ; Hibbs, Taintor, Vavrin & Rachlin, 1988 ; Stuehr & Nathan, 1989), especially since the cytotoxicity of activated macrophages is inhibited following endocytosis of haemoglobin or erythrocytes (Weinberg & Hibbs, 1977).

Activated macrophages are thought to exert their cytotoxic effects in a variety of ways:

1. Inhibition of deoxyribonucleic acid (DNA) replication. This is reflected in a reduction in the uptake of tritiated ([^{3}H]) thymidine in target cells (Krahenbuhl, 1980; Hibbs, Taintor, Vavrin, Granger, Drapier, Amber & Lancaster, 1991). It is thought likely that deoxyribonucleotide reductase, the rate limiting enzyme in DNA synthesis, is the target for macrophage-derived NO. The catalytic site on this enzyme contains non-haem iron which is potentially vulnerable to NO attack (Reichard & Ehrennberg, 1983).

2. Inhibition of the mitochondrial respiratory chain. The site of NO-induced inhibition of the electron transfer system has now been identified as the two proximal oxidoreductases, NADH:ubiquinone oxidoreductase and succinate:ubiquinone oxidoreductase (Granger & Lehninger, 1982). Both complexes contain Fe-S groups, known to be susceptible to NO binding. Adenosine triphosphate (ATP) synthesis, via the glycolytic pathway, is apparently unaffected when target cells are co-cultivated with activated macrophages (Granger, Taintor, Cook & Hibbs, 1980).

3. Inhibition of aconitase (a citric acid cycle enzyme). As with the electron transport chain enzymes inhibited by activated macrophages, aconitase contains a 4(Fe-S) prosthetic group at its catalytically active site (Kilbourne, Klostergaard & Lopez-Berestein, 1984). The inhibition of this, and the afore-mentioned enzymes, may be associated with the reported loss of a significant proportion of intracellular iron from target cells (Hibbs, Taintor & Vavrin, 1984), since the effects of NO can be reversed by incubation with ferrous ions (Drapier & Hibbs, 1986).

It is interesting to note that activated macrophages themselves are prone to the same cytotoxic effects of NO as found in target cells (Drapier & Hibbs, 1988). Activation of macrophages to release NO in large quantities is evidently an important immune response involved in the control of microorganisms and tumour cells. The non-specific cytotoxicity of NO is largely due to its inhibitory effects on enzymes involved in DNA synthesis, metabolism and respiration.

Attempts to characterise the enzyme responsible for NO synthesis in activated macrophages proved more difficult than in other systems. It became apparent that the enzyme was only expressed in macrophages once they had been activated with *Escherichia coli* -lipopolysaccharide (LPS) alone, or in combination with interferon-g (IFN-γ) (Hibbs *et al*, 1987), and that the process involved protein synthesis (Marletta *et al*, 1988). Evidently, activation of macrophages induces NOS synthesis within the cells. The enzyme was found to be inhibited by L-canavanine (Iyenger, Stuehr & Marletta, 1987) as well as LNMMA (the isoforms found in endothelial cells, platelets and the nervous system are not inhibited by L-canvanine). Hereafter, the isoform of NOS found in activated macrophages will be termed 'inducible' whilst that found elsewhere is 'constitutive'. Recently, a more intensive study of the various NO synthase isoforms has revealed further subdivisions of these two classes, some of which have been purified and characterised (Forstermann, Schmidt, Pollak, Sheng, Mitchell, Warner, Nakane & Murad, 1991). Table 1.1. shows the major differences between the six isoforms so far characterised:

	ISOFORM					
Characteristic	**Ia**	**Ib**	**Ic**	**II**	**III**	**IV**
Sol. / partic.	soluble	soluble	soluble	soluble	particulate	particulate
Cosubstrates/ Cofactors	NADPH, BH_4, FAD/FMN	NADPH	NADPH, BH_4, FAD	NADPH, BH_4, R-SH, FAD/FMN	NADPH, BH_4	NADPH
Regulated by:	Ca^{2+}/calmod	Ca^{2+}/calmod	Ca^{2+}	?	Ca^{2+}/calmod	?
M_r (denatured)	155 kDa	?	150 kDa	125-135 kDa or 150 kDa ?	135 kDa	?
Present in:	cerebellum	endothelium	neutrophils	macrophagses hepatocyte? Kupffer cells? VSM ? endothelium ? lung?liver?	endothelium	macrophages

Table 1.1. *Isoforms of NO synthase (after Forstermann et al., 1991). All isoforms use L-arginine as a substrate and are inhibited by LNMMA and LNIO.*

1.10 NO DONOR DRUGS

Considerable reference has already been made to so-called 'nitrovasodilators'. Hereafter, the term 'NO donor drug' will be used when referring to any compound capable of releasing NO under physiological conditions, either by enzymatic degradation or spontaneous decomposition. Figure 1.6 shows the structural formulae of some common nitric oxide donor drugs as well as those of two novel compounds.

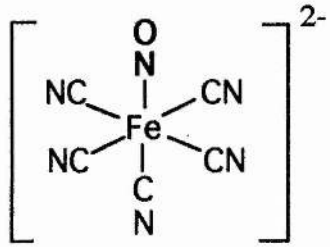
AMYL NITRITE

$(CH_3)_2CH\text{-}CH_2\text{-}CH_2\text{-}O\,NO$

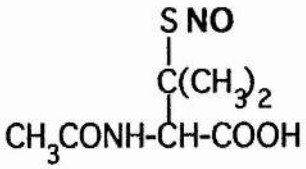
GLYCERYL TRINITRATE

$CH_2\text{-}O\text{-}NO_2$
$CH\text{-}O\text{-}NO_2$
$CH_2\text{-}O\text{-}NO_2$

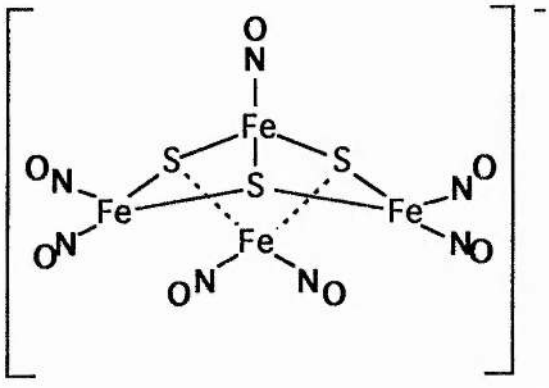
NITROPRUSSIDE
(NP)



S-NITROSO-N-ACETYL PENICILLAMINE
(SNAP)



ROUSSIN'S BLACK SALT
(RBS)



CUBANE
(CUB)

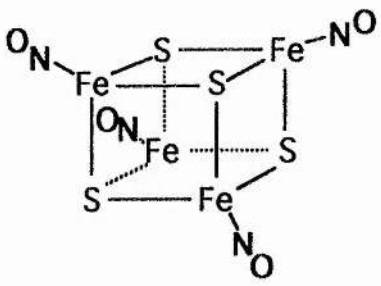


Figure 1.6. *Examples of NO donor drugs. Full chemical names: NP - pentacyanonitrosylferrate. RBS - heptanitrosyltri-μ_3-thioxotetraferrate. CUB. - tetranitrosyltetra-μ_3-suphido-tetrahedro-tetrairon.*

A. ORGANIC NITRATES

Both amyl nitrite and glyceryl trinitrate have been used for over a century in the treatment of angina (Brunton, 1867). The mechanism of action of these compounds remained a mystery until1973 when it was discovered that relaxation of precontracted aortic strips was dependent on the presence of -SH groups within the tissue (Needleman *et al,* 1973; Ignarro *et al,* 1981). Ignarro *et al,* (1981) went on to show that S-nitrosocysteine and S-nitroso-N-acetylpenicillamine have similar vasorelaxant properties to GTN, amyl nitrite and NP when -SH groups are present (also Kowaluk, Poliszczuk & Fung, 1987). As yet, the mechanism by which organic nitrates break down to release NO has not been satisfactorilly explained. Current hypotheses include breakdown of GTN to produce NO_2^- (possibly enzymatic), followed by acidification to produce HONO and ultimately NO. The shortfall of this hypothesis lies in the requirement for acid conditions; intracellular pH in smooth muscle cells has been estimated as between 5.9 and 7.0 (Adler, 1972; Stephens, Mitchell, Kroegger, 1977).

B. NITROPRUSSIDE

The sensitivity of vascular smooth muscle to sodium nitroprusside (NP) has been recognised for over a century (Leeuwenkamp, Van Bennekom, Van der Mark & Bult, 1986). Clinically, NP was used extensively in acute hypertensive crises and malignant hypertension (Kreye, 1980). The recent decline in its use is linked to sporadic reports of cyanide poisonings (Smith, Kruzyna, 1974; Butler, Glidewell, 1987). It is now widely accepted that cyanide is generated from NP on exposure to light in acid conditions (Arnold, Longnecker & Epstein, 1984) or in the presence of Hb (Smith & Kruzyna, 1974).

It has been long suspected that the nitrosyl group of NP is the vasodilatory component of the molecule. However, the apparent stability of NP when protected from light prompted investigation of the intracellular mechanism by which the iron-nitrosyl bond is cleaved *in vivo.* Early work using homogenates from various tissues suggested that the decomposition was non-enzymatic (Casinelli, 1956; Hill, 1942; Smith & Kruszyna, 1974). Degradation, however, was shown to be accelerated in the presence of cysteine and methionine (Page,

Corcoran, Dustan & Koppanyi, 1955). Leeuwenkamp *et al* (1986) suggested the process involved reduction of NP by an 'unidentified reductor'. Recent work on subcellular fractions of bovine coronary arteries showed that NO was readily generated from NP in subcellular fractions and that the dominant site of activity appeared to be in the membrane fractions (Kowaluk, Seth & Fung, 1992). The researchers went on to isolate two NO generating enzymes of Mr 4 kDa and 112-169 kDa using gel filtration.

Experiments in this laboratory, have shown that half-ventricular preparations of the frog heart do not respond to NP in the dark. However, when exposed to laser light, NP was shown to have a negative inotropic effect on contractility (Flitney & Kennovin, 1988). The inference from these experiments is that no NO generating enzyme exists in this particular tissue.

Recently, some doubt has been cast on whether NO production is exclusively responsible for the vasodilatory effect of NP. Otsuka, Dipiero, Hirt, Brennamen & Lockette (1988) found that endothelium-dependent relaxations are attenuated by agents that inhibit guanylate cyclase but that the same was not always true of NP. They found that maximal NP-induced relaxations were accompanied by a relatively small increase in intracellular cGMP. Similar elevations in cGMP levels by endothelium-dependent vasodilators result in relatively small relaxations. Elsewhere, it has been shown that low concentrations of both NP and GTN are capable of causing precontracted aortic rings to relax without increasing cGMP levels (Diamond & Chu, 1983). The efficacy of the drugs was also shown to be dependent on agonist (phenylephrine) concentration but cGMP levels were unaffected.

In addition, NP does not fit the correlation between guanylate cyclase EC_{50} and NO formation that holds true for other NO donor drugs (Feelisch, 1991). Accordingly, it is now thought that NP acts through several mechanisms, one or more of which is (are) cGMP-*in*dependent.

C. S-NITROSO-N-ACETYL PENICILLAMINE (SNAP)

SNAP is stable in the solid state, but thermally unstable once in solution. The rate of decomposition is dependent on pH (most unstable at pH7) and the type of buffer present, as well as on the ambient temperature (Butler & Askew, unpublished data). Breakdown appears to be non-enzymatic *in vivo,* since both L- and D-enantiomers give similar dose-dependent response curves (Butler, Askew & Flitney, 1993). Degradation of SNAP is accelerated on exposure to light (absorption maximum - 339 nm ; Butler & Askew, unpublished data).

D. IRON-SULPHUR NITROSYL CLUSTERS

RBS and CUB (see figure 1.6) belong to a class of compounds which have been known for over a century (Roussin, 1858). The chemistry of these compounds is unique, not least in respect of their synthesis, which arises by a process known as 'spontaneous self assembly' (Sung, Glidewell, Butler & Hoffmann, 1985). That is to say, under appropriate conditions, synthesis occurs from mononuclear starting materials without the detectable formation of any intermediates. Of all compounds in this class, RBS appears the most stable, as it is the end product of numerous reactions involving other iron-sulphur nitrosyl clusters. In the solid state, the compounds are relatively stable, particularly if kept under nitrogen and at -10°C. The present study (chapter 4) will show that in solution, both compounds have vasodilator properties, inhibitable by Hb and MB but not by LNMMA (Flitney, Megson, Butler & Clough, 1990; Flitney, Megson, Flitney & Butler, 1992). Evidently, once in solution, they decompose to release NO. Ultimately, the products of breakdown of RBS have been shown to be iron oxide and elemental sulphur, although it is unclear whether other intermediates exist (Butler & Askew, unpublished data). Both compounds are potentially potent vasodilators should they decompose to release their full complement of bound nitrosyl groups in solution.

1.11 PHOTORELAXATION

Furchgott, Ehrreich & Greenblatt (1961) first demonstrated that precontracted strips of rabbit aorta relax when exposed to light. Using a monochromator, they showed that the degree of relaxation was both intensity and wavelength dependent (shorter wavelengths eliciting larger photorelaxations). The amplitude of the response was independent of oxygen tension, though recovery in the dark was markedly slower under anaerobic conditions. Lowering the temperature from 37 to 20°C increased the amplitude of the response and prolonged its time course. The nature of the photosensitive material could not be established, though the way in which the response varied with wavelength (biological 'action' spectrum) led Furchgott *et al* to propose that it could be a 'complex of a metal, such as iron, with a protein'. Since then, research focussed on the mechanism responsible for photorelaxation.

It is known that the extent of photorelaxation is attenuated in the presence of GTN (Furchgott, 1971). The experimental procedure involved increasing the concentration of contractile agonist (phenylephrine) to compensate for the vasodilatory effect of GTN. Photorelaxations before and after GTN treatment could then be compared, in the knowledge that any differences found were not due to changes in pre-illumination tension. The inference from this result was that photorelaxation and GTN-induced relaxation involve a common pathway.

However, similar experiments showed that nitrite (NO_2^-), potentiated photorelaxation. Recently, a comprehensive study of the interaction of ultraviolet light with NO_2^- has shown that SOD enhances nitrite-potentiated photorelaxation of aortic rings but not unpotentiated responses (Matsunaga & Furchgott, 1988). Photorelaxation was shown to be inhibited by Hb (but not met Hb). Photoactivation of NO_2^- before superfusing the preparations also resulted in potentiation of the relaxation due to NO_2^-. This effect was augmented by SOD and inhibited by Hb. These results suggest that NO_2^- releases NO on exposure to light, resulting in enhanced relaxation.

Non-vascular smooth muscle does not undergo photorelaxation unless pre-treated with NO_2^-. Experiments by Furchgott (1984) seemingly eliminated the endothelium as a possible source of NO in the photorelaxation of vascular smooth muscle since endothelium-denuded preparations still relax when exposed to ultraviolet light.

However, recent work by Deliconstantinos, Villiotou & Fassitsas (1992) on cultured human endothelial cells and isolated NO synthase suggests that ultraviolet light stimulates NO synthesis by the enzyme since the effect was inhibited by LNMMA. The authors propose that vasodilation of microvessels in the skin due to increased NO synthesis is responsible for the redness caused by sunburn.

The involvement of NO in photorelaxation is disputed by work on crude preparations of soluble guanylate cyclase from mesenteric arteries (Karlsson, Axelsson & Andersson, 1984). Ultraviolet irradiation of the enzyme results in an elevation of cGMP, leading to claims that photorelaxation is due to direct stimulation of guanylate cyclase.

Controversy as to the involvement and source of NO is evident from these conflicting opinions. Previous experiments in this laboratory have shown that photorelaxation in the isolated rat tail artery is inhibited by pyrogallol and Hb but not by LNMMA. Detailed experiments are reported here, undertaken to determine (a) whether NO is responsible for photorelaxation and if so, (b) the possible source of NO released on exposure to light.

CHAPTER 2

MATERIALS AND METHODS

2.1 PREPARATION

Experiments were performed on 8 - 12 mm long segments of tail artery taken from normotensive adult Wistar rats (270 - 538 g). Animals were killed by cervical dislocation and the tail removed. The artery was exposed following removal of the overlying skin and connective tissue. Once exposed, the artery was cannulated (Portex cannula), dissected free and transferred to a perspex bath. Care was taken to identify and tie-off all side-branches.

2.2 APPARATUS

The apparatus is shown in Figure 2.1. The vessel (V) was perfused internally at a constant flow rate of 2ml min^{-1} driven by a peristaltic pump (P1: Gilson minipuls 2). The perfusate was pre-warmed to 32-33°C by passage through the heat exchanger (HE). Drugs were introduced into the lumen of the vessel by bolus injection (10μl) through a resealable rubber septum (RS). A differential pressure transducer (P: Sensym type SCX 150NC; Farnell Electronic Components, Leeds) detected changes in back pressure determined by arterial tone.

The outer surface of the vessel was superfused continuously (ca. 8ml min^{-1}) with solution driven by a second peristaltic pump (P2: BDH minipump). The solution was pre-warmed to 32-33°C by passage through a heat exchanger similar to that used in the internal circuit.

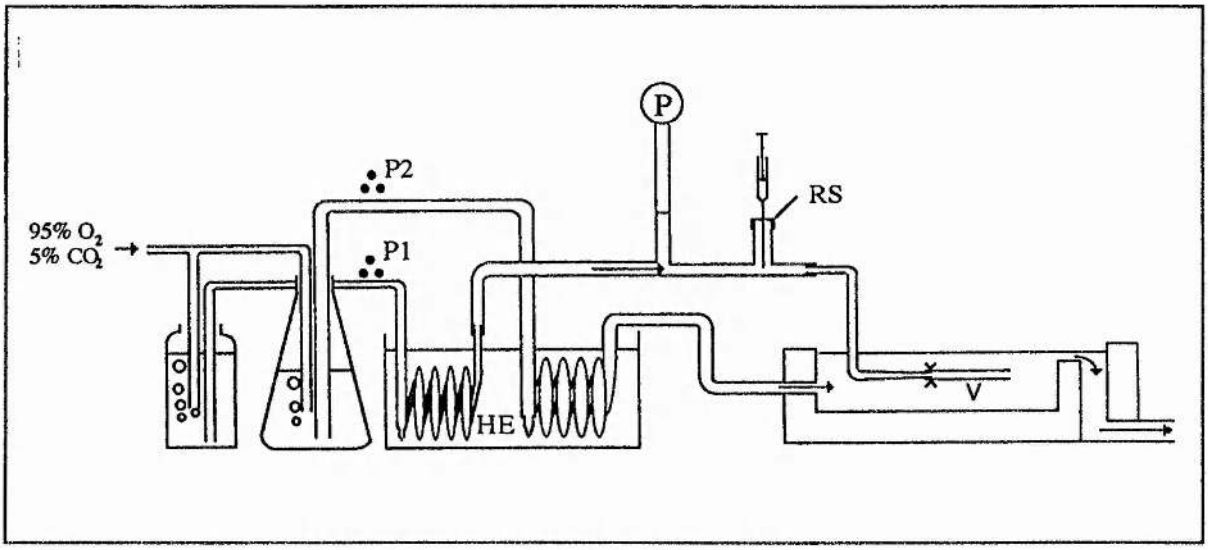


Figure 2.1 *Apparatus used for perfusing isolated segments of rat tail artery. See text for full description and explanation of lettering.*

Alterations made to this appparatus for particular experiments will be described in the Methods section of the relevent chapters.

2.3 EXPERIMENTAL PROTOCOL

Arteries were perfused internally and externally with oxygenated Krebs solution (composition mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.15, $CaCl_2$ 2.5, $MgCl_2$ 1.1, glucose 5.6, gassed with 95% O_2 / 5% CO_2 to maintain pH 7.4). Internal perfusion of the cannulated vessel was initially slow (flow rate ca. 0.2 ml min^{-1}). The working flow rate of 2ml min^{-1} was attained by gradually increasing the pump speed over a period of 10-20 mins. The preparation was then allowed to stabilise for a further 20-30 mins, at which time a 'passive' pressure of 5 - 24 mmHg was recorded. Precontraction was induced by addition of phenylephrine hydrochloride (PE) to both internal and external perfusates (figure 2.2).

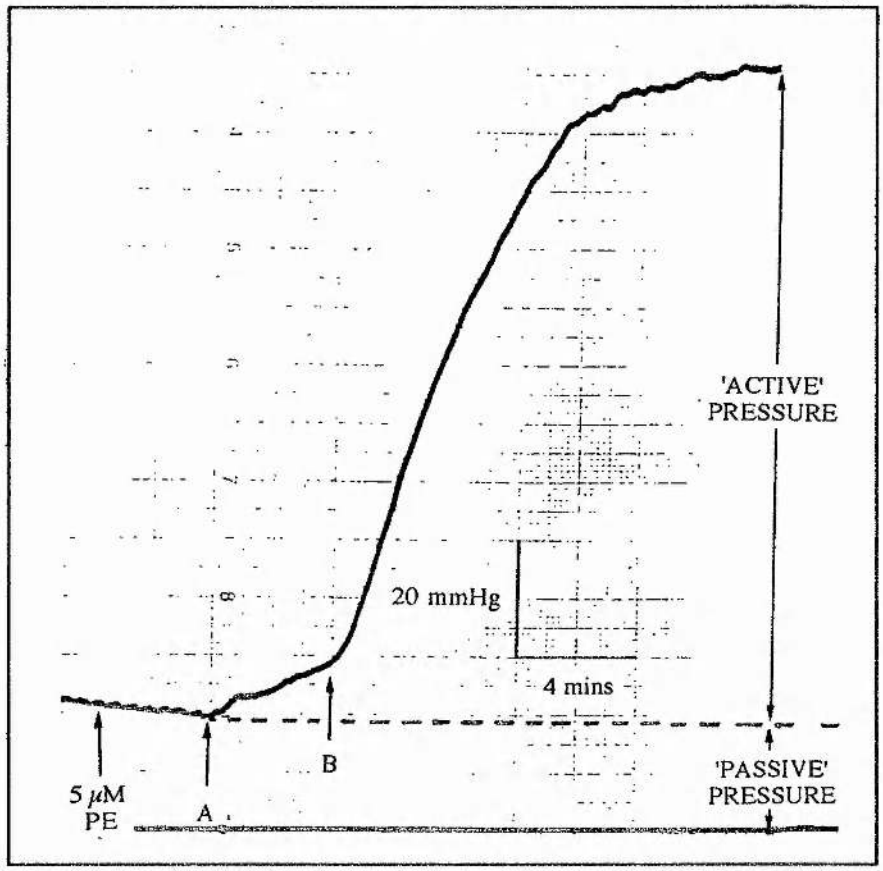


Figure 2.2. *Pressure recording showing precontraction with PE. 5μM PE was added to both internal and external perfusate reservoirs at the time indicated, reaching the vessel through the external perfusate at A and the internal at B. The pressure maintained by the vessel before PE addition is termed 'passive', whilst agonist-induced pressure is 'active'. Expt. № 920304.*

The PE concentration was adjusted to maintain a pressure of 90 - 138 mmHg above the passive pressure.

At this point in the experimental procedure, the laboratory lights were switched off, leaving a red safelight (60W) as the only means of illumination. This precaution served to prevent photorelaxation of the vessel and to protect any NO donor drugs in use from photolytic decomposition.

2.4 DRUGS

L-phenylephrine hydrochloride (Mr 203.7; Sigma Chemicals). Appropriate volumes of a 10^{-3}M stock solution were added to both the internal and external perfusates to produce concentrations ranging from 1 -12 μM.

Sodium nitroprusside (Mr 298; Sigma Chemicals). 10^{-2}M stock solutions were serially diluted to produce the desired range of concentrations immediately prior to use.

Ferrohaemoglobin (Mr 64500; Sigma Chemicals) was prepared by reduction of 100mls of 1mM bovine haemoglobin using 10mM sodium dithionite. The dithionite was dialysed out of solution against 3x 2 litre volumes of water at 0-5°C. The stock ferrohaemoglobin was then split into 3ml samples and frozen (-10°C). Samples were used within 2 weeks.

Methylene blue (Mr 320; Sigma Chemicals). Appropriate volumes of a 10^{-3}M stock solution were added to the internal perfusate to produce final concentrations of 10 - 200μM.

Carbamylcholine chloride (Mr 182; Sigma Chemicals). 10^{-2}M stock solutions were serially diluted to produce the desired range of concentrations immediately prior to use. Solutions were kept on ice during experiments.

N^G-Monomethyl-L-arginine (Mr 252; gift of RMJ Palmer & H Hodson, Wellcome Research labs., Kent). Appropriate volumes of a 10^{-3} M stock solution were added to the internal perfusate to produce a final concentration of 100μM.

S-nitroso-N-acetylpenicillamine (SNAP; Mr 220; prepared by S. Askew & A.R. Butler, Department of Chemistry, University of St. Andrews) was synthesised by reacting N-acetylpenicillamine with sodium nitrite (Field, Dilts, Ravichandran, Lenhert & Carnahan, 1978). 10^{-2}M stock solutions were serially diluted to produce the desired concentration immediately prior to use. Solutions were kept on ice during experiments.

Roussin's Black Salt (RBS; Mr 553) was prepared in this laboratory and by Dr. S. Glidewell (Department of Chemistry, University of St. Andrews) using the protocol detailed below. $5x10^{-3}$M stock solutions were serially diluted to produce the desired range of concentrations immediately prior to use.

Cubane (CUB; Mr 472) was prepared by Dr. S. Glidewell at the Department of Chemistry, University of St. Andrews using the protocol detailed below. Unlike RBS, CUB. is not soluble in water and as such cannot be dissolved in Krebs solution. The solid was first dissolved in dimethyl suphoxide (Sigma Chemicals) to produce a 10^{-2}M stock solution which was then serially diluted with Krebs solution to produce the desired range of concentrations.

'Authentic' NO. Saturated solutions of NO were prepared by bubbling NO gas, derived from the reaction of ascorbic acid with sodium nitrite, through distilled water. Extreme precautions were taken to exclude oxygen from the water which was double distilled under O_2-free N_2 and kept under argon throughout the entire procedure. A small sample (10ml) was transferred into a glass vial, already gassed with argon to drive out oxygen. The 3/4-filled vial was then sealed with a rubber septum through which two needles were passed. One of the needles was attached to a small, deoxygenated and sealed reaction flask containing ascorbic acid and sodium nitrite. NO produced from the reaction passed through the distilled water and out via the second needle. The process was allowed to continue for

approximately 30 mins, at which point the needles were removed, leaving a saturated solution of NO below a NO atmosphere.

Dilutions of the saturated solution were made by injecting a small volume (<10μl) into degassed, sealed vials containing known volumes of 'O_2-free' distilled water.

All procedures were carried out in a fume cupboard.

PREPARATION OF ROUSSIN'S BLACK SALT (Sodium heptanitrosyl-tri-μ_3-sulphido-tetraferrate : $Na[Fe_4S_3(NO)_7]$)

Figure 2.3 shows the appparatus used to synthesise RBS.

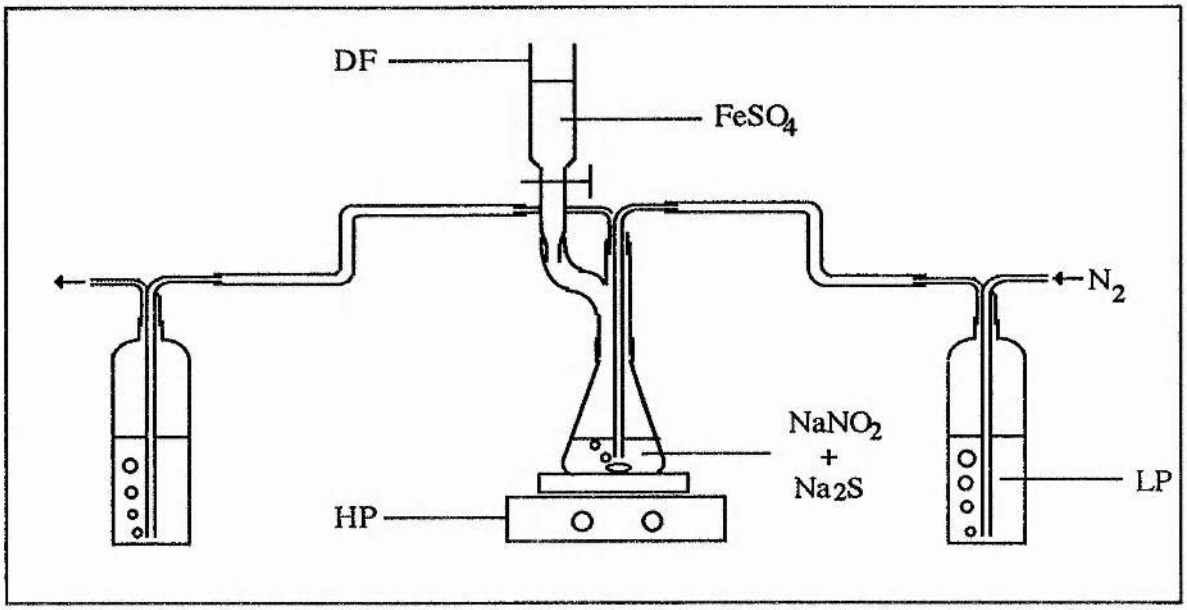


Figure 2.3. *Apparatus used in synthesis of RBS. Abbreviations: DF - dropping funnel; LP - liquid paraffin; HP - hotplate and stirrer.*

Sodium nitrite ($NaNO_2$) and sodium sulphide (Na_2S) were stirred and heated under nitrogen until boiling. Ferrous sulphate solution (FeSO4) was added from a dropping funnel (DF) to the accompaniment of black frothing, quelled by the addition of 20% ammonia solution. The resulting mixture was hot-filtered through pre-heated Hyflo to remove iron oxide and the residue washed with hot / boiling water. The filtrate was cooled on ice and crude RBS precipitated out of solution. The precipitate was filtered off, recrystallized from hot water and dried in a vacuum dessicator. RBS was stored in the dark,

under nitrogen and at -10°C. Purity was assesed using infra-red spectroscopy where three peaks are seen at 1795,1747 and 1707 cm^{-1} (Figure 2.4).

PREPARATION OF CUBANE (Tetranitrosyl tetra-μ_3-sulphido-tetrahedro-tetrairon: $Fe_4S_4NO_4$).

RBS and elemental sulphur (S_8) were heated under reflux in dry, redistilled toluene under nitrogen for 16 hrs. After cooling, the mixture was filtered through a packed bed of Hyflo supercel and the solvent removed (oil-pump vacuum). The residue was dissolved in the minimum volume of chloroform and introduced onto a silica chromatography column. The black band was eluted with chloroform. The column effluent was then evaporated to yield shiny black plates of the product. Purity of the product was assesed by melting point (95-97°C) and infra-red spectroscopy, where a single peak is seen at 1790 cm^{-1} (Figure 2.4).

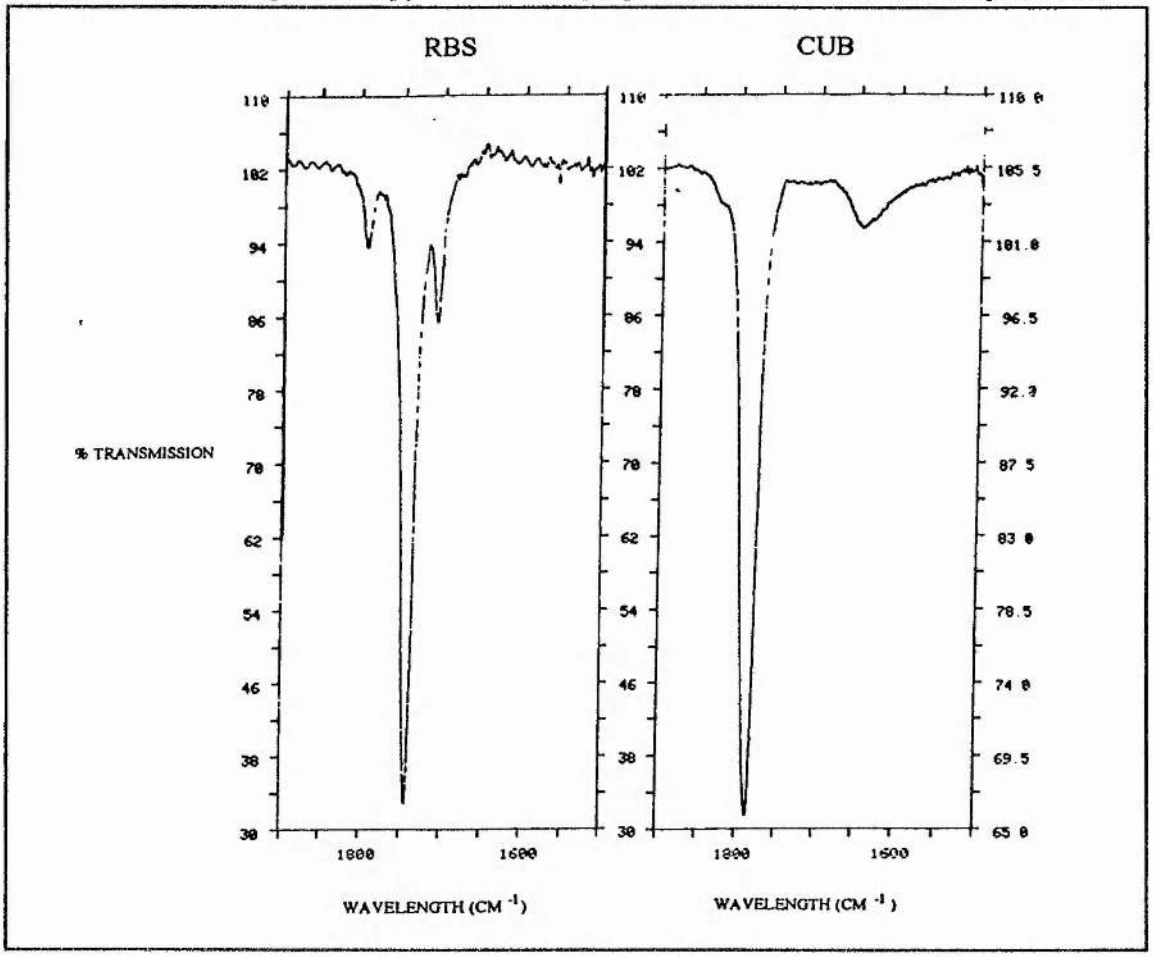


Figure 2.4. *Infra-red absorption spectra for RBS and CUB (1 mg ml^{-1}) dissolved in tetrahydrafuran (THF). Note the small, broad peak at ca. 1620 cm^{-1} in the CUB spectrum. This is due to the presence of a small amount of water in the sample. Spectra recorded on 19/07/91 (RBS) and 1/05/91 (CUB).*

CHAPTER 3

EFFECTS OF HB ON VASODILATOR RESPONSES

TO BOLUS INJECTIONS OF NP, SNAP, RBS AND CUB.

3.1 INTRODUCTION

The vasodilator properties of organic nitrates and nitrites have been used clinically for many years, without a clear understanding of the mechanism underlying their actions. Since the endogenous vasodilator (EDRF) was recognised as NO (Moncada *et al*, 1987), it became clear that the actions of these and other related compounds were mediated by NO. The term 'nitrovasodilator' was attributed to any such compound but here they will be referred to as NO-donor drugs.

Experiments were performed to determine the relative potency of authentic NO, NP and SNAP as well as two novel NO donors, RBS and CUB (all molecular structures shown in figure 1.7). The inhibitory effect of Hb, a recognised NO scavenger, was investigated. Preliminary experiments in this laboratory (Flitney *et al*, 1990), have shown that responses to bolus injections of NP, RBS and CUB are unaffected by the NOS inhibitor, LNMMA. In contrast responses to carbamylcholine chloride (CCh) were severely attenuated in the presence of LNMMA (Figure 3.1 (c)). However, responses to CCh, RBS and CUB, but not NP, were inhibited by both MB and Hb (figure 3.1 (b) and (d)).

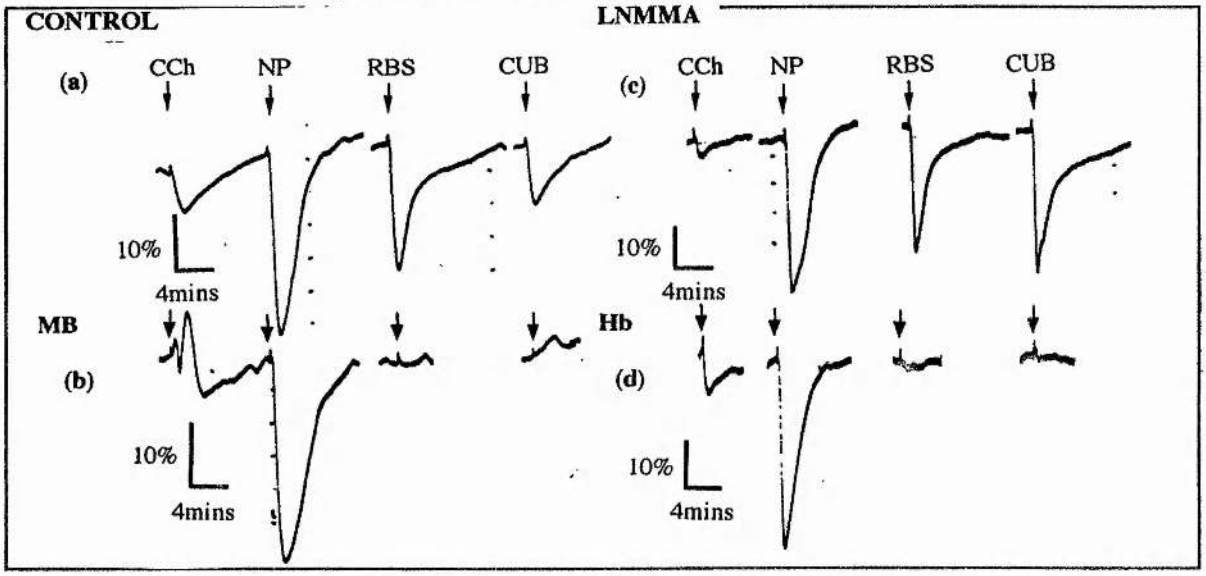


Figure 3.1 *(a) Vasodilator responses to 10µl test doses of CCh (10^{-2}M), NP ($2.5x10^{-4}$M), RBS ($5x10^{-4}$M) and CUB (10^{-4}M). Other traces show responses to test doses in the presence of internally perfused MB (75µM, (b)), LNMMA (100µM, (c)) and Hb (15µM, (d)). Experimental details (a,b): 274g male Wistar rat. 5µM PE produced an active pressure of 135mmHg. Basal tone = 12mmHg. Expts (c) and (d) 373g male Wistar rat. 4µM PE produced an active pressure of 114mmHg. Basal tone = 17mm Hg.*

3.2 MATERIALS AND METHODS

A. ANIMALS

Adult male Wistar rats (250-460 g) were killed by cervical dislocation, their tails removed and a length of artery (8-15 mm) cannulated and dissected free as described earlier.

B. APPARATUS

The apparatus shown in figure 2.2 was used for all experiments.

C. EXPERIMENTAL PROTOCOL

Vessels were precontracted with PE to produce agonist-induced perfusion pressures of 101 +/- 3.5 mmHg ([PE] = 6.5 +/- 0.5 μM; n=21). Responses to 10 μl bolus injections of increasing dose (0.1 μM - 10 mM for NP and SNAP; 0.1 μM - 5 mM for RBS and CUB) were compared to responses to the same doses injected into internal perfusate containing 15 μM Hb.

D. ANALYSIS OF RESULTS

The amplitude of responses are expressed as a % of the pre-injection pressure and plotted as a function of $\log_{10}$ dose concentration (M).

From the log-dose response curves, the dose required to elicit half-maximal responses in each case was estimated.

3.3 RESULTS

A. Responses to SNAP and NP.

Microinjections of either SNAP or NP produced immediate, dose dependent vasodilations. Responses consistently recovered to the pre-injection pressure, although the time taken to recover displayed a dose-dependence.

Perfusion of the vessel with 15μM Hb resulted in a reversible increase in pressure of 55.92 +/- 6.4% (n=15). This compares favourably with the increase in pressure observed when vessels are internally perfused with 100μM LNMMA (57.1 +/- 6.1%, n=10), a concentration which exceeds that required to produce total blockade of EDRF-induced relaxation in aortic rings (Moncada *et al*, 1986).

Figure 3.2 shows recordings from experiments using both compounds. The records show responses to increasing doses of SNAP or NP before the addition of 15μM Hb followed by corresponding responses in the presence of Hb. Vasodilations evoked by both compounds are transient, with those to SNAP showing more rapid recovery. In some cases, responses to high doses of either compound recover to a level above the pre-injection pressure and then return slowly to the expected level (figure 3.2, SNAP).

Addition of Hb to the internal perfusate produces a rapid rise in pressure. Injections of either drug in the presence of Hb are still capable of producing substantial vasodilator responses. However, responses to both SNAP and NP were found to be significantly attenuated in the presence of Hb, when expressed as a % of the pre-injection active pressure.

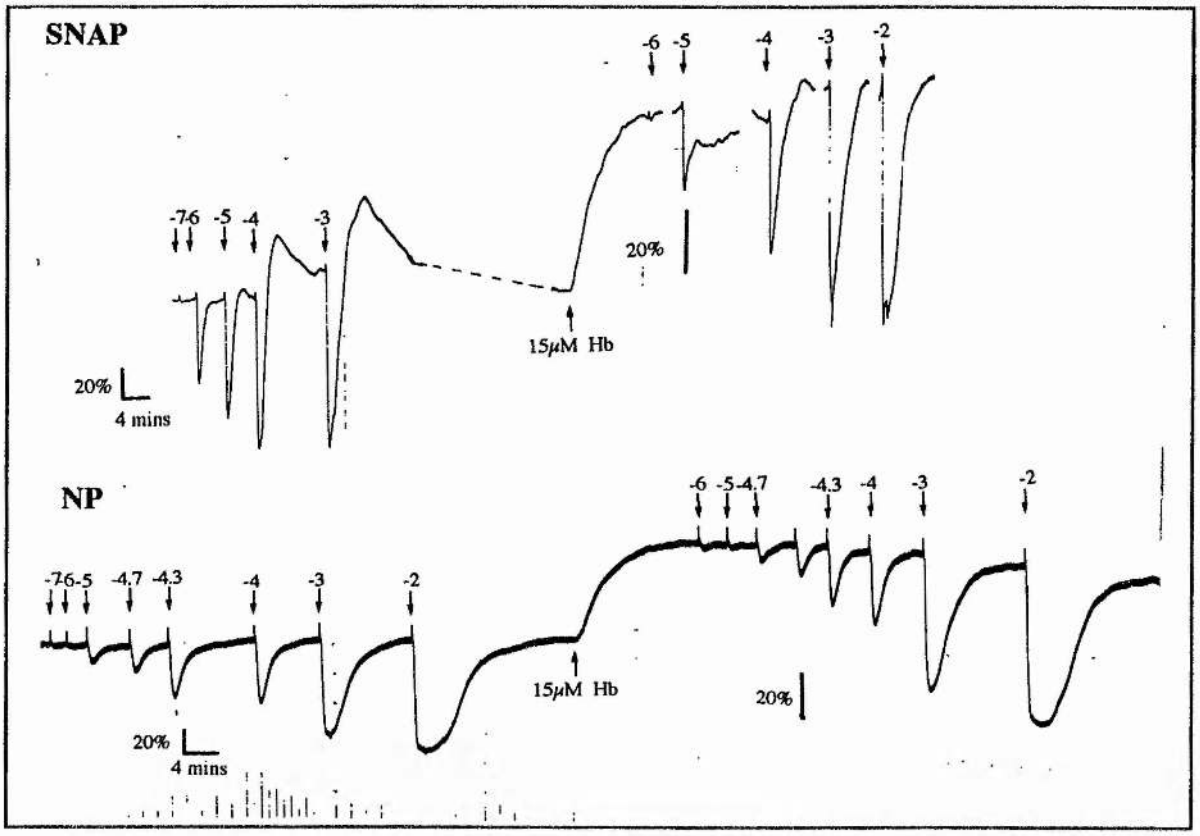


Figure 3.2. *Pressure recordings showing vasodilator responses to bolus injections (10μl) of increasing doses of SNAP or NP, before and during 15μM Hb perfusion. Concentrations indicated for each injection are log [dose](M). Note the change in vertical scalebar referring to responses in the presence of Hb (brought about by an increase in active pressure). Experimental details: SNAP, expt. No 930115. 304g, male Wistar rat; 1.5μM PE produced 120mm Hg active pressure. 15μM Hb caused a further 87% rise in active pressure. NP, expt. No 901016. 346g male Wistar rat. 5μM PE produced 113mm Hg active pressure. 15μM Hb caused a further 49% rise in active pressure.*

Figure 3.3 shows log dose response curves derived from experiments involving both compounds in the absence and presence of 15μM Hb.

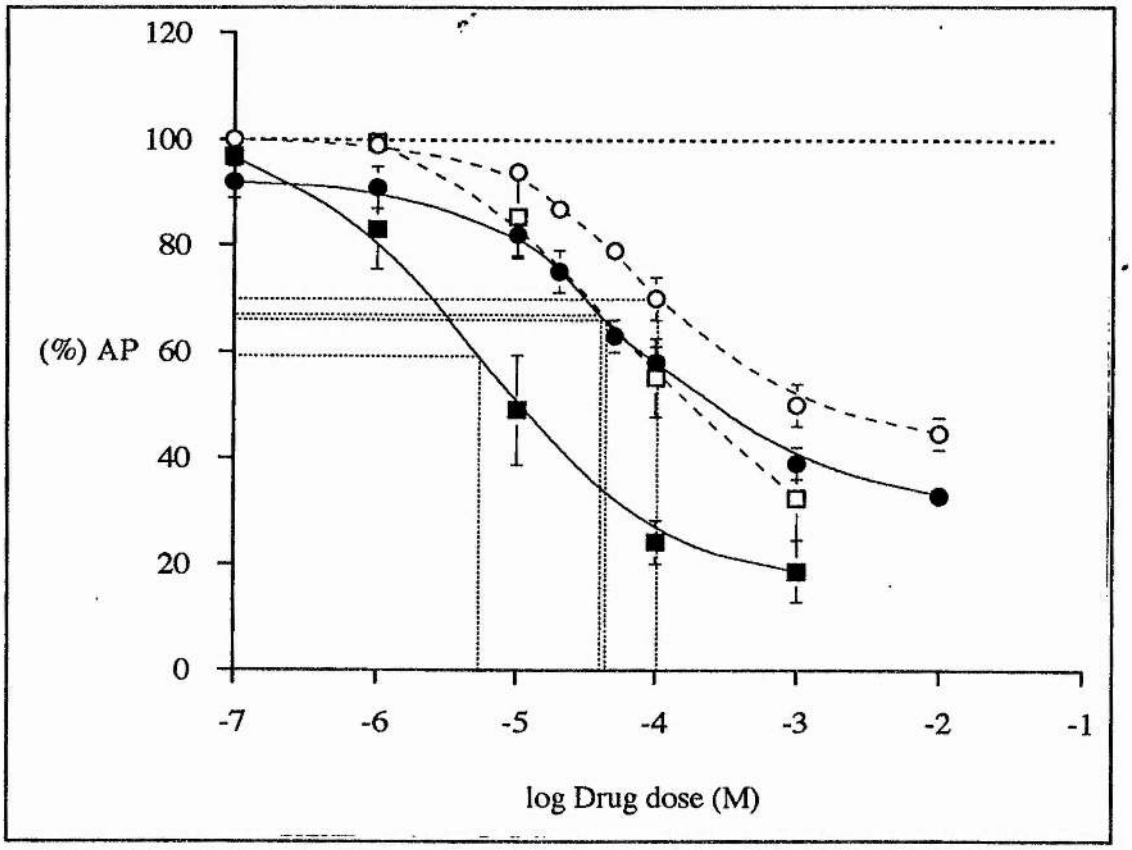


Figure 3.3. *Log dose response curves for SNAP (filled squares; n=5) and NP (filled circles; n=5). Corresponding log dose response curves for SNAP (open squares; n=4) and NP (open circles; n=5) in the presence of 15μM Hb are also shown.*

From the curves, it is clear that SNAP is the more potent vasodilator and that its effects are more severely inhibited by Hb. The inhibition was found to be significant for intermediate doses of both compounds (unpaired t-test; $P< 0.05$ for 10^{-5} - 10^{-4}M SNAP and 10^{-5} - 10^{-3}M NP, see appendix I and II for details). It is evident that high doses of either compound are still capable of eliciting responses which are not significantly different from control responses obtained before introduction of Hb (unpaired t-test; $P>0.05$ for 10^{-3}M SNAP and 10^{-2}M NP - see appendix I and II). ED50 values derived from graph 3.3 are shown in table 3.1.

	SNAP ED_{50}	**NP ED_{50}**
Before Hb	6 μM	52 μM
In Presence of Hb	40 μM	108 μM

Table 3.1. *ED50 values for SNAP and NP derived from graph 3.3.*

These values confirm that SNAP is a more potent vasodilator than NP in the dark and that Hb reduces the potency of SNAP by a factor of ca. 7, whilst that of NP is only reduced by a factor of ca. 2.

B. Responses to RBS and CUB.

Figure 3.4 shows recordings of responses to bolus injections of RBS and CUB in the absence and presence of 15μM Hb.

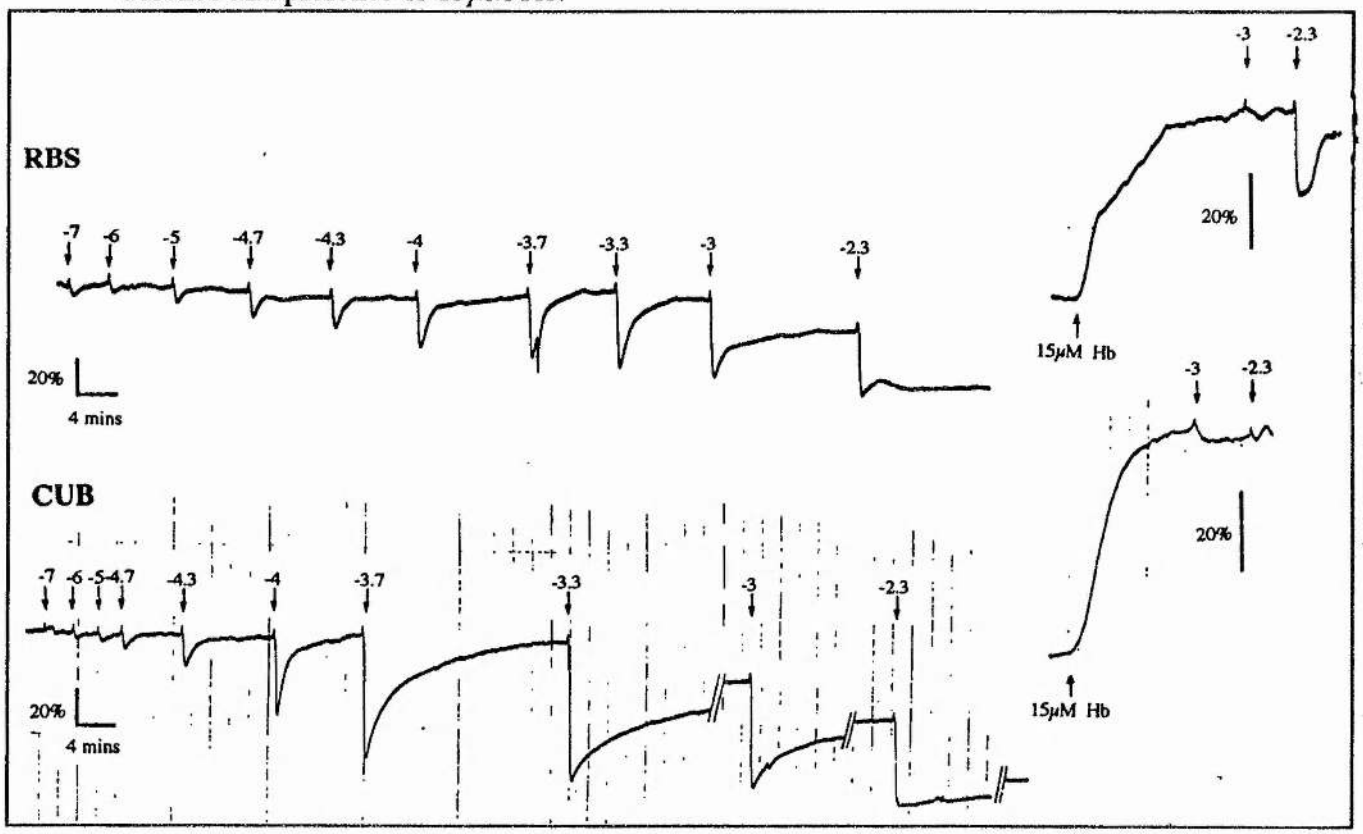


Figure 3.4. *Pressure recordings of experiments showing responses to 10μl injections of increasing doses of RBS or CUB. Also shown are experiments carried out on different preparations, in which 10^{-3}M and 5 x 10^{-3}M doses of both compounds were injected in the presence of 15μM Hb. Concentrations indicated for each injection are log [dose](M). Experimental details:* ***RBS****, Expt N^{o} 910121. 404g, male Wistar rat; 5μM PE produced 97mm Hg active pressure.* ***RBS + Hb****, Expt. N^{o} 910117. 419g male Wistar rat; 8μM PE produced 77mm Hg active pressure. 15μM Hb caused a 90% increase in pressure.* ***CUB****, Expt. N^{o}901210/1. 247g male Wistar rat.; 4μM PE produced 82mm Hg active pressure.* ***CUB + Hb****, Expt. N^{o} 901210/2. 247g male Wistar rat; 4μM PE produced 104mm Hg active pressure. 15μM Hb caused a 109% rise in pressure.*

Responses to RBS and CUB could not be directly compared with those elicited by SNAP and NP since responses to both RBS and CUB displayed an increasing tendency to fail to recover fully with increasing dose (Figure 3.4). In the case of the highest dose of RBS injected, the vessel consistently showed no recovery at all, even if left for up to 6 hrs. It was necessary, therefore, to employ an alternative method of quantifying the results for the purposes of plotting log-dose response curves. Figure 3.5 shows log-dose response curves for RBS and CUB where each point represents the peak vasodilation acheived by each successive dose (X) expressed as a % of the pressure before the first microinjection (Y). Experiments to investigate the effect of Hb were carried out on seperate preparations. Responses to RBS and particularly CUB were severely attenuated in the presence of Hb, with only the highest dose (5mM) in each case, able to cause significant vasodilation. Interestingly, the majority of responses obtained in the presence of Hb were fully reversible.

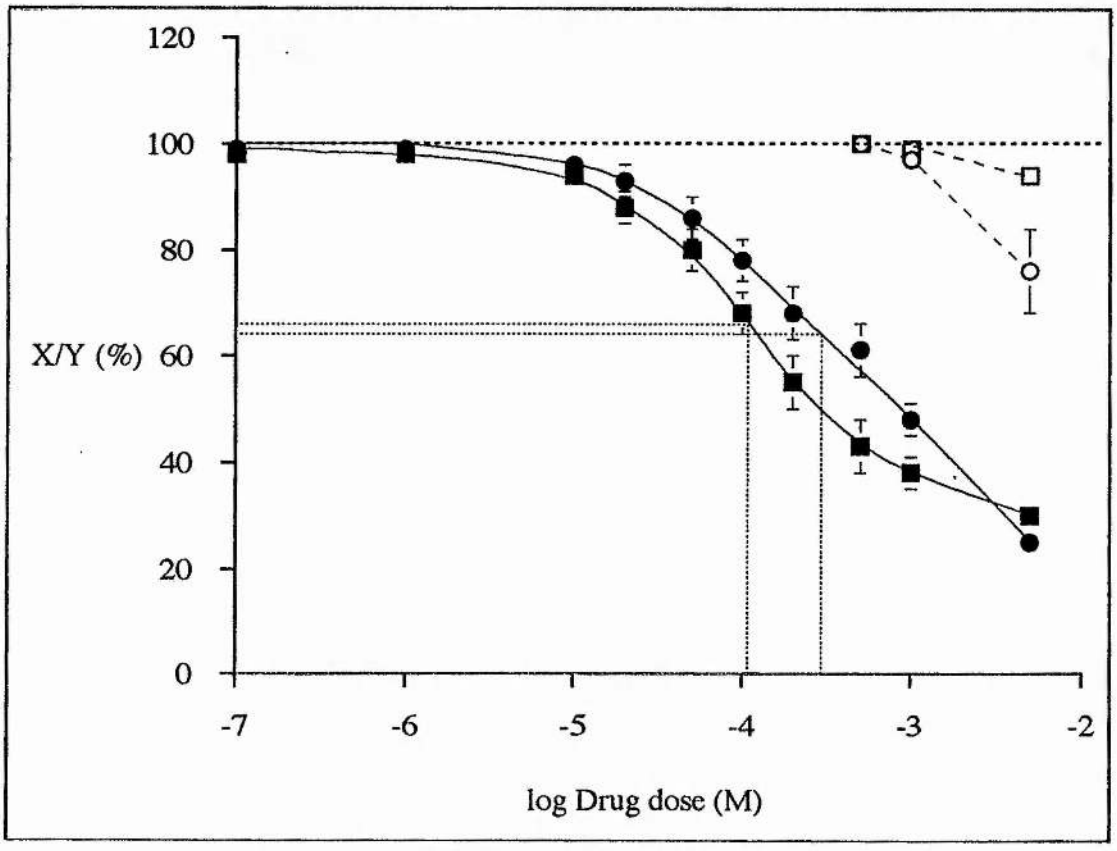


Figure 3.5. *Log-dose response curves for RBS (filled circles; n=5) and CUB (filled squares; n=6). Also shown are responses to 10-3 and 5 x 10-3M RBS (open circles n=6) and CUB (open squares; n=6) in the presence of 15μM Hb.*

From the log-dose response curves shown above, 'ED50' values were obtained for RBS and CUB (table 3.2). It was not possible to estimate comparable values for Hb-inhibited responses because of the extent of blockade.

RBS ED_{50}	CUB ED_{50}
300μM	112μM

Table 3.2. *ED50 values derived from the log-dose response curves shown in figure 3.5.*

These results are surprising in that the compound with the highest NO-capacity (RBS, 7 nitrosyl groups) is the least potent vasodilator in terms of initial amplitude of response, implying that RBS is the most stable of the compounds studied ***under the conditions of these experiments***.

3.4 DISCUSSION

A. SNAP and NP

The results show that bolus injections of either compound into the internal perfusate elicit transient responses which recover rapidly. Hb significantly inhibits, but does not abolish, the responses to either drug, with SNAP being more sensitive than NP. This finding suggests that NO is responsible for a proportion of vasodilation caused by both compounds. However, to explain the results fully, either NO-independent mechanisms are involved in SNAP and NP-induced vasodilation, or a proportion of NO released from these compounds is protected from Hb attack.

In the case of NP, considerable doubt has already been shed on the assertion that NO is solely responsible for its vasodilator properties. Otsuka *et al* (1988) found that guanylate cyclase inhibitors were not always capable of attenuating responses to NP and that maximal NP-induced relaxations were accompanied by a relatively small increase in cGMP. Similar results were obtained by Diamond & Chu (1983). These findings, coupled with irregularities with regards to the kinetics of NP stimulation of guanylate cyclase (Feelisch, 1991), suggest that NP acts through a number mechanisms, one or more of which is cGMP-independent.

In addition, Flitney & Kennovin (1988) showed that NP does not release NO spontaneously in the dark. Mounting evidence indicating the existence of a membrane-bound enzyme which 'strips' NO off NP (Leeuwenkamp *et al*, 1986 ; Kowaluk *et al*, 1992) suggests that NP-derived NO may never experience the intra-lumenal environment, thus avoiding Hb inactivation at this level.

The involvement of NO in NP-induced relaxation in the dark is further questioned by results obtained in this laboratory (Flitney *et al*, 1990; figure 3.1). The results show that responses to bolus injections of NP are not significantly inhibited by Hb or MB, lending further weight

to the argument that some of its vasodilator properties are mediated by a cGMP-independent mechanism.

SNAP is thermally unstable (Butler & Askew, unpublished data) and is likely to release some NO spontaneously in the lumen where it would be susceptible to Hb inactivation. Recent experiments (Butler *et al*, 1993) have shown that SNAP undergoes a process known as 'cross nitrosation' in the presence of -SH-containing compounds, such as cysteine. It is possible, therefore, that NO could be 'stripped' from SNAP in a similar way to NP. NO derived in this manner may not be susceptible to Hb inhibition. Potentially, therefore, SNAP can release NO by two mechanisms, only one of which is susceptible to Hb inhibition.

B. RBS and CUB

Both RBS and CUB, as high-capacity NO-containing compounds, have great potential as NO donor drugs. In terms of peak amplitude of response, this is clearly not the case, with SNAP and NP proving to be substantially more potent vasodilators. However, responses to bolus injections of RBS and CUB showed a tendency to fail to fully recover with increasing doses. The loss of pressure does not appear to be due to cytotoxic effects of the drugs themselves, since no sustained loss of pressure occured when even the highest dose of either compound was injected in the presence of Hb. Cytotoxic effects of NO cannot be ruled out, although this too seems unlikely, since similar effects were not seen with SNAP. Instead, it appears that the effect is due to genuine, Hb-inhibited vasodilation. Bolus injections of moderate to high doses of either iron-sulphur cluster nitrosyl are capable, therefore, of producing NO-mediated vasodilator responses which persist long after the bolus passes through the vessel.

A more detailed investigation was undertaken to determine the source of NO responsible for protracted vasodilator responses produced by bolus injections of RBS and CUB, the results of which are reported in chapters 4 and 5.

CHAPTER 4

IRON-SULPHUR CLUSTER NITROSYLS, A NOVEL CLASS OF NO GENERATOR.

4.1 INTRODUCTION

The results from experiments described in the previous chapter establish that both iron-sulphur cluster nitrosyls (RBS and CUB) cause vasodilation of the isolated rat tail artery. The vasodilation was shown to be NO-mediated on account of its susceptibility to Hb inhibition. The NO involved had previously been shown not to be endothelium-derived since responses were unaffected after inhibition of NOS using LNMMA. (figure 3.1; Flitney *et al.*, 1990).

However, as intimated in the previous chapter, both compounds displayed characteristics which were qualitatively different from NP and SNAP in that the vasodilation caused by a single bolus injection, in contact with the artery for less than 0.5 secs., showed a tendency to only partially recover to the pre-injection pressure. This tendency became more pronounced at higher concentrations and, in the case of RBS, there was no recovery following microinjection of the highest dose used ($5x10^{-3}M$).

Here, these characteristics are studied in more detail. Experiments were carried out to establish that the smooth muscle cells were still capable of contracting once vessels had been injected with high doses of either compound and that the responses obtained were not due to a cytotoxic effect of the compounds. Hb and MB were used to show that the responses were due to long-term stimulation of guanylate cyclase by NO. A computer model was used to determine the underlying components responsible for the unique response profiles.

4.2 MATERIALS AND METHODS

A. ANIMALS

Adult male Wistar rats (250-460 g) were killed by cervical dislocation, their tails removed and a length of artery (8-15 mm) cannulated and dissected free as described earlier.

B. APPARATUS

The apparatus shown in figure 2.1 was used for all experiments.

C. EXPERIMENTAL PROTOCOL

Vessels were precontracted with PE to produce agonist-induced perfusion pressures of 101 +/- 3.5 mmHg ([PE] = 6.5 +/- 0.5 μM; n=21). Responses to 10 μl bolus injections of RBS and CUB were compared to those evoked by NP and SNAP. The effects of adding 15 μM Hb or >10 μM MB to the internal perfusate were examined.

D. ANALYSIS OF RESULTS

For the purposes of these experiments it was necessary to record the pressure attained at the peak of a response (figure 4.2; X) and at intervals during recovery to establish a response profile. The final pressure attained once the vessel had ceased to recover (figure 4.2; Z) was also recorded. Modified log-dose response curves for all the compounds (figure 4.3) show the extent of both relaxation (X/Y % where Y is the agonist-induced pressure before the first injection), and recovery (Z/Y %) for all the drugs tested.

4.3 RESULTS

Microinjections of NP and SNAP produce transient responses.

Pressure recordings made following injections of increasing doses of these compounds confirm that they produce transient responses only (figure 4.1).

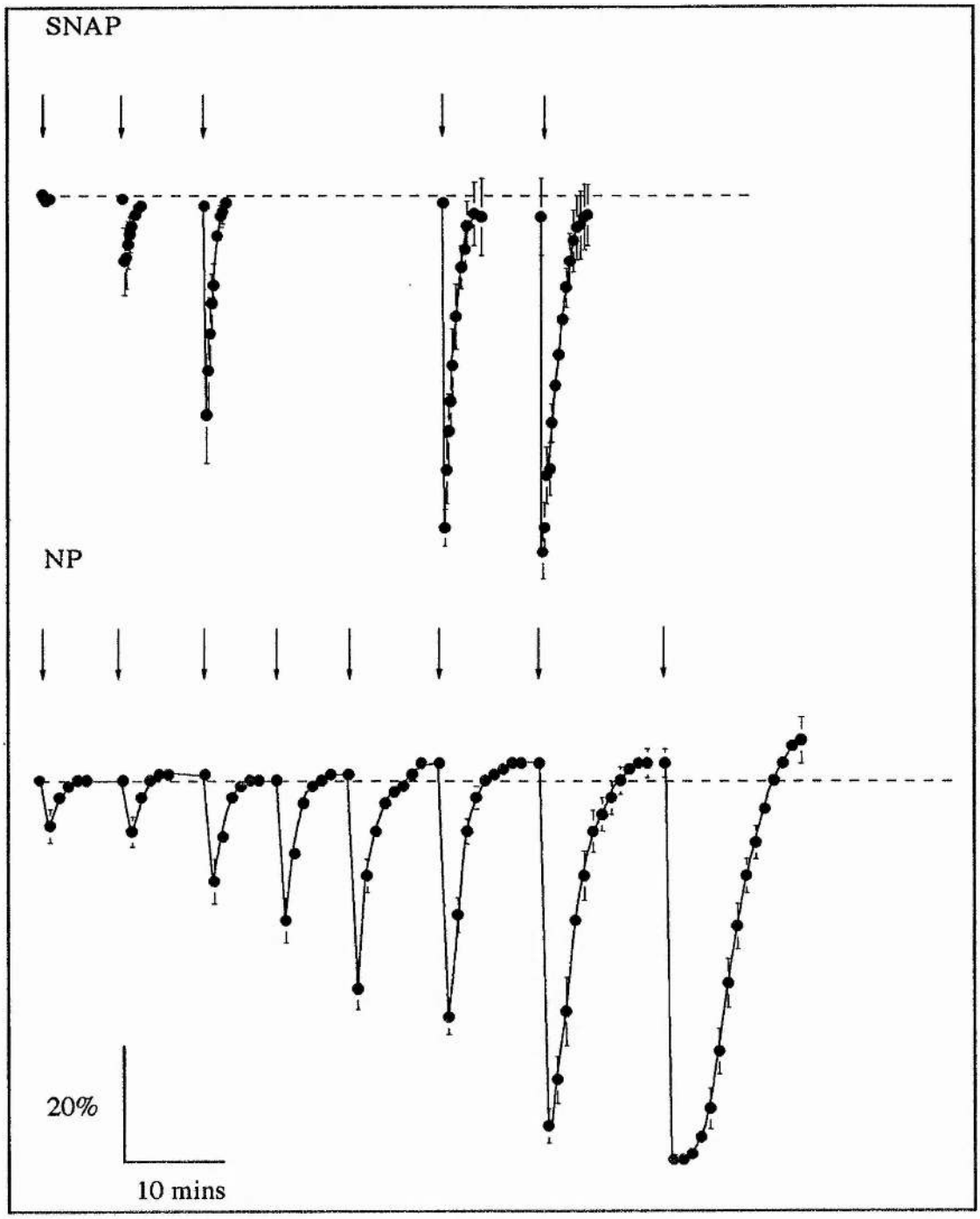


Figure 4.1. *Meaned pressure recordings (+/- s.e., vertical bars) obtained by successsive injections of SNAP(n = 5) and NP (n =5). 10 µl injections (10^{-7}M to 10^{-3}M for SNAP and 10^{-7}M to 10^{-2}M for NP) were administered as indicated (vertical arrows).*

There is, however, an interesting difference between the response profile obtained for each compound: responses to SNAP recover significantly faster (ca. 3 mins for the highest dose) than those to NP (ca. 10 mins for the highest dose). This may be indicative of the different mechanisms involved in the transport of NO derived from the two compounds from the lumen to guanylate cyclase in the smooth muscle.

Microinjections of RBS and CUB produce sustained responses

Pressure recordings obtained for RBS and CUB (figure 4.2) show that both compounds produce transient (T-type) responses when injected in low concentrations.

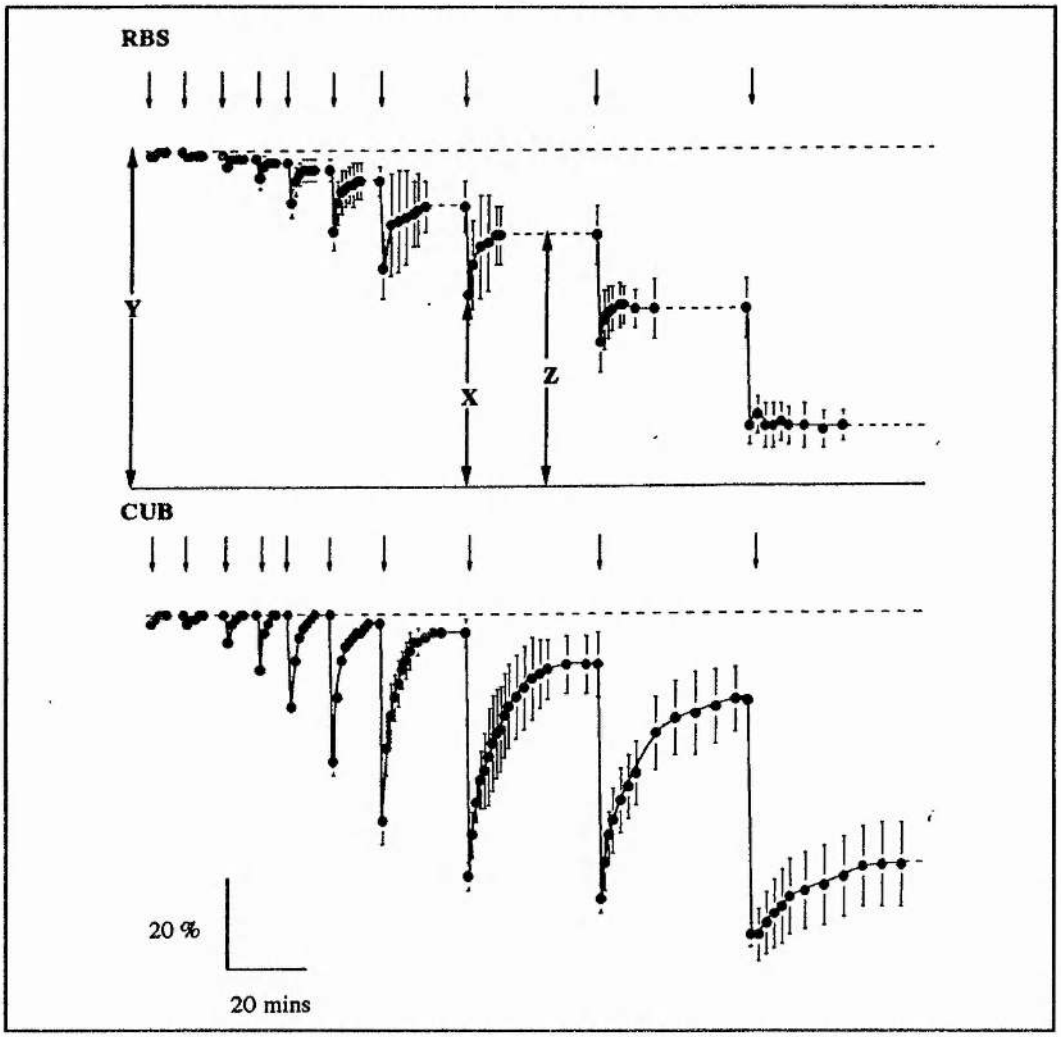


Figure 4.2. *Meaned pressure (+/- s.e., vertical bars) recordings obtained following successsive injections of RBS (n=5) and CUB (n=6). Parameters X, Y and Z are defined for RBS responses. Note that injection doses above the Dt for both compounds (see text) produce progressively more pronounced sustained responses. This is in contrast to the responses shown in figure 4.1 which are all transient.*

However, when the injected dose exceeds a critical threshold concentration (*Dt*; 1-10 μM for RBS and 20-100 μM for CUB), both the rate and extent of recovery become progressively reduced, resulting in a stepwise and persistent loss of tone.

Figure 4.3 shows perfusion pressure minima (X; filled circles) immediately following injection of each drug and the steady-state pressures attained after recovery (Z; open circles) both expressed as a % of the initial agonist-induced pressure (Y) and plotted as a function of $\log_{10}$ injected dose for each drug used. The ED_{50} values for each drug were listed previously in tables 3.1 and 3.2.

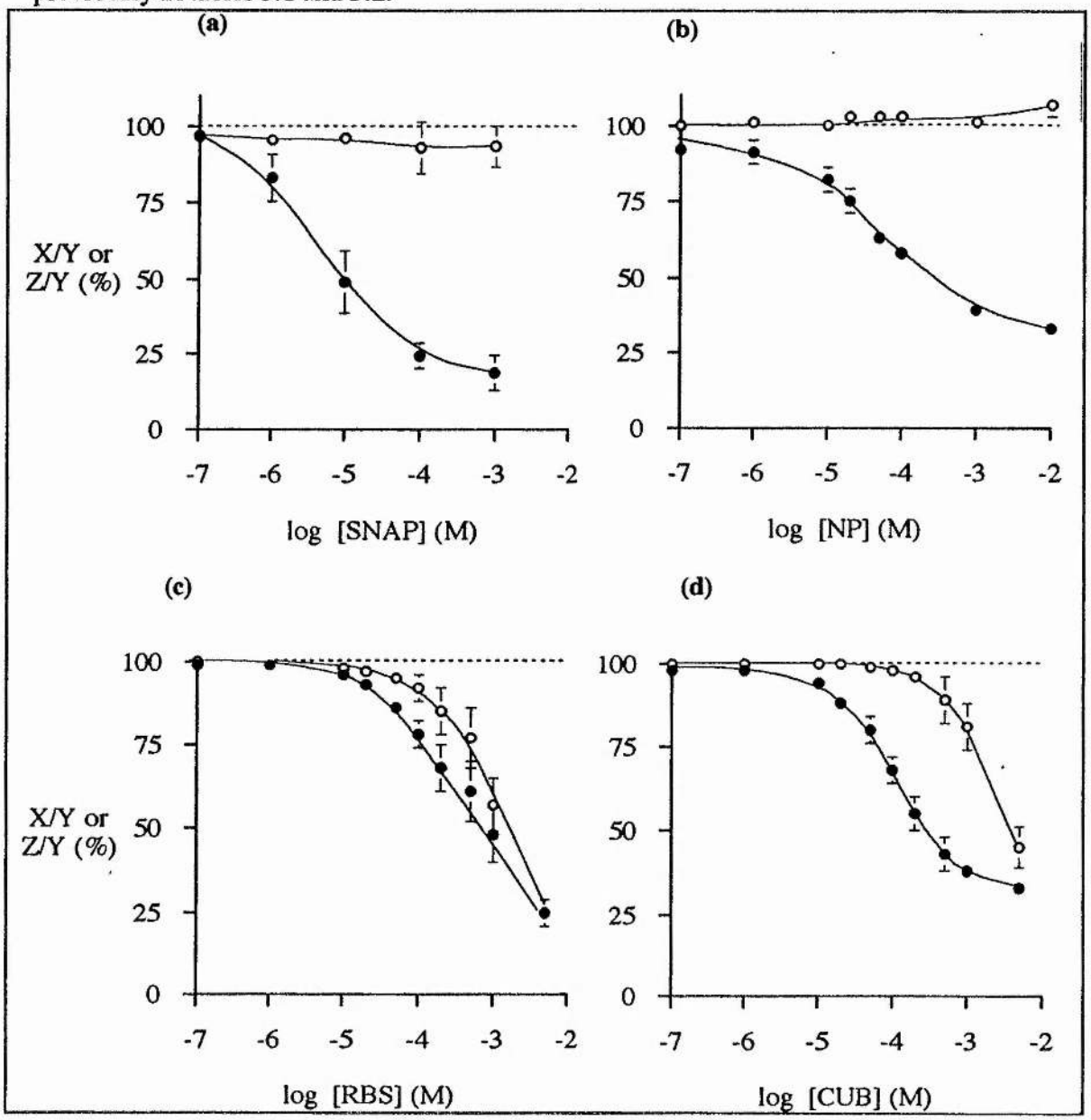


Figure 4.3. *X/Y (filled circles) and Z/Y (open circles) plotted as a function of $\log_{10}$ injected dose of SNAP (a), NP (b), RBS (c) and CUB (d).*

The Z/Y curves reflect the tendency of iron-sulphur cluster nitrosyls to generate sustained (S-type) responses. The effect is more pronounced for RBS than for CUB as shown by the closer proximity of the X/Y and Z/Y curves.

Abolition of the 'plateau' phase of S-type responses by Hb or by MB

The S-type responses obtained following 5 mM microinjections of either RBS or CUB were remarkable in that prolonged (5 hr.) perfusion (washout) with PE-Krebs solution failed to induce any further vasoconstriction, once the plateau phase was established. However, the addition of either 15 μM Hb or >10μM MB to the internal perfusate initiated a prompt and complete restoration of all agonist-induced tone; indeed as figure 4.4 clearly shows, the perfusion pressure rises well above the pre-injection level (Y). Subsequent removal of Hb from the perfusate causes arteries to redilate fully. This is not the case for MB since inhibition of guanylate cyclase induced by MB is irreversible.

These observations suggest that extremely brief exposures of arteries to RBS and CUB is sufficient to establish a durable source of NO within the tissue.

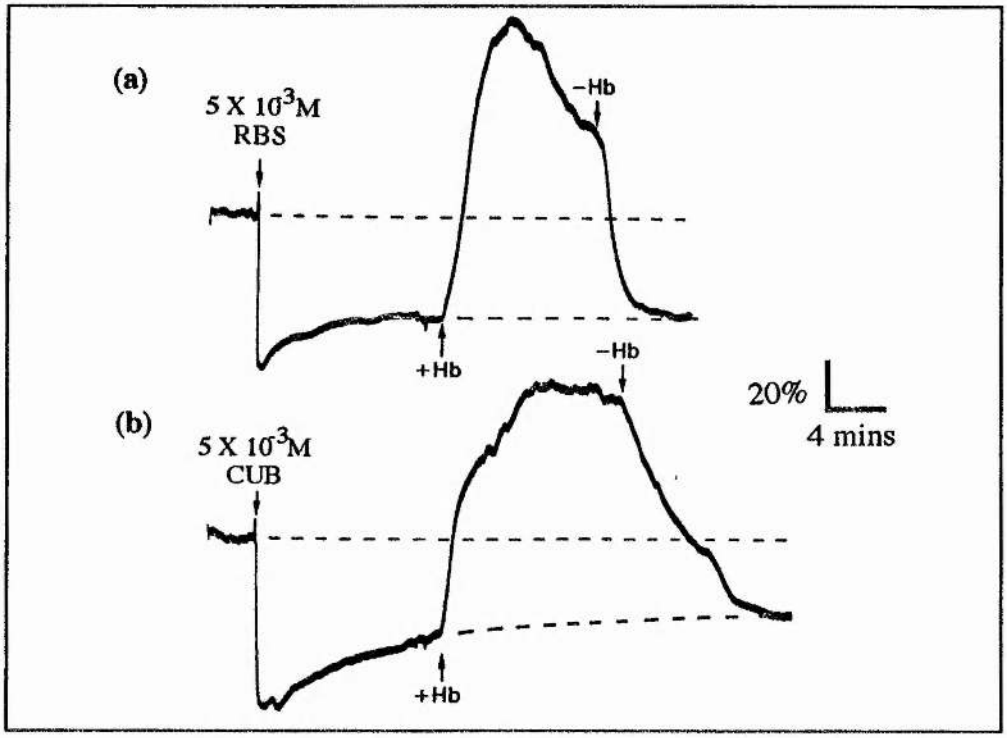


Figure 4.4. *Pressure recordings from two preparations showing responses to 15μM Hb added to, and then removed from, the internal perfusate during the plateau phase of sustained responses to RBS (a) and CUB (b).*

4.4 DISCUSSION

These results establish iron-sulphur cluster nitrosyls as a novel class of NO donor drug with unusual vasodilator properties. The compounds investigated here are able to generate two distinct kinds of response, transient and sustained. In this respect they differ from SNAP and NP which give transient responses only. The ability of Hb and MB but not NOS inhibitors to block both transient and sustained responses shows that they are mediated by NO derived from each cluster and not by enhanced EDRF production through stimulation of the endothelial L-arginine:NO - pathway.

Composite nature of S-type responses

The results shown here suggest that S-type responses represent the sum of two distinct vasodilator components: a reversible component (V_1), attributable to NO generated by the spontaneous decomposition of the clusters in solution and present at the time of injection, superimposed on a 'non-recoverable' component (V_2), perhaps caused by the delayed release of NO from clusters which have penetrated and remained in the tissue. Component V_2 presumably reverses eventually, when the 'store' of cluster becomes depleted, but there is no indication of this happening up to 5 hrs after injection.

An estimate of the relative contributions from V_1 and V_2 can be obtained from the data presented in figure 4.3, as follows. The amplitude of V_2 for the nth injection is given by:

$$V_2n = (Z/Y_{n-1} - Z/Y_n) \qquad (1)$$

and V_1n when there is no V_2 component by:

$$V_1n = (Z/Y_{n-1} - X/Y_n) \qquad (2)$$

Eqn.1 gives V_2 unambiguously, provided sufficient time is allowed for the plateau phase to become fully established. Eqn. 2 gives the correct value for V1 for injection doses <Dt, but

overestimates it when the dose >Dt. For simplicity, an exponential recovery is assumed for V_1 ($P = P_0e^{k_1t}$) and also for the time course of the developing component V_2 ($P = 1-P_0e^{-k_2t}$), where P is the perfusion pressure, k is the rate constant (min^{-1}) and t is time (min). Simulated pressure recordings were obtained by summing V_1 and V_2 using an iterative computer programme, varying the amplitude of V_1 (but not V_2) and the rate constants k_1 and K_2. No attempt was made to reproduce the time courses exactly (cf fig 4.2), though this could be done, by suitably adjusting k_1 and k_2. However, the iteration was allowed to run until the calculated values for both X/Y and Z/Y were identical to the mean values obtained in our experiments.

Computer simulations of sustained responses

Figure 4.5 shows computer simulations obtained with k_1 and k_2 set at $0.24.min^{-1}$ and $0.4.min^{-1}$ (CUB) and $0.24.min^{-1}$ and $0.18.min^{-1}$ (RBS).

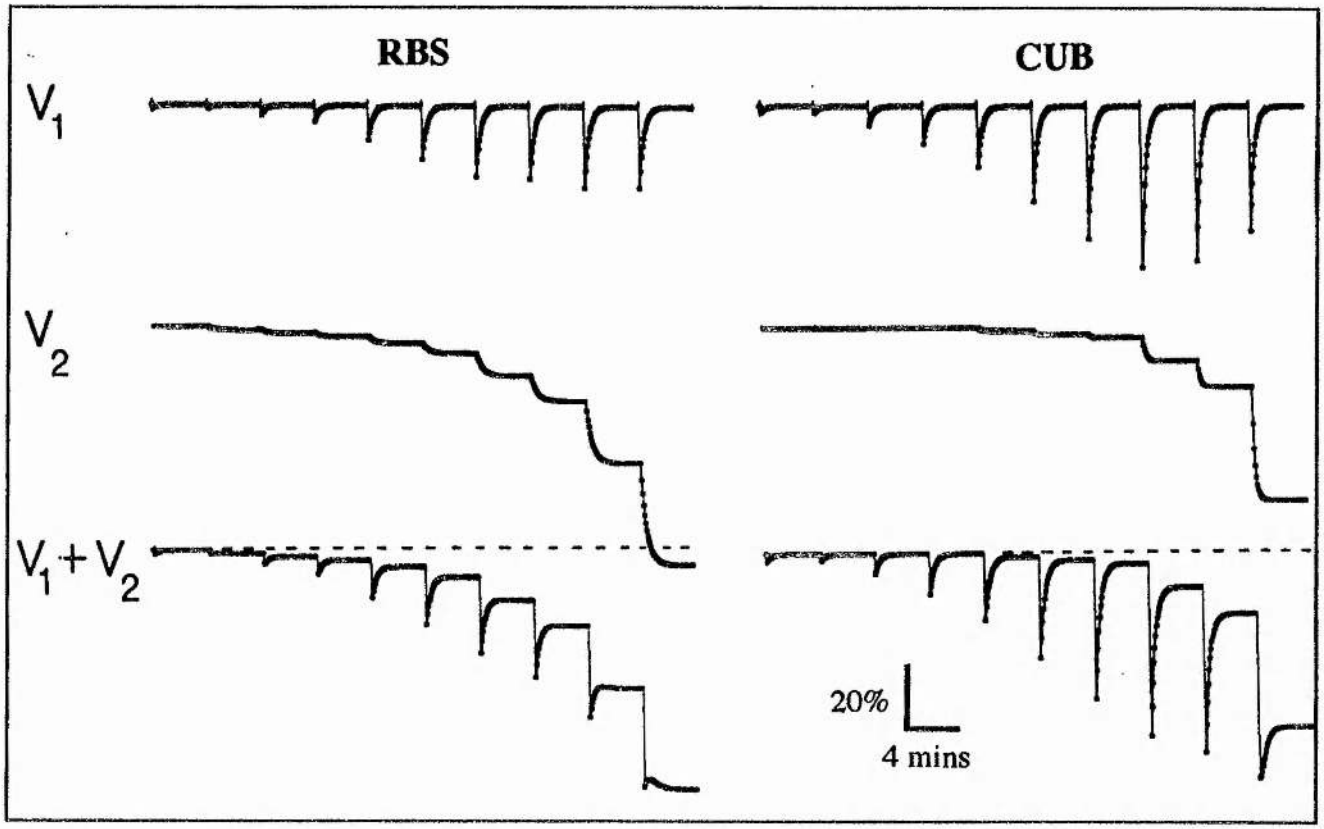


Figure 4.5. *Computer simulations of sustained responses. The simulation allows V1 and V2 to be segregated in an effort to examine the two response profiles responsible for sustained responses. Values for K_1 and K_2 were adjusted until response amplitudes matched those shown in figure 4.2.*

The amplitudes of V_1 and V_2 used in the reconstructions are plotted as a function of the injection dose in figure 4.6. The ED_{50} values for component V_1 for CUB and RBS are estimated to be 78 μM and 62 μM respectively. The maximum amplitudes for V_1 are 0.52 and 0.27 respectively.

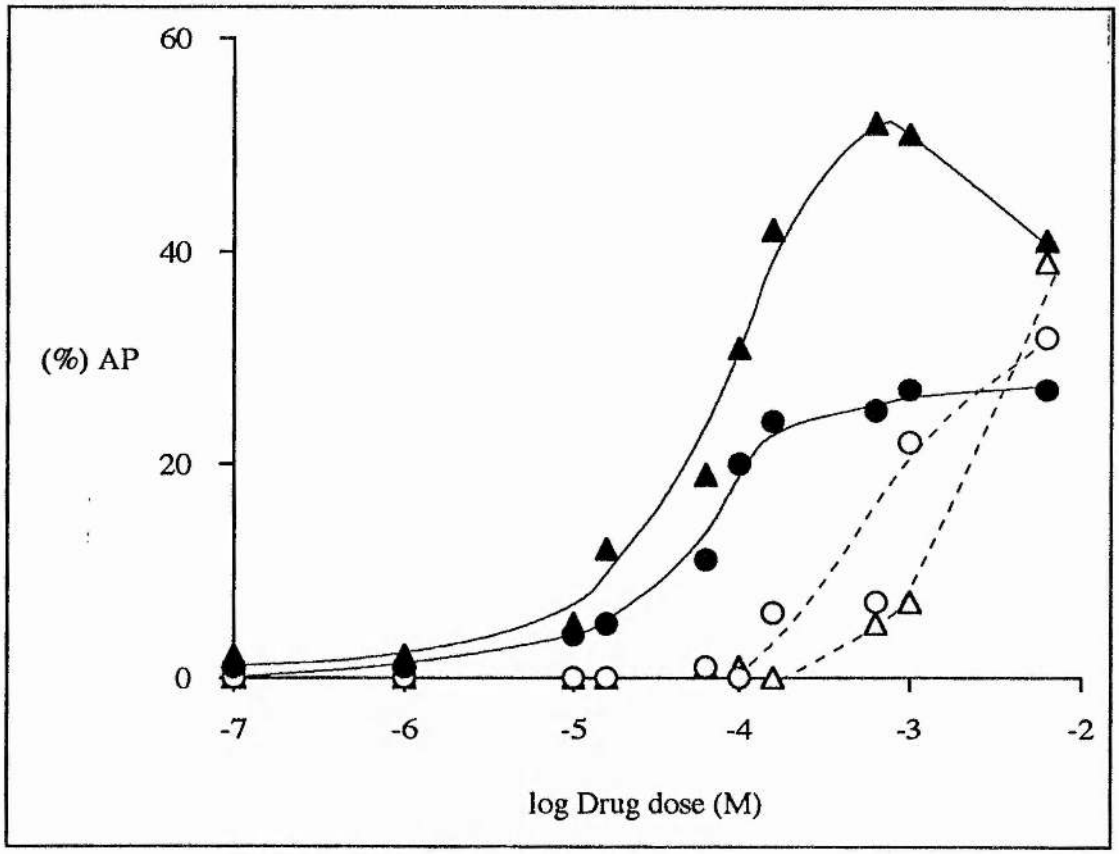


Figure 4.6. *V1 (filled symbols) and V2 (open symbols) values plotted as a function of $\log_{10}$ injection dose of RBS (circles) and CUB (triangles).*

These results suggest that CUB is less stable in solution than RBS. The V_1 log dose response curve for RBS can be made to coincide with that for CUB by scaling all CUB values by a factor of 1.9x. A statistical comparison of the scaled V_1 values for RBS with those for CUB (paired students' t-test) shows no significant difference between the two data sets at the 95% confidence level (t=0.165 ; 9 degrees of freedom). Since RBS contains 7/4 the number of nitrosyl groups as CUB, it can be estimated that approximately 3.5 molecules of CUB degrade in physiological solution for every molecule of RBS.

In contrast, the V_2 curves in figure 4.6 show this component to be more pronounced in the case of RBS than that of CUB, reflected in the Dt values for the two compounds.

A possible explanation for the V_2 component of the responses observed is that bolus injections in which the concentration exceeds Dt for that compound lead to the uptake of small quantities of the drug into the tissue. Once there, NO is released at a rate determined by the intracellular environment. The amplitude of V_2 would therefore dependent on:

1. The amount of drug taken up by the tissue.
2. The rate of NO release from inside the cell.

With this in mind, a study of the histochemistry of vessels perfused with RBS or CUB. was carried out. The results, detailed in chapter 5, suggest that the hypothesis was correct, a fact confirmed by microanalysis on frozen sections of the tissue.

Vanin (1991) has shown that iron-dinitrosyls $(FeNO)_2$ complexed with low molecular weight thiols (e.g. cysteine or glutathione) can vasodilate isolated blood vessels and also inhibit platelet aggregation. Interestingly, the vasodilator action exhibits two-phase kinetics when tested on intact animals, resembling the sustained responses described here: that is, an initial, rapid decrease of arterial pressure, followed by a a persistent (several hours) hypotensive action. The rapid and sustained components are attributed to to NO released from (i) iron-dinitrosyl low - Mr thiol complexes (rapid phase) and (ii) from iron-dinitrosyl protein-thiol complexes (sustained phase) formed within the tissue. The latter are thought to result from the transfer of $Fe(NO)_2$ groups from low Mr-compounds to protein-borne ligands, forming a long-lasting molecular 'store' of NO in the vascular bed.

Suppression of endogenous EDRF production did not occur during the plateau phase of S-type responses.

The effects of Hb administered during established sustained responses suggest that the formation of endogenous NO from L-arginine is not suppressed by either cluster and that its continued release from endothelial cells helps to sustain the plateau phase. Thus, perfusing vessels with Hb does not merely re-establish the pre-injection perfusion pressure, but

instead drives it to a level substantially greater than this (figure 4.4). The inference to be drawn is that Hb scavenges NO from both exogenous (cluster-derived) and endothelial sources.

Physicochemical properties of iron sulphur cluster nitrosyls

The physicochemical properties of iron-sulphur cluster nitrosyls (Butler, Glidewell & Min-Hsin, 1988) provide some insight into their vasodilator actions. Both compounds are potentially able to deliver large quantities of NO (figure 1.7). Extended Huckel orbital calculations show that both compounds are electron precise (Sung, Glidewell, Butler and Hoffman, 1985), that is to say that addition or removal of an electron is sufficient to cause the iron-sulphur framework to disintegrate, releasing all the ligated NO groups as free NO (7 per molecule of RBS and 4 per molecule of CUB). The redox status of the immediate environment is therefore likely to be a crucial factor in determining the rate of release of NO from these compounds.

CHAPTER 5

HISTOCHEMICAL AND X-RAY MICROPROBE EVIDENCE FOR ACCUMULATION OF IRON-SULPHUR CLUSTER NITROSYLS IN THE ENDOTHELIUM

5.1 INTRODUCTION

Experiments reported in the previous chapter suggest that the unusual vasodilator responses produced by bolus injections of either RBS or CUB are due to a combination of two distinct responses:

1. Transient responses, due to the spontaneous release of NO in solution.
2. Sustained responses, possibly due to slow release of NO from clusters trapped in the tissue.

Experiments were therefore undertaken to investigate whether RBS and CUB are able to accumulate in vessels. To this end, arteries were perfused with both compounds and later examined using histochemical techniques. In addition, vessels perfused with RBS were subjected to X-ray microprobe analysis in an attempt to pinpoint the locality of clusters trapped within the tissue.

5.2 MATERIALS AND METHODS

A. PROTOCOL FOR HISTOCHEMICAL STUDIES

Lengths of artery were cannulated and perfused with Krebs solution as described previously (sections 2.1, 2.2 and 2.3). Following precontraction, vessels were internally perfused with 10^{-4}M RBS or CUB for 5 mins followed by a 15 min washout with PE-Krebs. In both cases, pressure dropped to <25% of its original value and showed no signs of recovery during the washout period. Vessels were then removed from the perfusion system, frozen and transversely sectioned (20 μm). Fixation of the tissue was not possible since the process (even vapour fixation) invariably led to destruction of the iron-sulphur cluster nitrosyls. Instead, sections were viewed under the microscope unstained, and following staining for 7hrs with bathophenanthroline followed by 5 min counterstaining with MB. Bathophenanthroline is a recognised stain for iron (II) (Pearse, 1972). Control (untreated) vessels did not stain with bathophenanthroline.

B. PROTOCOL FOR MICROANALYSIS

Vessels were treated with 10^{-4}M RBS for 10 mins followed by a 10 min washout. Once removed from the perfusion system, a small length (<1mm) was removed and placed on a lead weight which was plunged into liquid N_2. Rapid freezing produced in this way reduces the chance of ice-crystal development which can cause distortions within cells. Samples of frozen tissue were taken to Glasgow University where Dr H. Elder and J. Pediani analysed sections of the tissue using an X-ray microprobe to determine relative amounts of iron and sulphur present in endothelial cells and in the adjacent elastic lamina.

5.3 RESULTS

Figure 5.1 shows transverse sections of a freshly-frozen vessel following perfusion with RBS. The unstained section (A) shows a brown discolouration, predominantly in the cells surrounding the lumen. The discolouration appears to extend into deeper layers of the tissue but this may be a result of diffusion after mounting since the sections were unfixed.
Section B shows the effect of bathophenanthroline staining. The red/brown stain is clearly confined to the same area of tissue as the brown discolouration in (A).
High magnification of a section counterstained with MB only (C) clearly shows that the discolouration is confined to the endothelial layer.

Figure 5.2 shows the chemical composition detected by the X-ray microprobe, in outer smooth muscle cells (a), cells in the inner elastic lamina (adjacent to the endothelium; (b))and in endothelial cells (c), in an RBS-treated vessel. The results clearly show that in RBS-treated vessels, both the Fe and the S levels are significantly higher in endothelial cells than in cells located deeper in the tissue, where both exist only as trace elements. The detected Fe/S ratio approximates that expected for RBS (4/3).

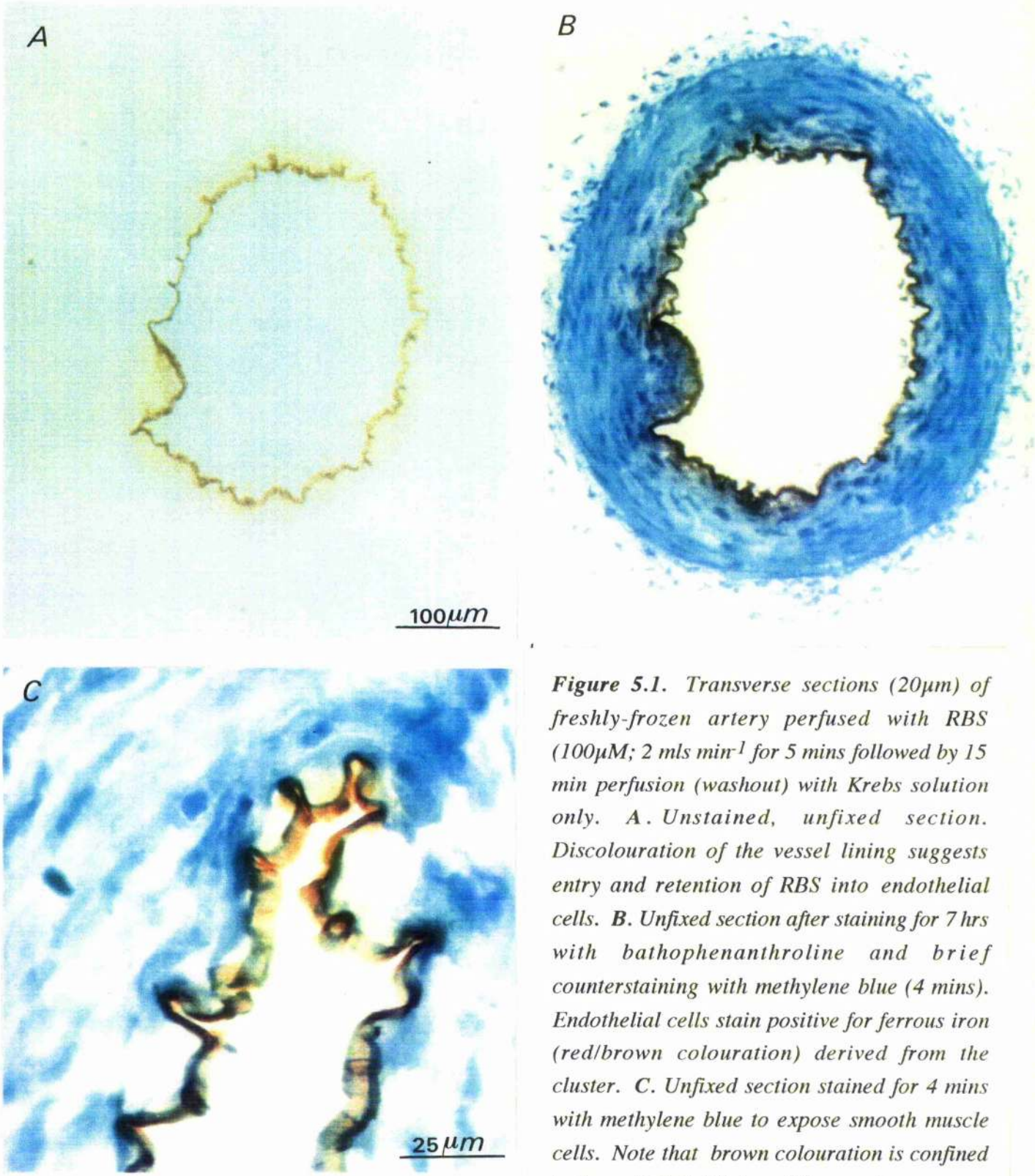


Figure 5.1. *Transverse sections (20μm) of freshly-frozen artery perfused with RBS (100μM; 2 mls min^{-1} for 5 mins followed by 15 min perfusion (washout) with Krebs solution only.* ***A.*** *Unstained, unfixed section. Discolouration of the vessel lining suggests entry and retention of RBS into endothelial cells.* ***B.*** *Unfixed section after staining for 7 hrs with bathophenanthroline and brief counterstaining with methylene blue (4 mins). Endothelial cells stain positive for ferrous iron (red/brown colouration) derived from the cluster.* ***C.*** *Unfixed section stained for 4 mins with methylene blue to expose smooth muscle cells. Note that brown colouration is confined to the endothelial lining of the vessel.*

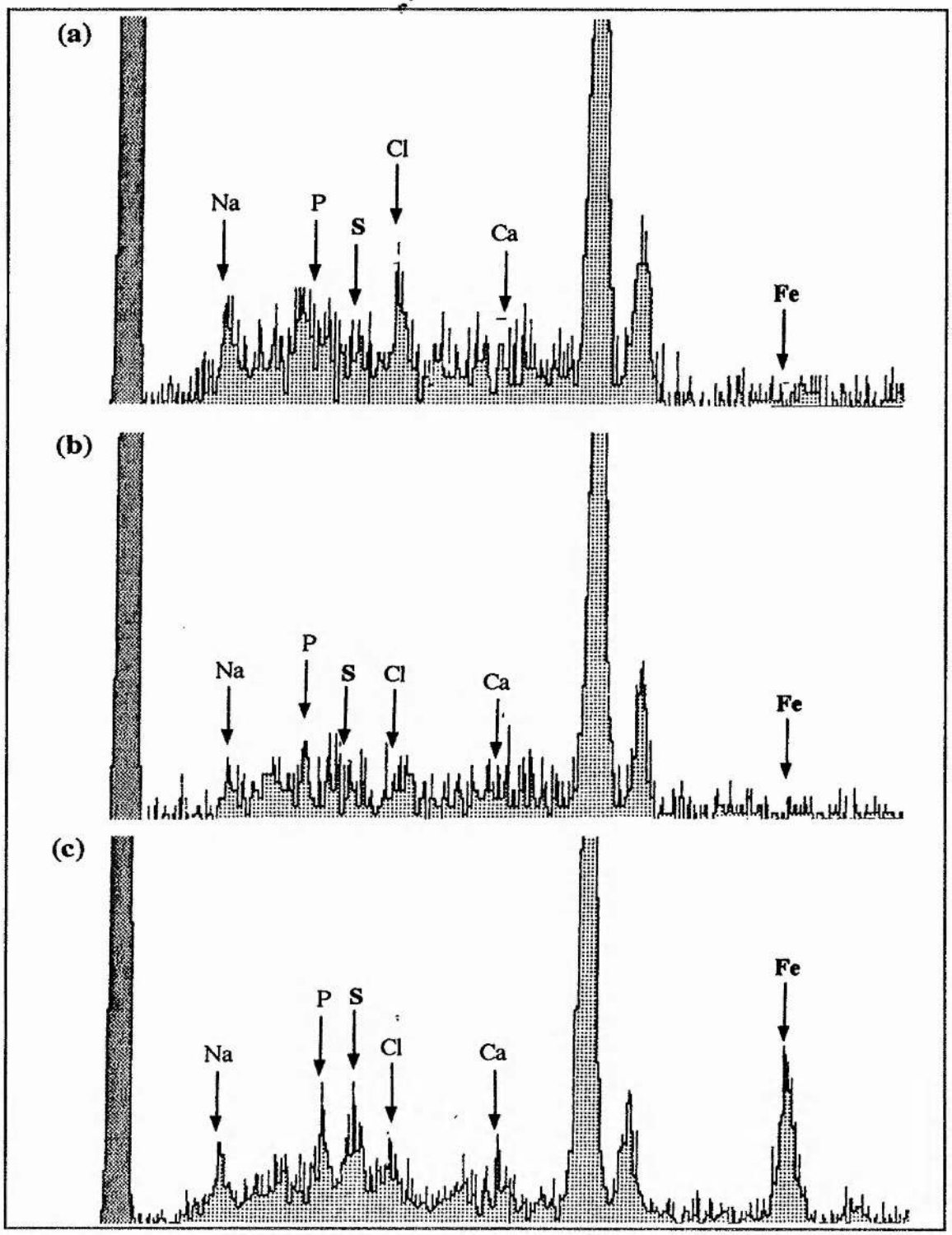


Figure 5.2. *Relative quantities of ions detected by X-ray microprobe analysis in (a) smooth muscle cells in the outer region of the vessel wall, (b) cells immediately adjacent to the endothelium and (c) endothelial cells in vessels treated with 10^{-4}M RBS for 10mins followed by a 10min washout period. Note that Fe and S levels are significantly higher in (c) than in (a) or (b). Presumably, this is due to the uptake of RBS into the endothelium. Experimental details: Expt 910301. 100 KeV, 30^0 tilt, x 6,600 magnification, 4 x 10^{-9}A beam current.*

5.4 DISCUSSION

The results shown above strongly support the hypothesis proposed in the previous chapter whereby sustained vasodilator responses to iron-sulphur cluster nitrosyls which persist long after the drug has cleared the lumen, are due to the entry of a small quantity of drug into the tissue. Once there, the compounds decompose slowly, releasing NO and maintaining a 'vasodilator tone'. Moreover, the results of the histochemical study and especially the results obtained from the X-ray microprobe, suggest that the clusters accumulate exclusively in the endothelium.

Importantly, the accumulation of either compound does not appear to affect the ability of the endothelium to synthesise NO, a point clearly illustrated by the effects of Hb on vessels treated with 5 mM RBS or CUB (figure 4.5). Not only did Hb abolish drug-induced sustained responses, but also caused an additional vasoconstriction, attributable to inhibition of an on-going, EDRF-induced vasodilation.

The apparent ease with which both clusters are able to penetrate the endothelial cell membrane is probably related to their high solubility in non-polar solvents (Butler, Glidewell & Min Hsin, 1988). RBS is especially interesting in that it is anionic (CUB is neutral) and therefore soluble in polar solvents too.

It should also be noted that preliminary experiments using sephadex columns have shown that RBS binds non-specifically to both albumin and Hb, a result which may have some bearing on the effectiveness of Hb in inhibiting RBS responses. Intracellular proteins, therefore, may also be capable of binding these compounds and may contribute to their accumulation within endothelial cells, as well as intracellular lipids.

CHAPTER 6

LASER-INDUCED POTENTIATION OF VASODILATOR RESPONSES TO IRON-SULPHUR CLUSTER NITROSYLS.

6.1 INTRODUCTION

The findings reported in the previous three chapters, show that the iron-sulphur cluster nitrosyls, are potent vasodilators with unusual pharmacological properties. Bolus injections of either compound into pre-contracted lengths of rat tail artery were found to generate two kinds of response. Doses below a critical threshold (<Dt) evoked fully reversible (T-type) responses, as seen with conventional NO donor drugs. However, doses >Dt produced extraordinarily long-lasting (S-type) responses, comprising an initial, rapid drop in pressure, which then either failed to recover or showed partial recovery only. S-type responses were characterised by a plateau of reduced tone which could persist for several hours. Both T- and S-type responses were shown to be mediated by NO. Histochemical studies showed that the plateau component of the S-type response is caused by the protracted release of NO from clusters which become trapped within the endothelium.

The experiments described in this chapter will show that both RBS and CUB are also photosensitive. Irradiation either of vessels during the plateau phase of sustained responses, or of the internal perfusate containing low concentrations of RBS or CUB, *en route* to the preparation, potentiates their vasodilator actions. The potentiating effects of light are attributed to the accelerated release of ligated nitrosyl groups as free NO, since potentiation can be prevented by Hb (but not by NOS inhibitors) and enhanced by superoxide dismutase (SOD).

6.2 MATERIALS AND METHODS

A. ANIMALS

Adult male Wistar rats (340 - 495g) were killed by cervical dislocation, their tails removed and a length of artery (9.6 +/- 0.3mm) cannulated and dissected free as described earlier.

B. APPARATUS

Figure 6.1 shows the modified apparatus used in these experiments.

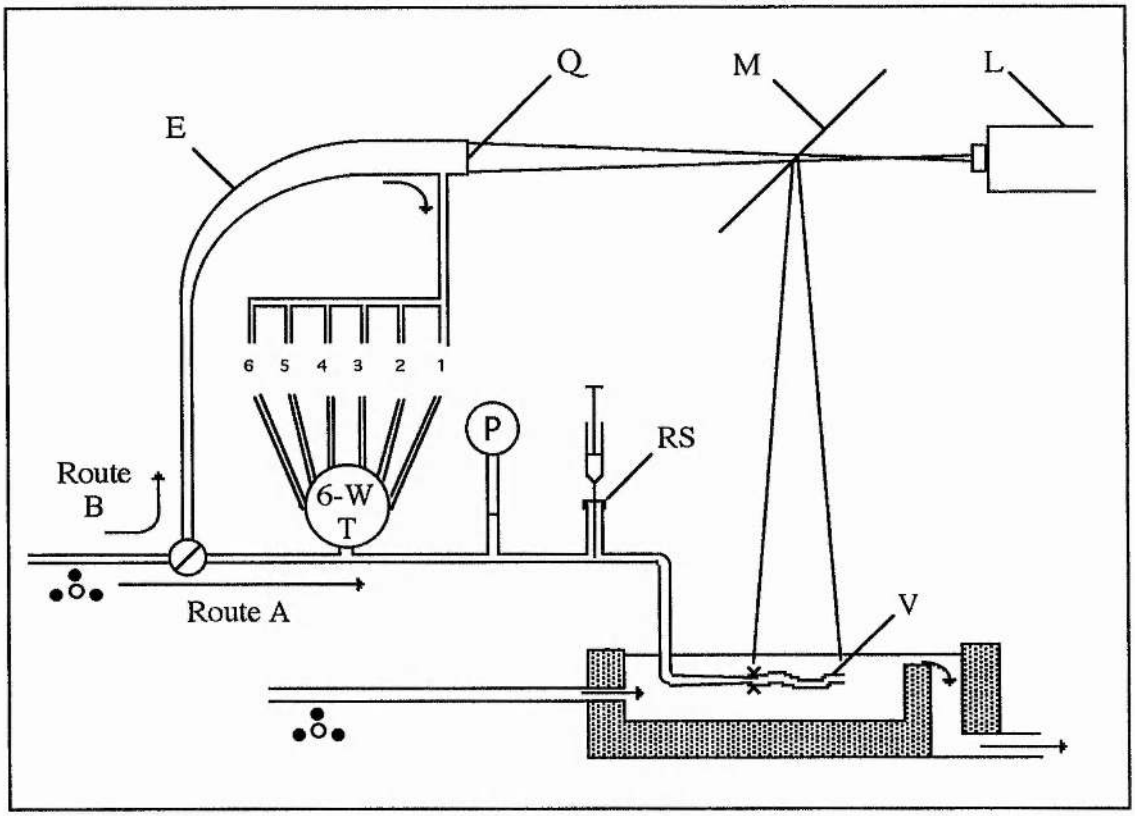


Figure 6.1. *Apparatus used to irradiate the vessel (V) or the perfusate en route to the preparation in the exposure tube (E) through the quartz window (Q). Time delay for outlet tubes: 1 - 0.73min. 2 - 1.05min. 3 - 1.33min. 4 - 1.69min. 5 - 2.54min. 6 - 3.12min. See text for full details and explanation of lettering.*

Light from an argon ion laser (L; Spectra Physics, type 160-8) could be directed onto the vessel by reflecting the expanded beam off a 45°, front-silvered mirror (M). The perfusate in these experiments followed route A. 10μl injections could be made through the rubber septum (RS).

Alternatively, the perfusate could be diverted through a curved glass tube (E; route B). Laser light could be directed in to the tube through a quartz end window (Q) to irradiate solutions *en route* to the preparation. The tube was tapered and its outer surface coated with silver to optimise the dispersion of light within. Six PVC outlet tubes (1-6) of different lengths were connected to the cannulated artery via a 6-way tap (6-WT). Each could be selected in order to vary the time delay between formation of photolytic products in tube E and arrival at V (0.72 - 3.12 mins).

During the perfusion experiments, it became clear that the tubing used to convey the perfusate to the preparation rapidly became contaminated with RBS and CUB. It was necessary, therefore, to change the tubing frequently to avoid cross-contamination between experiments.

C. EXPERIMENTAL PROTOCOLS

Vessels were precontracted with PE to produce agonist-induced perfusion pressures of 102.7 +/- 5mmHg ([PE] = 11.7 +/- 1.3μM; n=29).

Two experimental protocols were used:

(i) Potentiation of photorelaxation during the plateau phase of sustained responses.

Following precontraction, vessels perfused via route A were directly exposed to laser light by reflecting the laser beam off a front-silvered mirror (M, figure 6.1; beam diameter at preparation: 2cm). The illumination conditions (0.2 or 2 mW; = 514.5nm; 1 min exposures) were selected to produce small-amplitude (<10% of active pressure) photorelaxant responses (Furchgott *et al*, 1961). A single bolus injection (10 μl) of RBS or CUB was then introduced into the internal perfusate, through the rubber septum (RS, figure 6.1). The concentrations of RBS (250 μM) and CUB (500μM) were sufficient to induce moderate sustained responses. Vessels were again exposed to laser light (as above) at 12 min intervals, for periods of up to 5 hrs. The photosensitivity of vessels was enhanced by this treatment.

(ii) Perturbation of steady-state vasodilator responses to RBS or CUB by laser irradiation of solutions en route to preparations.

Vessels were perfused with PE Krebs solution via route B (figure 6.1). Pressure recordings were made during continuous infusions of RBS or CUB (0.3 μM or 0.9 μM), initially in the dark, and then during intermittent laser irradiation of the incoming solution (in tube E, figure 6.1). Exposure of solutions to light (457.9 nm or 514.5 nm; 5 mins) produced an additional vasodilation during the period of illumination. This is termed the *light-induced vasodilator response* (LIVR).

LIVRs were recorded under different illumination conditions (beam output energy and wavelength) and in the presence of either Hb (5 μM), SOD (150 Units. ml^{-1}) or the NO synthase inhibitor LNMMA (100 μM). The decrease in pressure, measured at the end of the illumination period, is expressed as a fraction of that recorded immediately prior to illumination.

(iii) Photodecomposition of RBS.

Photolysis of RBS was studied under conditions which simulated those used for recording LIVRs as closely as possible. Solutions (0.3 or 0.9μM) were exposed to laser light in the photolysis tube (flow rate: $2ml.min^{-1}$; 457.9 nm and 514.5 nm) and collected in a spectrophotometer cuvette (10 cm pathlength). It was necessary to make solutions in distilled water rather than oxygenated Krebs solution since bubbles and/or precipitate produced in Krebs solution distorted the absorption spectra recordings (Cecil double-beam UV-VIS spectrophotometer; type CE 594). The decrease in absorbance at 360nm (ε = 14, 940 $M^{-1}.cm^{-1}$) was measured to determine the quantity of RBS photolysed. The results are used to calculate the efficiency of NO production at source.

The rate of inactivation of NO *en route* to the vessel was estimated by recording the decrease in size of LIVRs (produced by standardised illumination conditions) with increasing transit times between exposure (in tube E) and the vessel (V). The results are used to relate the amplitude of the LIVR to the [NO] reaching the vessel.

6,3 RESULTS

Enhanced Photorelaxation Produced By Irradiating Vessels During Sustained Responses.

Furchgott *et al* (1961) showed that exposure to light causes pre-contracted vascular smooth muscle to relax (see also sections 1.11 and 7.1). Vessels which had been injected with RBS or CUB exhibit enhanced photorelaxant responses (EPR's) when irradiated during the plateau phase of sustained responses.

Typical recordings of EPR's are shown in figure 6.2 (a) and (b).

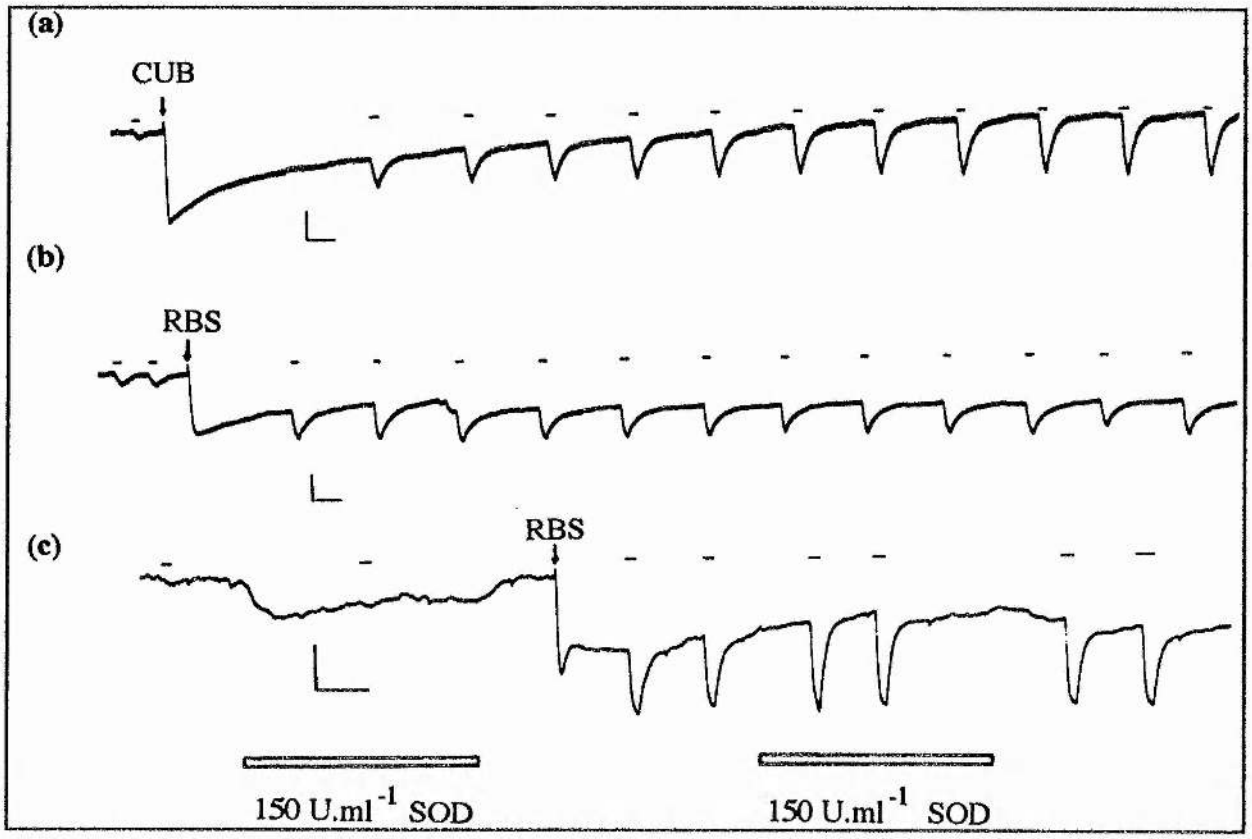


Figure 6.2. *Pressure recordings showing enhanced photorelaxant responses obtained following 10µl injections of (a) and (b) 250µM CUB 500µM RBS. Irradiations (0.2 mW; 514.5 nm; 1 min) are indicated by horizontal bars. Trace (c) shows the effect of 150 U.ml^{-1} SOD perfused before and after a 10µl injection of RBS. Irradiations of the vessel in the presence of SOD following an RBS injection show further enhancement. Scalebar: vertical - 20%; horizontal - 5 mins. Expt. N^{o}s (a) 910701, (b) 910704 and (c) 910725.*

Control photorelaxations were elicited before a single bolus injection (10μl) of either 500μM RBS or 250μM CUB was administered into the internal perfusate. Typically, vessels showed a rapid dilation followed by partial recovery to a level below the pre-injection pressure. Once in this sustained phase of the response, vessels were subjected to repeated periods of laser irradiation . This treatment produced a marked and long-lasting (up to 5hrs) potentiation of the photorelaxant response (figure 6.2 (a), (b)). The addition of SOD (150 U.ml^{-1}) to the internal perfusate further enhanced the sensitivity of vessels to light (figure 6.2 (c)).

Figure 6.3 shows histograms comparing control photorelaxations (open columns) to EPRs.

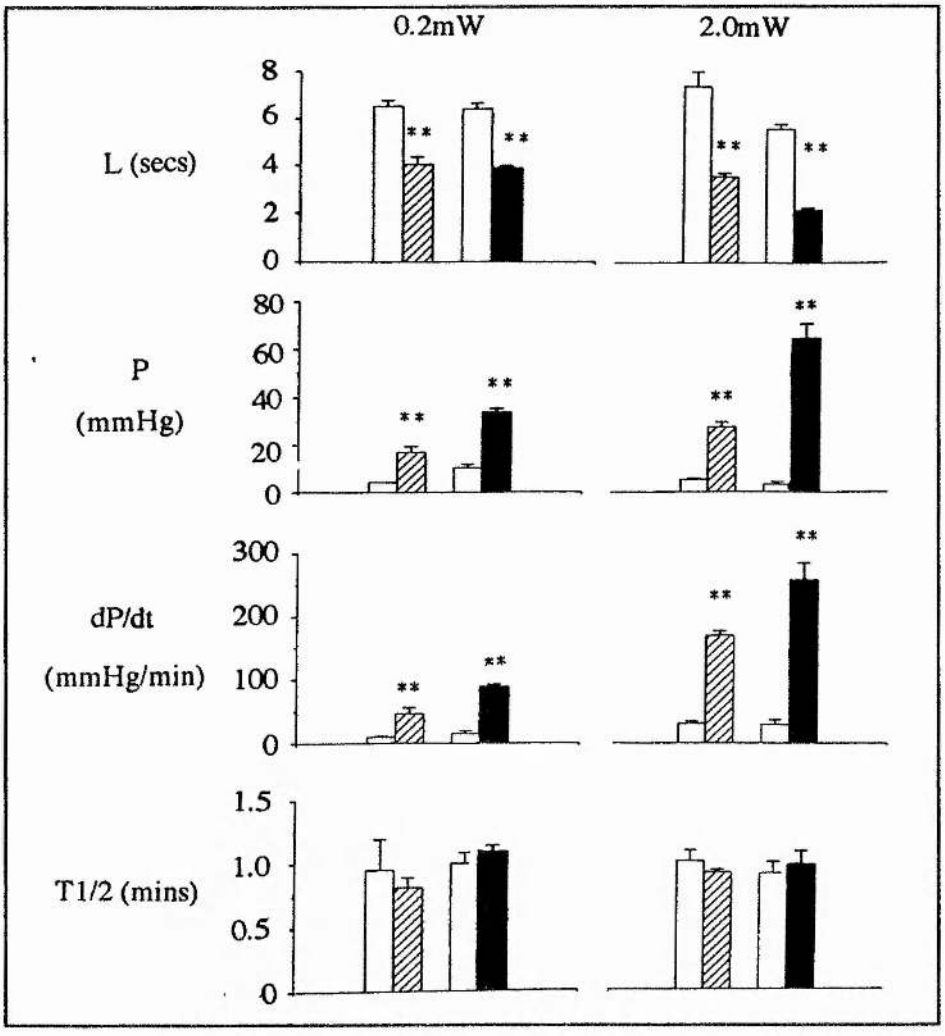


Figure 6.3. *Histograms showing the effect of bolus injections of 500μM RBS (black columns) or 250μM CUB (hatched columns) on photorelaxations (controls, open columns) evoked by 0.2 mW or 2mW. Parameters of photorelaxation investigated; latency of onset (L), amplitude (P), rate of drop of pressure (dP/dt), and rate of recovery ($T^1/_2$). ** indicates a significant difference from the control at P = 0.01 confidence level.*

Four parameters were measured: the time interval between the start of illumination and the earliest detectable drop in pressure (latent period, L; secs); the maximum rate of drop in pressure (dP/dt; $mmHg.min^{-1}$); the maximum amplitude of the photorelaxant response (P; mmHg); and the half-time of recovery in the dark ($T^1/_2$; mins).

The results show, first, that L was decreased and both dP/dt and P were increased following injection of either drug. There was no significant effect on the rate of recovery (T1/2) in the dark. Second, enhancement of the photorelaxant response was generally greater for RBS (black column) than for CUB (hatched column). This was probably due to the fact that 500μM RBS was used as opposed to 250μM CUB. Third, increasing the beam energy (from 0.2 to 2.0 mW) increased the potentiating effect of both compounds.

Perturbation of Steady-State Vasodilator responses to RBS and CUB by laser irradiation of solutions en route to the vessel.

Constant infusion of 0.3μM RBS caused a sustained drop in pressure of 17.3 +/-2.3% (n = 12) whilst the equivalent value for 1.6μM CUB was 9.0 +/-0.2% (n = 11).

A. CHARACTERISTICS OF THE LASER-INDUCED VASODILATOR RESPONSE (LIVR).

The main features of the LIVR are illustrated in figure 6.4. The horizontal lines associated with each pressure recording indicate the period (5min) of laser irradiation and the beam energies (mW) used. It is important to emphasise that vessels were carefully shielded from stray light throughout, to avoid evoking photorelaxant responses.

LIVRs produced by irradiating perfusate containing 0.3 μM RBS under different illumination conditions (beam energy; wavelength) are shown in figure 6.4. The LIVR typically comprises an initial, rapid drop in pressure, commencing after a short delay (here ca. 0.73 min; outlet tube 1).

This fast component was complete within ca. 1 min. Thereafter, pressure either stayed constant throughout the remainder of the illumination period, or it continued to fall but at a greatly reduced rate. After illumination ceased, the vessel reconstricted; pressure increased rapidly at first and then more slowly, terminating at (or near) the pre-illumination level.

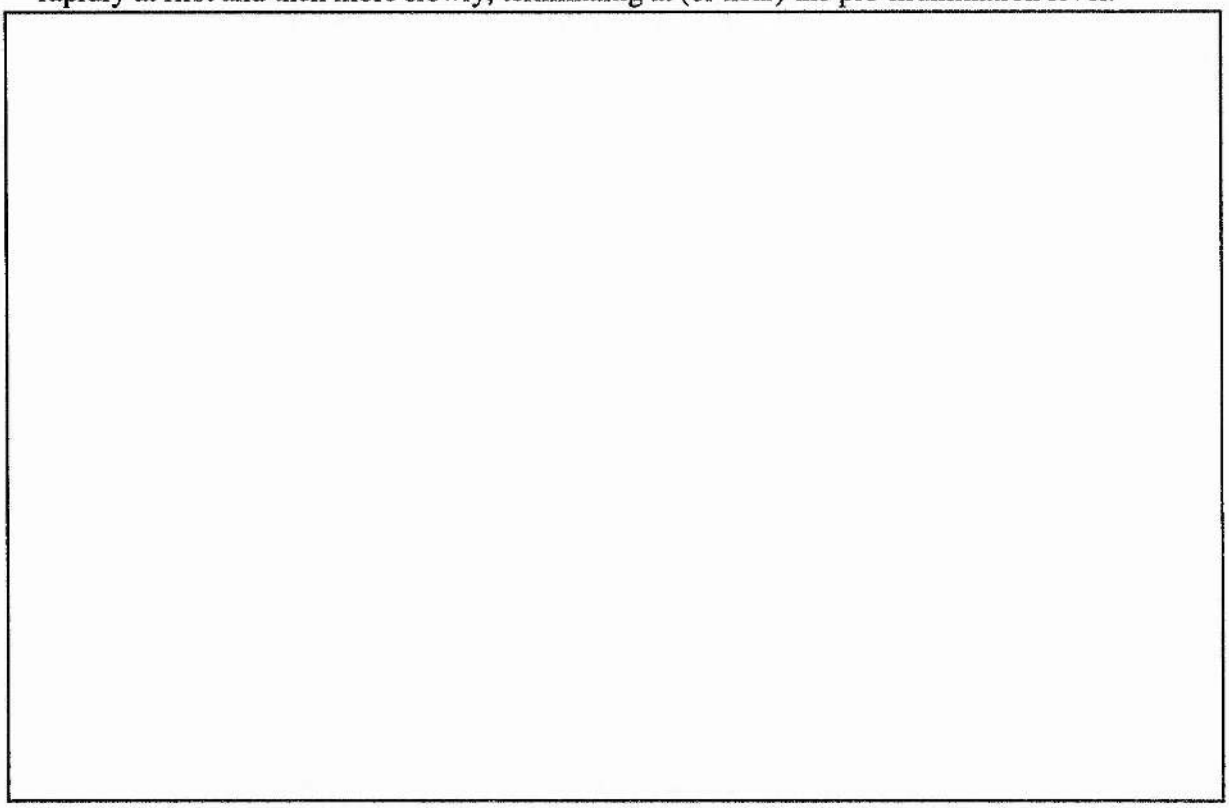


Figure 6.4. *LIVRs produced by illuminating solutions of 0.3μM RBS en route to the preparation. Trace (a) shows responses to 457.9 nm laser light, trace (b) responses to 514.5 nm laser light. Expt. N^{o} (a) 920324, (b) 920309.*

The results from experiments of this type for both RBS and CUB are summarised in figure 6.5. The amplitude of the LIVR is plotted as a function of beam energy. The reulting curves show that LIVRs increase in size with increasing beam energies, rising asymtotically towards a maximum value and that irradiating solutions with light at 457.9 nm is more effective at producing LIVRs than at 514.5nm. RBS is more light-sensitive than CUB and increasing the concentration of RBS increased the LIVR amplitude produced under standard illumination conditions.

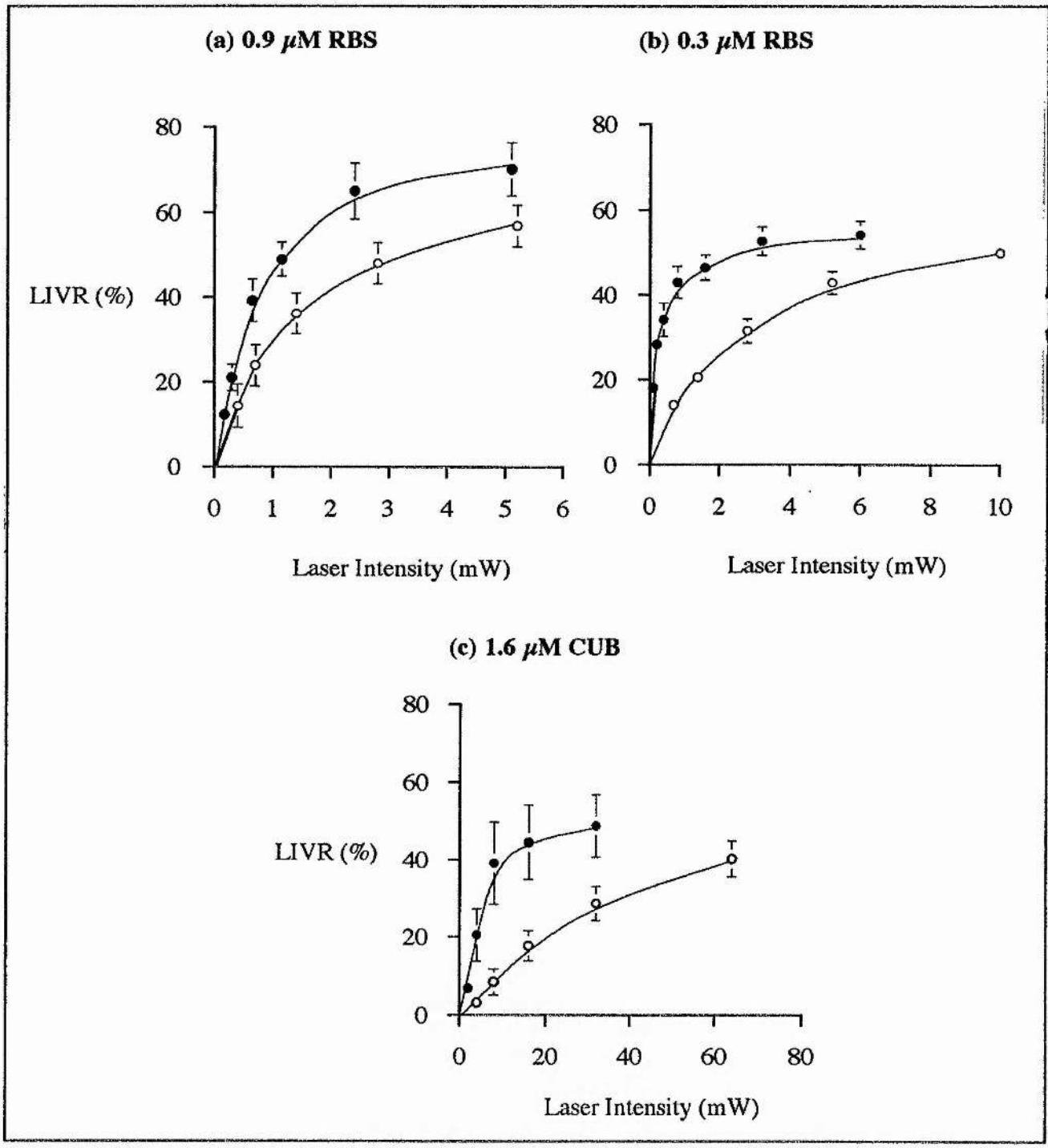


Figure 6.5. *Plots of LIVR amplitudes (% of active pressure) for (a) 0.9 μM RBS (n = 5), (b) 0.3μM RBS (n = 6) and (c) 1.6 μM CUB (n = 6). Responses to 514.5nm (open symbols) and 457.9 μM (filled symbols) are shown for all three graphs.*

Figure 6.6 is a reciprocal plot of the results obtained with RBS. This shows that 1/LIVR increases in direct proportion to 1/beam energy.

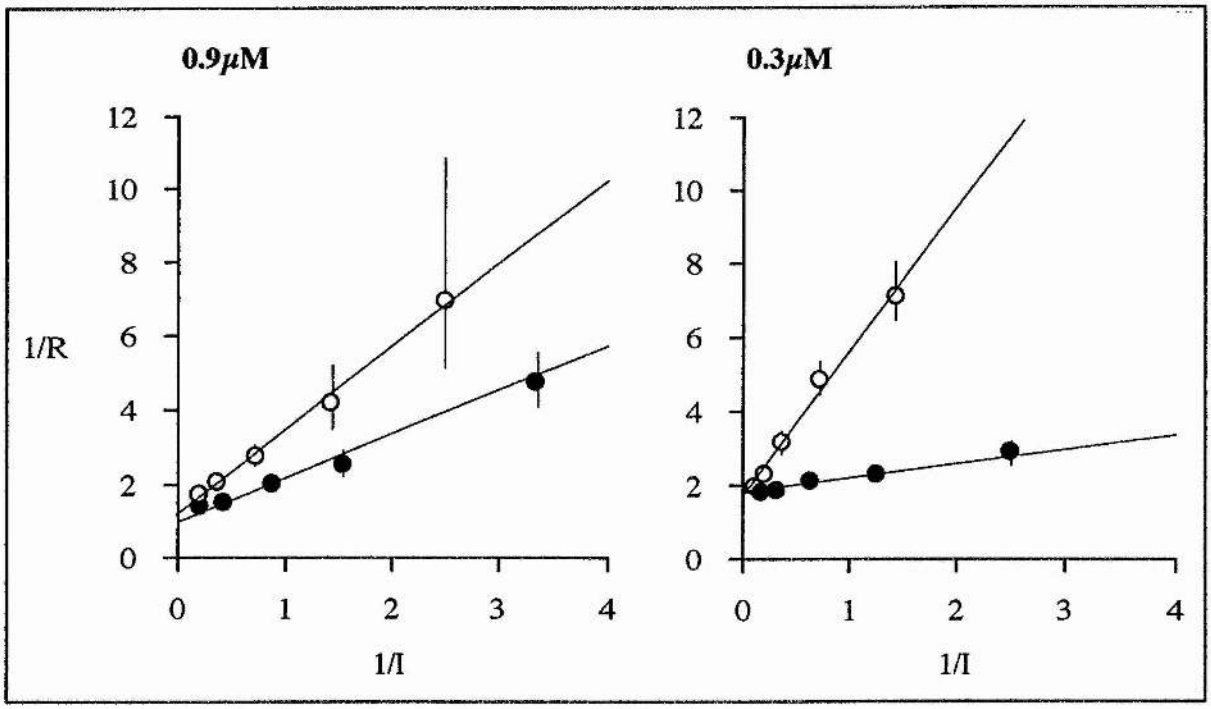


Figure 6.6. *Double reciprocal plot of 1/intensity (1/I) against 1/response (1/R) for 0.3 μM and 0.9 μM RBS solutions. Responses to 457.9nm laser light are represented by filled symbols whilst those for 514.5nm light are represented by open symbols.*

Linear regression analyses of the data gave the following results:

514.5nm; 0.9μM	$y = 2.23x + 1.22$	($r^2 = 0.995$)	(1)
514.5nm; 0.3μM	$y = 3.91x + 1.72$	($r^2 = 0.989$)	(2)
457.9nm; 0.9μM	$y = 1.19x + 0.99$	($r^2 = 0.995$)	(3)
457.9nm; 0.3μM	$y = 0.38x + 1.83$	($r^2 = 0.993$)	(4)

where y = 1/LIVR and x = 1/beam energy.

The maximum amplitudes of the LIVR attainable (Pmax) under the different experimental conditions (for x = 0 in equations 1-4) and the beam energies required to reduce the perfusion pressure by 50% ($I_{50\%}$ values) are listed in table 6.1.

λ (nm)	[RBS] (μM)	Pmax (%)	$I_{50\%}$ (mW)
514.5	0.9	82	2.9
514.5	0.3	58	14.1
457.9	0.9	101	1.17
457.9	0.3	55	2.28

Table 6.1. *maximum amplitudes (P max (%) - as calculated from double reciprocal plots in figure 6.6) and I_{50}% values for RBS under the conditions indicated.*

The I_{50} values suggest that a considerably higher intensity of light is required to produce 50% relaxation with 514.5nm laser light than 457.9nm. The situation is particularly clear with the lower [RBS] (0.3μM) where the I_{50} is 7x greater for 514.5nm laser light. Pmax is greater for the higher [RBS] with both wavelengths (ca. 100% with 457.9nm; 0.9μM RBS).

LIVRs are mediated by NO released photochemically.

Figure 6.7 shows that LIVRs are mediated by exogenous NO, generated by photodecomposition of the clusters.

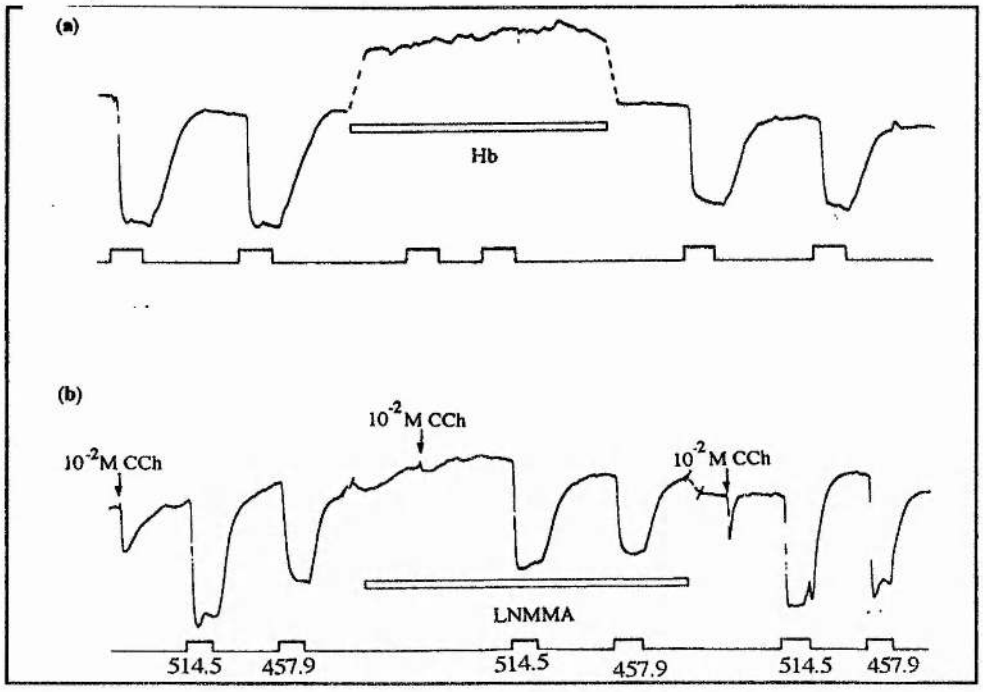


Figure 6.7 (a). *Pressure recordings showing the fully reversible, inhibitory effect of 5μM Hb on LIVRs induced in RBS-treated vessels (0.3μM; 3.3mW, 457.9nm and 10mW, 514.5nm). Trace* ***(b)*** *shows that LIVRs are unaffected by 100μM LNMMA (3.3mW, 457.9nm and 10mW, 514.5nm). Periods of irradiation are indicated by horizontal bars. Scale bars: vertical - 20% relaxation; horizontal ; 4 mins.*

First, LIVRs were suppressed by adding 5μM Hb to the internal perfusate (6.7(a); the effect was reversible. In contrast, the addition of 100μM LNMMA, which inhibited basal release of NO, as reflected in a rise in pressure of 57.1% +/-6 (n = 10), failed to inhibit the LIVR; in some cases a small reduction was observed, but more often the response was found to be potentiated by LNMMA.

Second, LIVRs were progressively reduced in amplitude when the interval between solution leaving the photolysis tube and entering the vessel (delay time) was extended by increasing the length of the outlet tube (figure 6.8 (a)). SOD was also found to potentiate sub-maximal LIVRs (figure 6.8 (b)).

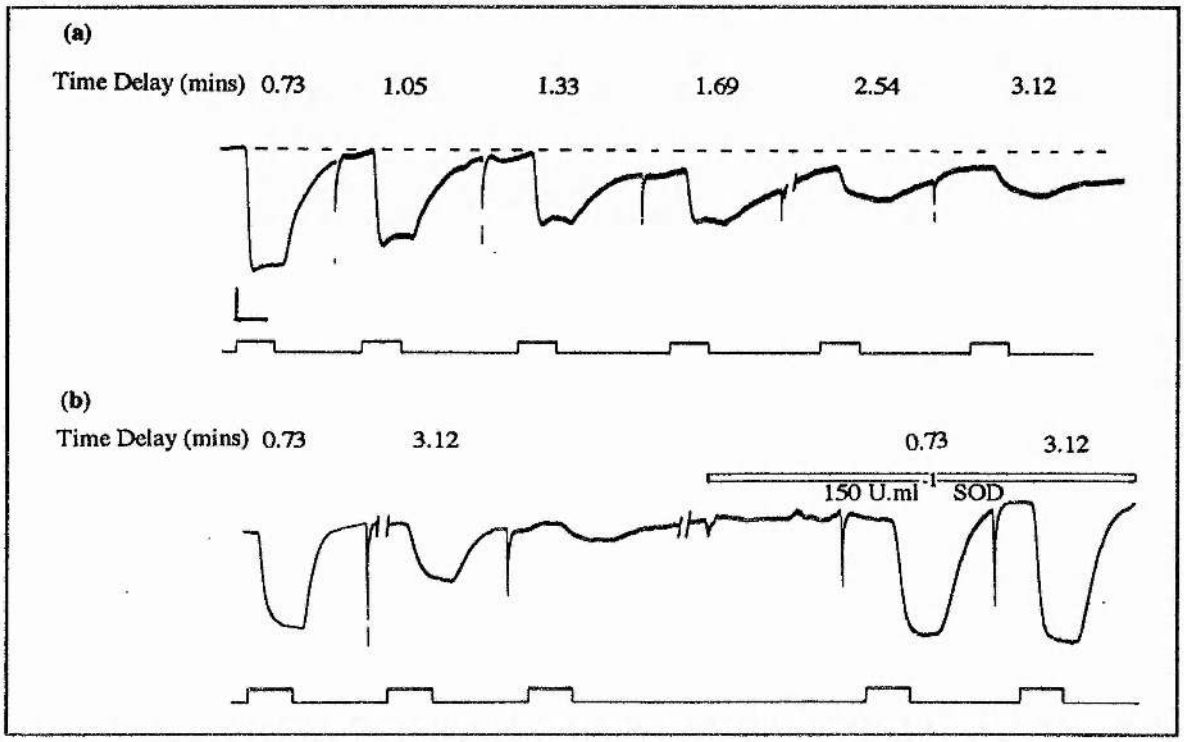


Figure 6.8(a) *Effect of increasing delay time between perfusate leaving the exposure tube and reaching the vessel on LIVRs (0.3μM RBS; 514nm laser light). Delay times for each outlet tube are indicated above the corresponding LIVR.* ***(b)*** *Effect of 150 U.ml^{-1} SOD on the LIVR produced using outlet tube 6 (c.f. (a) above). 0.3μM RBS; 514nm laser light.*

Exposure of 150 Uml^{-1} SOD alone (i.e. in the absence of RBS) *en route* to the preparation on occasion produced small vasodilations. 457.9nm laser light was unlikely to have a direct effect on SOD since spectrophotometric measurements showed SOD not to absorb light at

this wavelength. The results were therefore attributed to RBS contamination of the glass exposure tube (E).

Spectrophotometric measurements of the photodecomposition of RBS; photochemical efficiency of NO production.

Figure 6.9 shows computer-averaged spectra (n = 5 - 7) for unexposed solutions (control, upper spectrum) and for solutions illuminated with laser light (514.5 nm) at the beam energies indicated (lower 3 spectra).

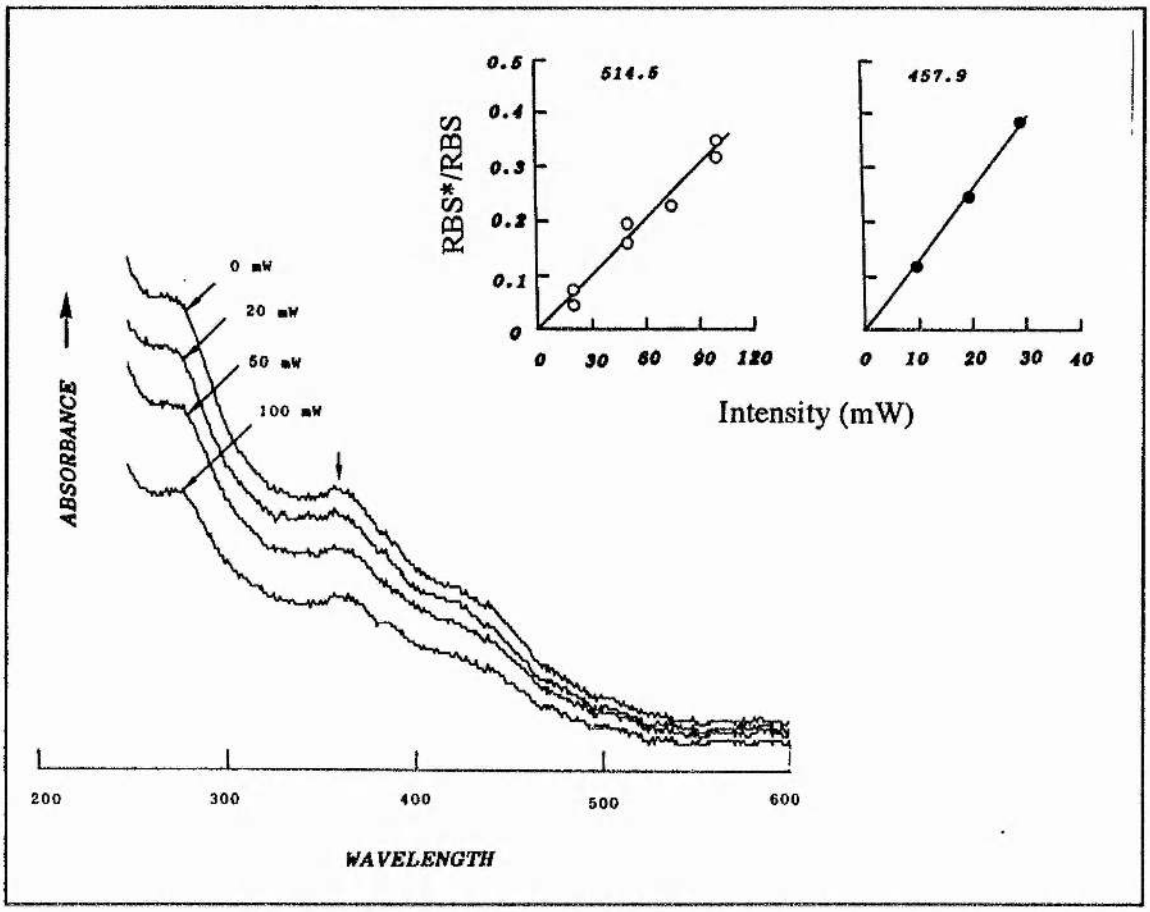


Figure 6.9. *Computer-averaged absorption spectra for 1μM unexposed RBS (upper trace n = 7). The lower traces show the equivalent spectra for RBS exposed to 514.5nm laser light as indicated (n = 5). Figure 6.9 (inset) shows the proportion of RBS broken down (RBS*/RBS) with increasing intensities of both 514.5nm and 457.9nm laser light.*

Exposure to light resulted in a proportional decrease in absorbance across the range of wavelengths 290-600nm. The spectrum was unaltered in all other respects, since the fact that the products of RBS breakdown are all insoluble (Fe_2O_3 and S_8; Butler & Askew, unpublished data).

Figure 6.9 (inset) shows the breakdown of RBS vs. the beam energy (514.5 nm, open circles; 457.9 nm, filled circles). The photodecomposition increased linearly with increasing energies, up to ca. 100mW at 514.5 nm and ca. 30mW at 457.9 nm, ranges which far exceed those used for recording LIVRs.

Table 6.2 lists values for the fractional decomposition of RBS per mW of light (f) for the different experimental conditions used in the study.

	0.9 μM RBS (n =9)		**0.3μM RBS (n = 6)**	
λ (nm)	**f* (mW^{-1})**	**E ($nM.mW^{-1}$)**	**f* (mW^{-1})**	**E ($nM.mW^{-1}$)**
457.9	14.06×10^{-3} (0.43)	88.6 (2.73)	20.57×10^{-3} (0.77)	43.2 (1.62)
514.5	3.64×10^{-3} (0.23)	22.96 (1.43)	4.10×10^{-3} (1.03)	8.6 (2.15)

Table 6.2. *Photochemical efficiencies of NO formation. All values are means (S.E.M. in brackets).*

The efficiency of the photolytic process (E), expressed in terms of the steady-state [NO] formed at source ($nM.mW^{-1}$), is given by:

$$E = fcn \qquad (5)$$

where f = the fractional breakdown of RBS (mW^{-1}) at wavelength 457.9, 514.5 nm

c = the concentration of RBS (nM) used;

and n = the number of moles of ligated nitrosyl groups released as free NO per mole of RBS photolysed.

The mean +/- SEM values for E obtained for two concentrations of RBS (c = 0.3 μM and 0.9 μM) at each wavelength appear in table 6.2. A value of n = 7 has been assumed in calculating the data (see discussion). These results show (a) that E is considerably greater at the shorter wavelength, by a factor of 4-5x; and (b) that increasing the concentration of RBS from 0.3 - 0.9 μM results in a 2-3 fold increase in E.

Inactivation of NO released photochemically.

NO reacts rapidly with oxygen-derived free radicals, such as O_2^- (Moncada *et al*, 1986) and the short-lived hydroxyl ($OH^\bullet$) radical. In the perfusion system used here, the concentration of NO which survives passage through each outlet tube is given by the following equation:

$$[NO] = E\lambda I p_n \qquad (6)$$

where $E\lambda$ = photochemical efficiency of NO production at wavelength λ (eqn. 1)
I = beam energy (mW) at wavelength λ
p_n = proportion of NO generated at source which reaches the artery for the nth outlet tube.

The time course for the inactivation of NO was determined in the following manner:
First, LIVRs were recorded at increasing delay times, as illustrated in figure 6.8(a). Solutions (0.3μM) were irradiated at 457.9 nm and 514.5 nm, using standardised beam energies of 3.3mW and 10mW respectively. Figure 6.10 is a semi-logarithmic plot of the LIVR amplitude as a function of the delay time.

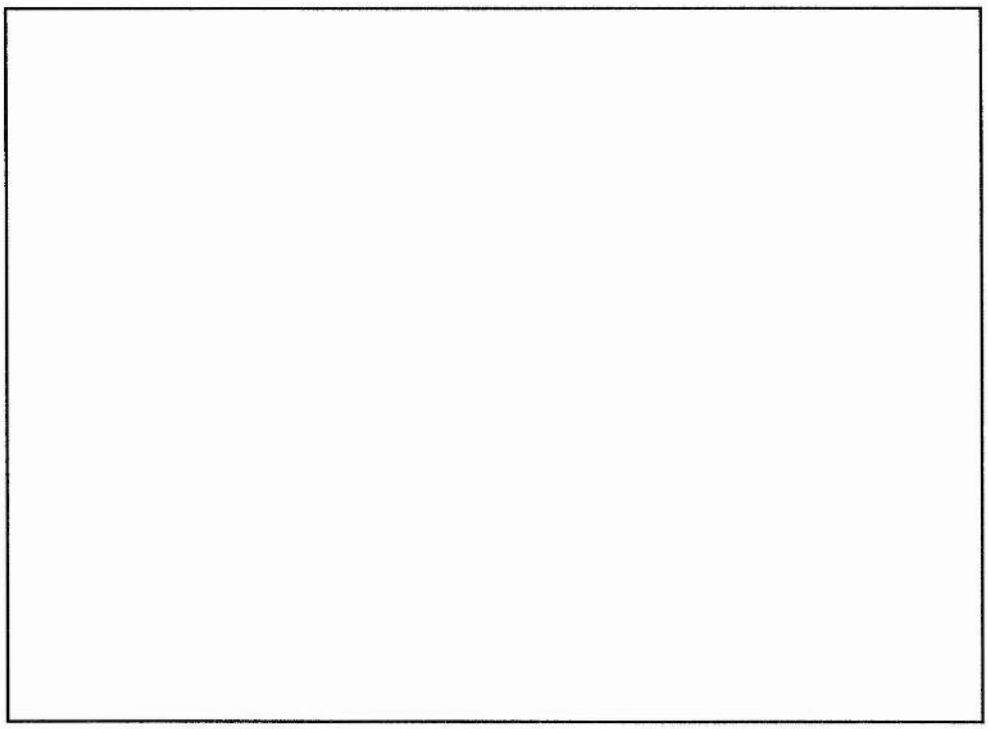


Figure 6.10. *Effect of increasing delay between exposure of the perfusate containing 0.3μM RBS (514.5nm, open circles, n = 5; 457nm, closed circles, n = 5) and the preparation. Addition of 150 U.ml^{-1} SOD to the perfusate abolished the effect of increasing delay on LIVR amplitude (filled triangles, n = 3).*

The responses decay exponentially, falling to 50% of their maximum values after 58.9 secs (514.5 nm; open circles) and 57.9 secs (457.9 nm; filled circles). Note that the addition of SOD to the internal perfusate (filled triangles) prevented attenuation of the LIVR (see also figure 6.8(b)).

Linear regression analyses of the data gave the following results:

$$\lambda = 514.5\text{nm}: \quad y = -0.307x - 0.151 \quad (r^2 = 0.985) \quad (7)$$

$$\lambda = 457.9\text{nm}: \quad y = -0.312x - 0.111 \quad (r^2 = 0.968) \quad (8)$$

where $y = \log_{10}$ LIVR amplitude

and x = delay time (mins).

The reationship between LIVR amplitude and [NO] is not linear as can be inferred from the results of figure 6.5, and so eqns 7 and 8 do not describe the rate of inactivation of NO directly. This information was aquired by first evaluating eqns 7 and 8, for x values ranging from 1 - 3.4 mins (0.2 min intervals). The resulting y values (LIVR amplitudes) were then inserted into eqns 2 (514.5 nm) and 4 (457.9 nm) to determine the beam energy corresponding with each LIVR amplitude. Lastly, the interpolated energy values were multiplied by the appropriate E value (table 6.2) to estimate the [NO] reaching the vessel.

The results reveal that the rate of inactivation of NO depends on the illumination conditions. Figure 6.11 shows that the time courses for the inactivation process at the two wavelengths are described by the sum of two exponential components e_1 and e_2.

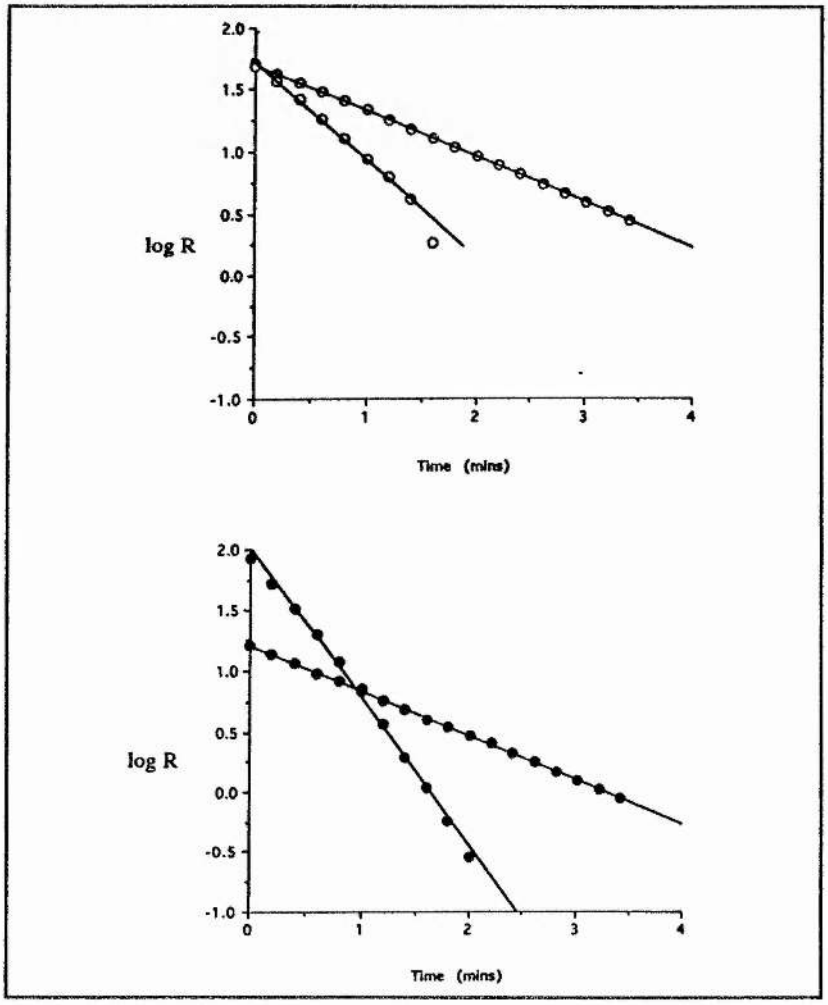


Figure 6.11. *Exponential components e_1 and e_2 for (a) 514.5nm and (b) 457.9nm LIVRs. [RBS] = 0.3μM.*

The amplitude (a) and time constant (k) for the rapid component (e_1) were greater for 457.9nm (a = 0.83 and k = 0.39 mins) than for 514.5nm (a = 0.49 and k = 0.63 mins), producing a faster rate of inactivation at the shorter wavelength. The time constants for the slow component (e_2) were the same for both wavelengths (k = 1.15 min). The possible reason for this difference is considered in the discussion.

The proportion of NO (P) reaching the vessel for each outlet tube is listed in table 6.3.

Tube Nº	Delay Time (mins)	P (514.5nm)	P (457.9nm)
1	0.73	0.413	0.242
2	1.05	0.289	0.132
3	1.33	0.210	0.076
4	1.69	0.134	0.045
5	2.54	0.059	0.018
6	3.12	0.036	0.017

Table 6.3. *The effect of increasing delay time on the proportion of NO (P) reaching the vessel for 514.5nm and 457.9nm laser light.*

LIVR log-dose response curves.

Figure 6.12 is the log-dose response curve relating the amplitude of LIVRs to the [NO] calculated to be reaching the vessel. LIVRs recorded under all experimental conditions used (wavelength, concentration and outlet tube delay times) lie on a single curve, with a threshold [NO] of ca. 1nM and an ED_{50} value of 14.1 nM, with a range of 6.6 - 27 nM.

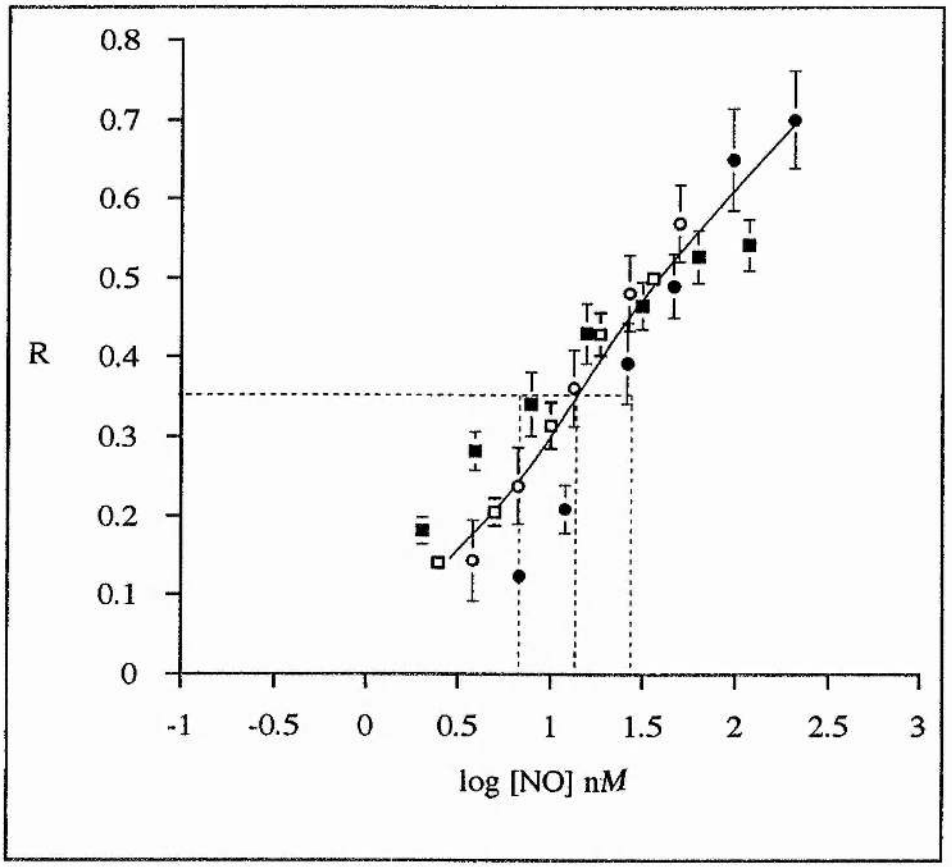


Figure 6.12. *Log-dose response curve for NO, calculated by the method described in the text. Curves derived from experiments using 514.5nm (open symbols) and 457.9nm (filled symbols) laser light at both 0.3 (squares) and 0.9μM RBS (circles) are shown.*

6.4 DISCUSSION

Vasodilator responses produced by nitrosylated iron-sulphur clusters are enhanced by light: this applies to sustained responses, resulting from single bolus injections (figures 6.3), and to 'steady-state' responses, generated by continuous infusions of RBS or CUB (figures 6.4).

The potentiating effects of light are greater at the shorter wavelength and higher beam energy (figure 6.4, table 6.1 and figure 6.5); they are facilitated by exogenous SOD (figure 6.8(b)), the enzyme which dismutes O_2^- (McCord & Fridovich, 1969), a known inactivator of endothelium-derived NO (Gryglewski *et al*, 1986; Moncada *et al*, 1986); and they are blocked by Hb (figure 6.5), a scavenger for NO (Antonini and Brunori, 1971; Martin *et al*, 1985).

These observations show that both RBS and CUB are photodegradable and that photolysis accelerates the release of ligated nitrosyl groups as free NO. The failure of LNMMA, a stereospecific inhibitor of NO synthase (Rees *et al*, 1990), to suppress LIVRs (figure 6.7 (b)) and enhanced photorelaxations confirms that neither type of potentiation is caused by stimulation of endothelium-derived NO synthesis.

'Inactivation' of NO generated photochemically and the role of O_2^-

NO is known to react readily with O_2^- to form peroxynitrite and ultimately NO_2^- and NO_3^- (Furchgott et al, 1990). SOD prevents this reaction sequence by catalysing the dismutation of O_2^- to H_2O_2 (McCord & Fridovich, 1969). The ability of SOD to prevent attenuation of the LIVR (figure 6.10) clearly implies that inactivation of RBS-derived NO is due to it reacting with O_2^-.

The time course of the inactivation process (figure 6.10) is more complex than previously supposed. The disappearance of NO was more rapid at the shorter wavelength, even though the beam energy was only one third of that used at the longer wavelength. McCord & Fridovich (1973) showed that photolysis of oxygenated water generates O_2^- and hydroxyl

(OH•) free radicals in equimolar amounts. The observations reported here could be explained if we postulate that the shorter wavelength light is more effective at producing O_2^- by this means. However, for theoretical reasons, it is unlikely that irradiation of oxygenated water with either of the two wavelengths used here could have generated significant quantities of O_2^-. The inactivation is nevertheless related to the wavelength and/or beam energies used to generate NO. It is therefore possible that O_2^- is formed as a consequence of reactions between dissolved molecular O_2 and one or more of the products of RBS decomposition. A likely candidate is Fe^{2+}, produced by fragmentation of the FeS framework, since this is known to catalyse the production of O_2^- from O_2 (Matsunaga & Furchgott, 1989).

Significantly, the time constant for the slow component of the inactivation process was found to be independent of the illumination conditions (k = 1.15 mins). This may represent the rate of reaction of NO with dissolved **molecular** oxygen, though recent calculations would suggest that this would be slow with the low concentration of NO involved (Butler & Williams,1993).

Sensitivity of vessels to NO released photochemically.

The log-dose response curve of figure 6.12 shows that the [NO] required to produce a detectable (threshold) LIVR is in the region of 1nM. The ED_{50} value for LIVRs of 14.1 nM (ranging from 6.6 - 27μM) is somewhat less than that quoted for enhanced dilator responses by Jacobs *et al* (1990; rabbit aorta; 63 nM) and by Sakuma *et al* (1990; 20 - 250 nM for a variety of arteries) but greater than that cited by Kelm *et al* (1988; 5nM).

There are two possible sources of error in the method of calculation used to interpret the results reported here.

First, LIVRs are necessarily superimposed on a 'background' vasodilator response, due to the spontaneous release of NO **in the dark** (Flitney et al, 1992; see also chapters 3 and 4). This may reduce the sensitivity of vessels to further NO produced photochemically.

Second, the estimates of photochemical efficiency (E values) of NO formation under different illumination conditions (equation 1; table 6.2) assume that all 7 ligated nitrosyl groups are released during photolysis. This assumption is supported by chemical theory although there is no direct experimental evidence for it to be so. RBS has an electron precise configuration (Sung *et al*, 1985). The addition or removal of an electron will destabilise the FeS framework causing it to disintegrate into NO, insoluble iron oxides and elemental sulphur (Butler & Askew, unpublished data).

Dual mode of action of iron-sulphur cluster nitrosyls.

The plateau of sustained responses is due to spontaneous release of NO (in the dark) from clusters trapped within the endothelium (Flitney *et al*, 1992). The ability to enhance photorelaxation by irradiating vessels previously injected with either RBS or CUB provides further circumstantial evidence for this hypothesis; exposure of treated vessels to light would be expected to accelerate the release of NO from clusters trapped within the endothelium and cause an additional vasodilation. This interpretation is supported by the finding that EPRs are further potentiated by SOD (figure 6.2(c)).

Relative photosensitivities of RBS and CUB

RBS invariably produces larger LIVRs and EPRs than CUB under comparable illumination conditions. The concentration of CUB chosen for the LIVR experiments was such that the potential NO concentration produced by complete degradation was the same as for RBS (i.e. [CUB] = 7/4 x [RBS]). The inference from this is that RBS is more photosensitive than CUB.

It was not possible to make a photometric study of the photodecomposition of CUB, for direct comparison with RBS, because its absorption spectrum showed marked, time dependent changes, even when solutions were kept in the dark. Unlike RBS, CUB is insoluble in water, which required that it be dissolved first in dimethyl sulphoxide (DMSO) prior to use. DMSO is a co-ordinating solvent which has recently been shown to cause

fragmentation of CUB and subsequent reassembly as RBS, the more stable of the two configurations. The reaction proceeds through the formation of mononuclear (dinitrosyl) solvo-complexes. The transformation of CUB to RBS by DMSO has been detected by infra-red spectroscopy under conditions which simulated those used here (Glidewell & Glidewell, unpublished data).

Therapeutic potential of iron-sulphur cluster nitrosyls

Haemodynamic studies on experimental animals and human volunteers, using stereospecific inhibitors of NO synthase, have established that endothelium-derived NO is a major determinant of peripheral resistance *in vivo* (Vallance et al, 1989; Chu *et al*, 1991; Aisaka *et al*, 1989; Gardiner et al, 1990; Rees *et al*, 1989). Significantly, endothelium-dependent relaxations are attenuated in hypertension (Luscher & Vanhoutte, 1986; Winquist et al, 1984; Otsuka et al, 1988; Tesmafariam & Halpern, 1988; Sunano et al, 1989) and in atherosclerosis (Forstermann *et al*, 1988; Harrison *et al*, 1987; Chappel *et al*, 1987; Henry et al, 1987; Guerra et al, 1989). The ability of conventional NO donor drugs to lower blood pressure is due to release of NO into the vascular bed, either spontaneously or after metabolic biotransformation (Feelisch & Noack, 1987; Waldman & Murad, 1984). However, their hypotensive actions are generally short-lived, so that continuous drug infusions are necessary to produced sustained responses (Kreye, 1980). In the previous chapter, it was shown that RBS and CUB readily penetrate endothelial cells and accumulate therein, releasing NO slowly to produce protracted vasodilator responses. The *photosensitivity* of these compounds adds an important new dimension to their therapeutic potential. Their ability to enter endothelial cells and to release NO more rapidly on exposure to light presents the possibility of causing ***local*** vasodilation with the use of fibre optic light guides and the potential to deliver cytotoxic quantities of NO to specific target sites.

CHAPTER 7

EVIDENCE FOR A PHOTOLABILE NO 'STORE' IN VASCULAR SMOOTH MUSCLE

7.1 INTRODUCTION

Pre-contracted vascular smooth muscle relaxes when exposed to visible or ultra-voiolet light (Furchgott *et al*, 1961). The effect is reversible and complete recovery occurs in the dark. Photorelaxation was shown to depend on both the intensity and wavelength of light used. The amplitude of the response was independent of oxygen tension, though recovery in the dark was markedly slower under anaerobic conditions. Lowering the temperature from 37 to 20°C increased the amplitude of the response and prolonged its time course. The nature of the photosensitive material could not be established, though the manner in which the response varied with wavelength (biological 'action' spectrum) led Furchgott *et al* to propose that it could be a 'complex of a metal, such as iron, with a protein'.

Photorelaxation is not seen with non-vascular smooth muscle, save in the presence of NO_2^- (Furchgott & Matsunaga, 1989), and it can be elicited in endothelium-denuded preparations (Furchgott *et al*, 1984). Recently, it has been postulated that ultraviolet light causes vasodilation by activating endothelial NO synthase (Deliconstantinos et al, 1992). In addition, irradiation of partially purified guanylate cyclase, the 'target' enzyme for endothelium-derived NO in vascular smooth muscle cells (Waldman & Murad, 1987), has been shown to activate cGMP synthesis both *in vitro* (Karlsson *et al*, 1985) and *in vivo* (Karlsson *et al* , 1984).

Previous work in this laboratory (Flitney, Watson & Megson, unpublished data) showed that exposure of segments of rat tail artery to low level laser irradiation (< 10 mW; 514.5nm or <4mW; 457.9nm) produced vasodilator responses similar to those reported by Furchgott and his colleagues. Exposure to higher light intensities produced responses whose amplitude was not increased but which showed signs of recovery *during the period of illumination.*

In the present study, photorelaxant responses were elicited in both PE- and high potassium (80mM) - contracted arteries and their amplitude, expressed as a % of active pressure,

appeared to be independent of the pre-illumination pressure. Photorelaxations typically displayed profiles similar to that shown in figure 7.1 (c).

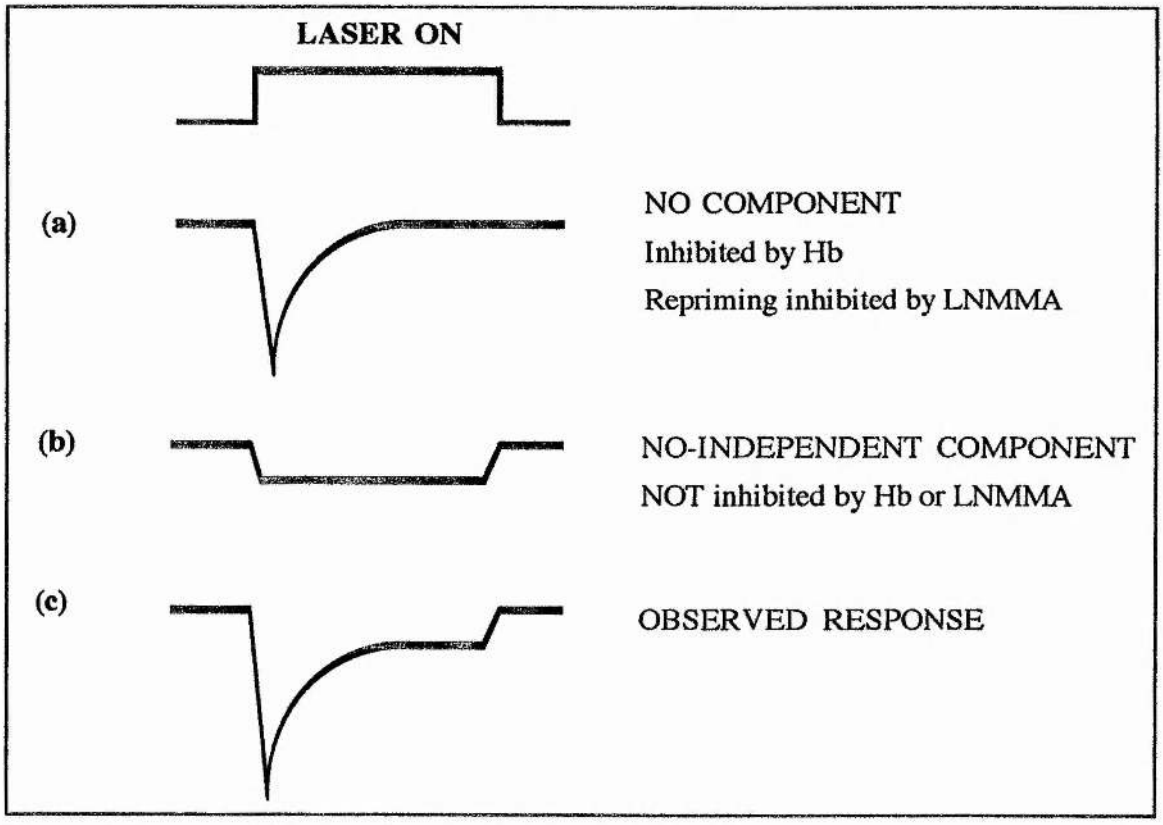


Figure 7.1. *A typical photorelaxant response profile and its theoretical components. The illumination period is indicated by the horizontal bar.*

The present investigation will show that the photorelaxant response (7.1 (c)) represents the sum of two components.

First, a component caused by the release of NO from a photolablile, molecular store within the tissue (7.1 (a)). The store can be depleted by exposing arteries to moderately high intensity laser light (6.4mW. cm^{-2}) for 4-6mins. Thus, the first photorelaxation in a series typically comprises a rapid vasodilation followed by vasoconstriction ***during the period of illumination.*** The amplitude of subsequent photorelaxations depends upon the time interval between successive exposures. The underlying process involved will hereafter be described as 'repriming'.

Second, a component that is NO-independent, accounting for the small responses seen in the presence of Hb and LNMMA (7.1 (b)). This study will focus on the NO-dependent component but a possible mechanism for the NO-independent component will be discussed later.

7.2 MATERIALS AND METHODS

A. ANIMALS

Adult male Wistar rats (270- 327g) were killed by cervical dislocation, their tails removed and a length of artery (8-12 mm) cannulated ***at both ends*** and dissected free.

B. APPARATUS

Modifications were made to the apparatus shown in figure 2.1 to facilitate laser irradiation of vessels. Figure 7.2 shows how light from an argon ion laser (L; Spectra Physics Ltd., type 168-09) was passed through an expanding lens and reflected onto the preparation by means of a 45° front-silvered mirror (M) to produce a uniform, 2 cm diameter beam at the preparation. The vessel (V) was cannulated at both ends for these experiments in order to hold it within the beam. Each irradiation was of a standard intensity (6.4mW. cm^{-2}), wavelength (514.5nm) and duration (6 mins). Pressure was monitored by a pressure transducer (P) as described in section 2.2.

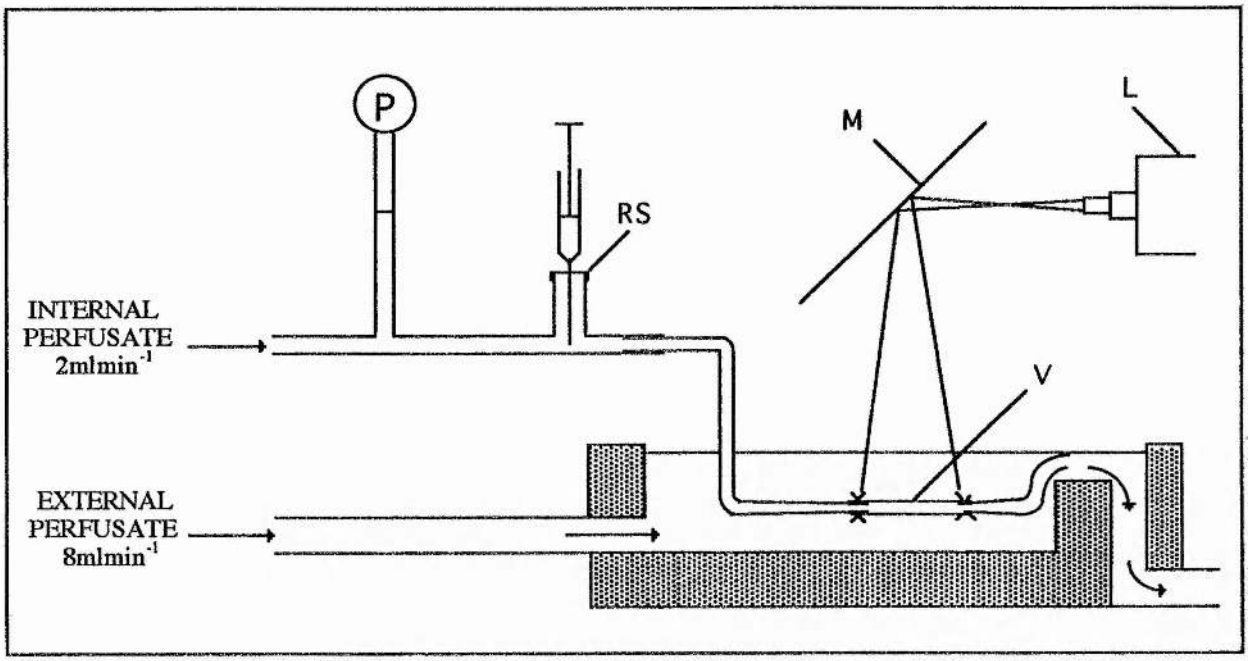


Figure 7.2. *Apparatus used to study the effects of laser light on isolated lengths of rat tail artery. See text for full details and explanation of lettering.*

C. EXPERIMENTAL PROTOCOL

Vessels were precontracted with PE to produce agonist-induced perfusion pressures of 101.7 +/- 4.4 mmHg ([PE] = 6.7+/- 0.5 μM; n=29). Once pre-contracted, vessels were irradiated as described above for 6 mins. The first exposure produces a transient vasodilator response which is referrred to as the depletion response (DR). The initial drop in pressure is followed by spontaneous recovery during the period of illumination. The peak amplitude of the first in a series of photorelaxations is variable (7.3 - 46.6%), and the perfusion pressure returns to a level slightly below the pre-illumination pressure within 4-6 min of commencing laser treatment. Full recovery is seen when illumination ceases.

An identical period of laser irradiation, delivered shortly after the first, succeeds only in producing a photorelaxant response equivalent in amplitude to the component which fails to recover ***during*** the previous exposure. This component of the response only recovers once laser illumination ceases. Importantly, the peak amplitude of subsequent photorelaxations depends upon the ***time interval (TI)*** between successive periods of laser irradiation. However, all photorelaxations elicited under the conditions described were of similar form to the initial response (DR).

Figure 7.3 shows pressure recordings made for the depletion exposure and after TIs of 10, 20, 40, 72 and 150 mins recovery in the dark (control). Also shown are corresponding recordings made in the presence of LNMMA or Hb.

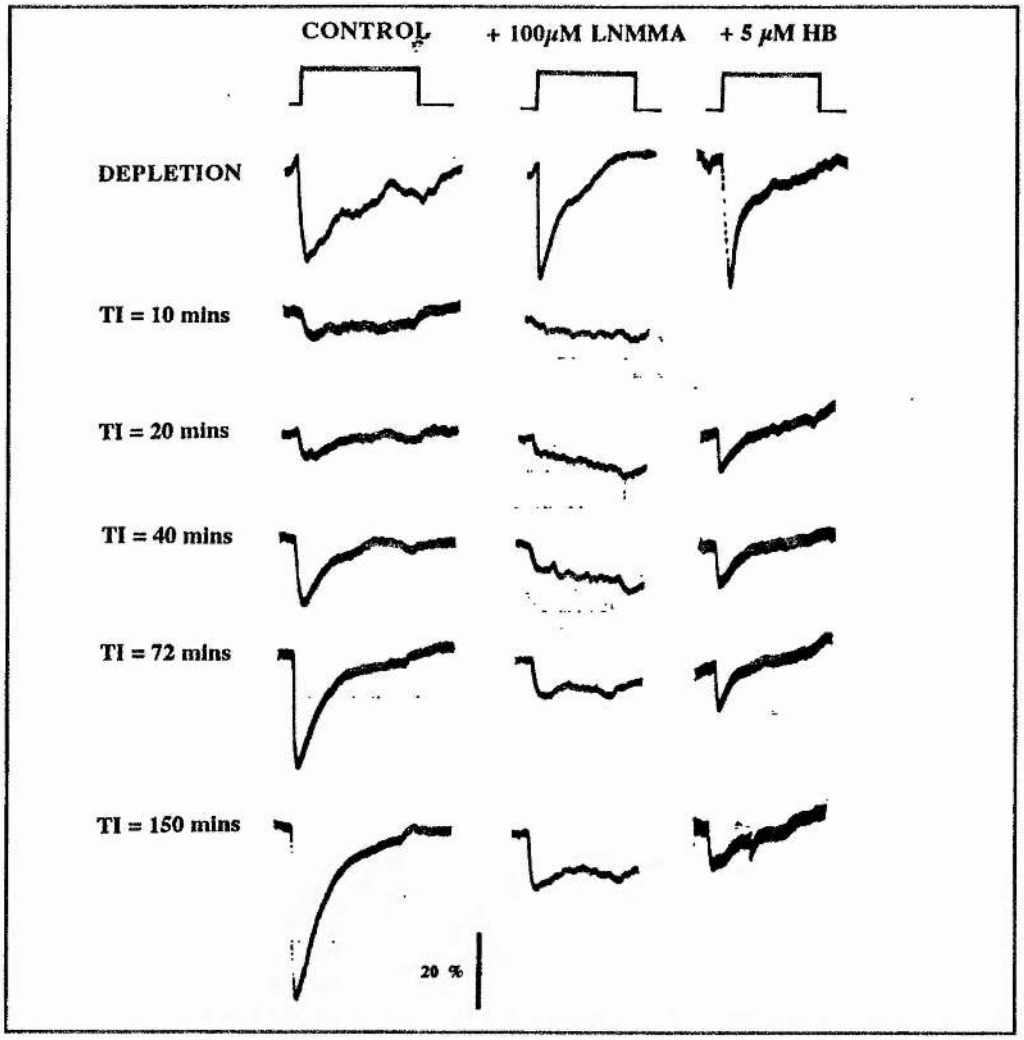


Figure 7.3. *Pressure recordings showing photorelaxations, following the time intervals indicated, in the absence of inhibitors (control), in the presence of 100 μM LNMMA and in the presence of 5 μM Hb. Periods of illumination are indicated by the horizontal bars. All responses have been scaled to the vertical scalebar (% amplitude). Experimental details: (a) Expt. No 930208: 286g male Wistar rat; 4 μM PE produced 116 mmHg active pressure. (b) Expt. No 930218: 317g male Wistar rat; 5 μM Hg produced 89 mmHg active pressure. (c) Expt. No 930401; 394g male Wistar rat; 4 μM PE produced 109 mmHg active pressure. Irradiations; 514.5 nm , 6.4 mW. cm^{-2}, 6 mins.*

Experiments were performed to establish the following:

(i) The timecourse of the repriming process. This was acheived by eliciting responses to identical irradiations after TIs of 10, 20, 40, 72 and 150 mins. Responses after TIs of 300 mins were carried out on seperate preparations.

(ii) The effect of internally perfused LNMMA (100 μM) on the repriming process. LNMMA treatment was initiated either immediately, or 20 mins after a depletion exposure. Some LNMMA treated vessels were subsequently perfused with 10^{-3}M L-arginine.

(iii) The effect of internally perfused 5 μM Hb on photorelaxations.

(iv) The effect of pretreatment with internally perfused 100 μM ethacrynic acid (EA) on photorelaxations.

(v) The effect of interposed 10μl injected doses of SNAP or NP on the amplitude of the TI = 20 mins photorelaxations.

D. ANALYSIS OF RESULTS

(i) The peak amplitudes were recorded for responses attained after each TI. In addition, response profiles were constructed by taking pressure recordings every 24 seconds throughout exposures.

(ii) The peak amplitude of responses attained after each TI in the presence of LNMMA were recorded. In order to compensate for differing sensitivities in preparations, the TI = 20 mins response obtained before LNMMA perfusion was compared to the mean TI = 20 mins response obtained in (i), and responses in the presence of LNMMA were scaled accordingly. In preparations where the introduction of LNMMA was delayed by 20mins, the amplitude of the first response obtained in the presence of the inhibitor was compared to the equivalent response before LNMMA perfusion, allowing 20 mins for LNMMA to take full effect (TI = 40 mins).

(iii) Results for Hb experiments were treated in the same way as for LNMMA described in (ii).

(iv) The peak amplitude of vasodilator responses to increasing doses of SNAP or NP injected immediately after a DR were measured. The amplitude of photorelaxations obtained at TI = 20 mins were also measured and compared to the control TI = 20 mins response elicited before the first drug injection. Log-dose response curves showing both the vasodilator responses and the drug-induced enhancement of photorelaxations were plotted.

7.3 RESULTS

(i) Repriming of Photorelaxation.

Figure 7.4 shows the averaged photorelaxations following various TIs, as shown. The peak amplitude increases with increasing TI, with exposures after TIs of 300 mins causing the perfusion pressure to fall by ca. 35%. Figure 7.4 also illustrates that responses plateau at a level 3-8% below the pre-illumination pressure until irradiation ceases.

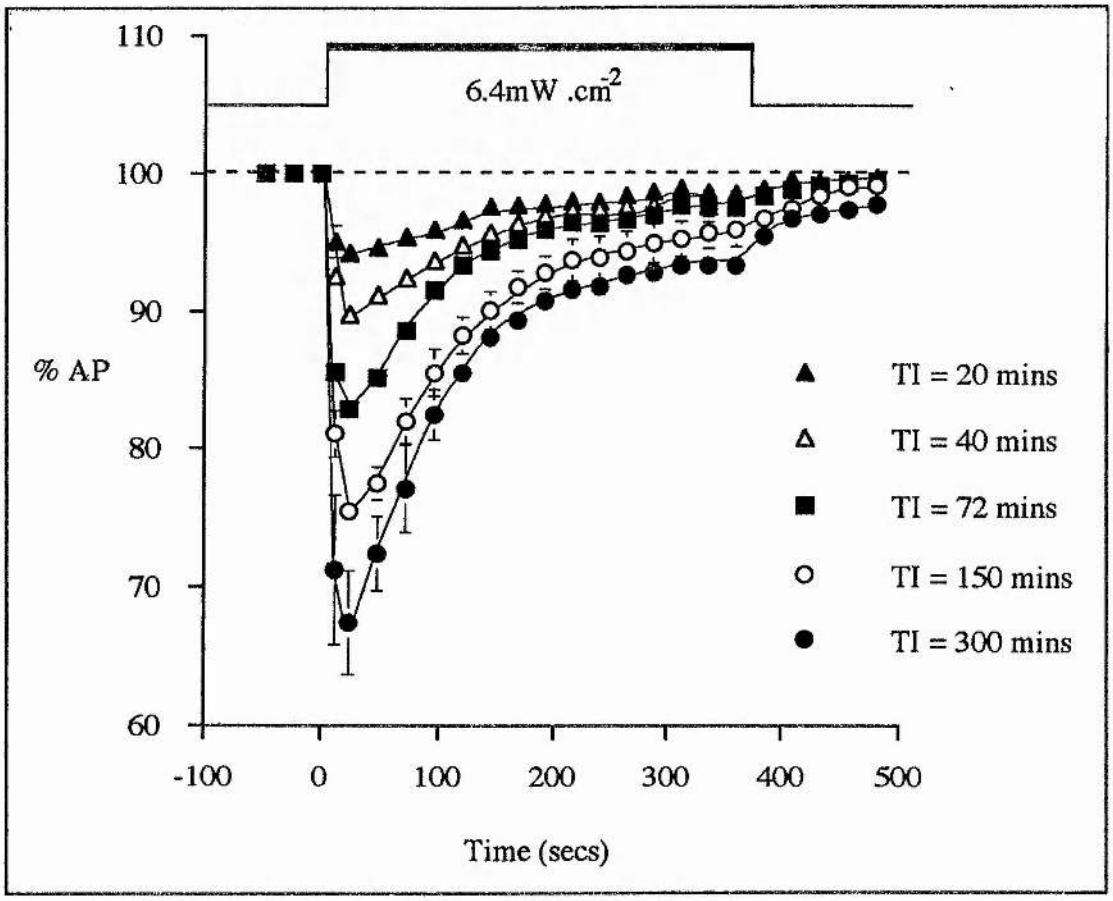


Figure 7.4. *Meaned response profiles recorded after the TIs shown (n = 5 for TI = 20, 40, 72 and 150 mins; n = 3 for TI = 300 mins).*

A plot of peak amplitude against TI is shown in figure 7.7 (filled circles), from which, $TI_{1/2}$ for repriming is estimated as 104 mins.

(ii) Effect of LNMMA on Repriming.

Figure 7.7 (open squares) shows the effect on the repriming process of perfusing vessels with 100 μM LNMMA immediately following a DR; responses are severely inhibited with photorelaxations of <5% being elicited, even after TI=150 mins. Pressure profiles closely resemble those of responses elicited immediately after DRs in untreated vessels. The amplitude of LNMMA-inhibited responses is also comparable to the element of photorelaxant responses that fails to recover during the period of irradiation noted in (i) above. This suggests that control response profiles shown in figure 7.4 comprise an LNMMA resistant vasodilator element (NO-independent) superimposed on a vasodilator element which is dependent on functional NO synthase.

In vessels where the introduction of 100 μM LNMMA was delayed until 20 mins had elapsed following a DR, responses elicited at TI = 40 mins were significantly greater in amplitude than the equivalent TI = 40 mins control responses (figures 7.5 and 7.6).

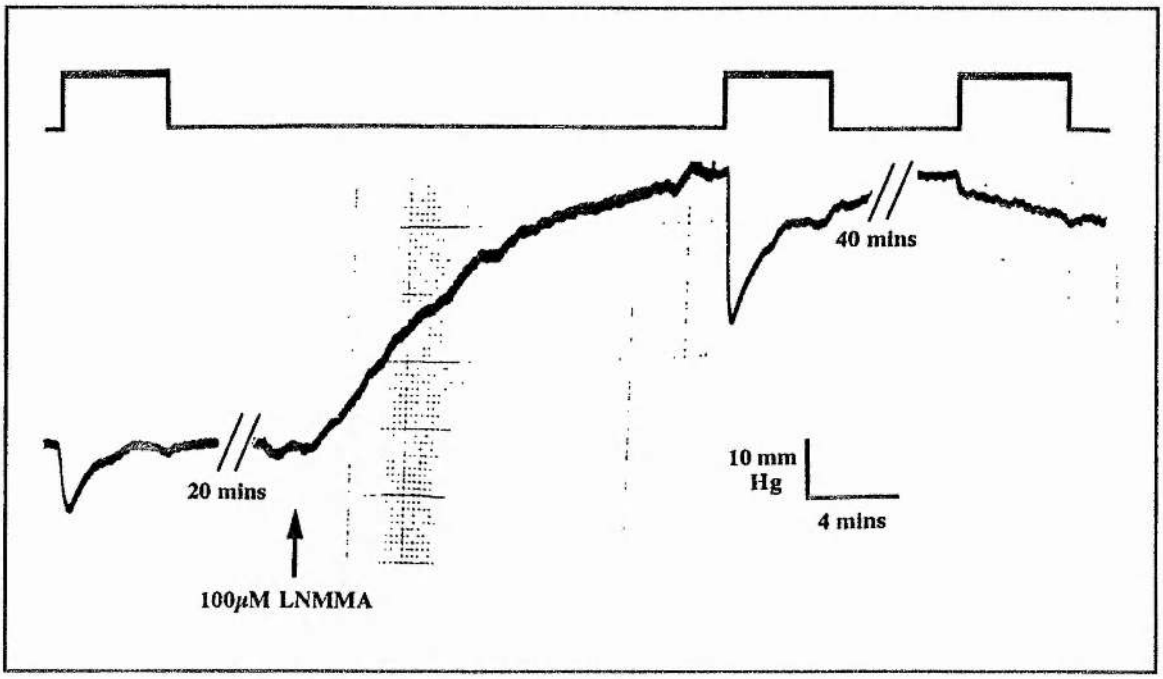


Figure 7.5. Pressure recording showing the effect of delayed infusion of 100 μM LNMMA on the amplitude of the first and second TI = 40 mins responses in the presence of the inhibitor. Expt. No 930218: 317g male Wistar rat; 5 μM Hg produced 89 mmHg active pressure. Irradiations; 514.5 nm . 6.4mW. cm^{-2}, 6 mins.

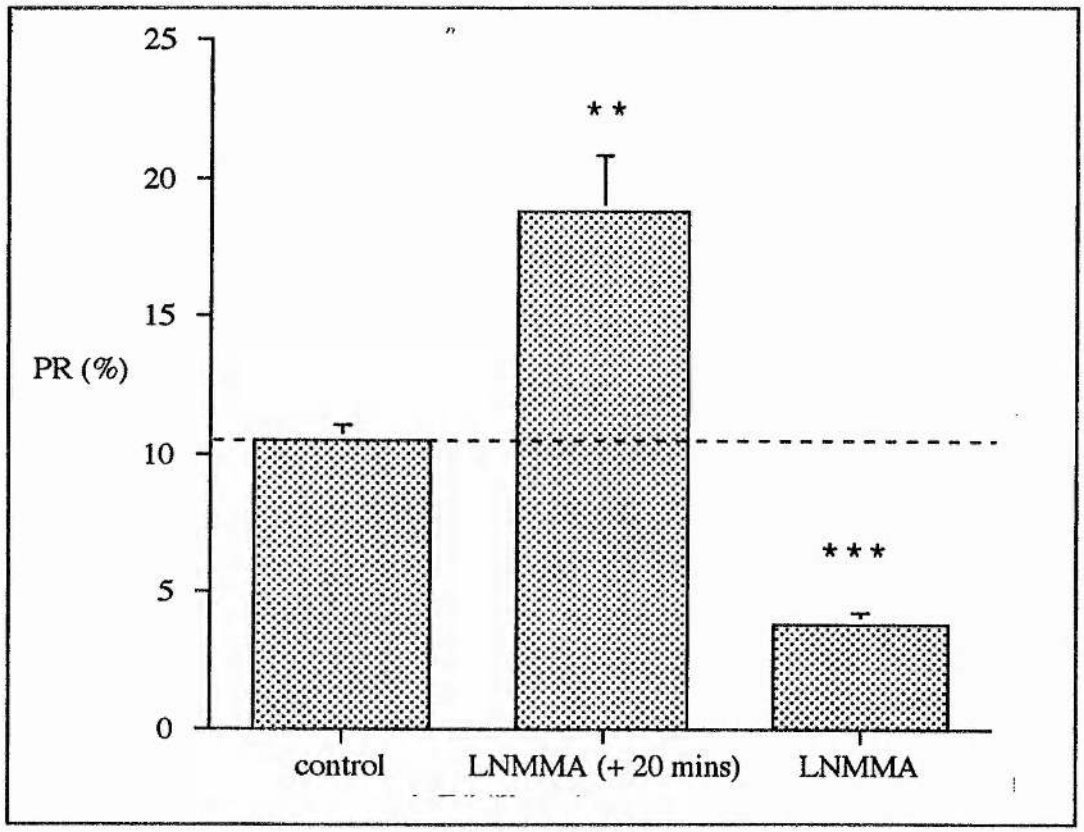


Figure 7.6. *Histogram showing control response for TI = 40 mins (n = 6) compared to the equivalent response in the presence of LNMMA (LNMMA; n = 4) and that where LNMMA introduction was delayed by 20 mins (LNMMA + 20 mins; n = 3). ** signifies a significant difference at the P = 0.01 confidence level and *** at the P = 0.001 confidence level.*

The result is more surprising since NO synthase was only functioning for 20 of the 40 mins following the DR. Responses obtained at TIs after this enhanced response in the presence of LNMMA were severely inhibited as detailed above (figures 7.4 and 7.5).

Figure 7.7 (open circles) shows photorelaxations obtained in the presence of 1 mM L-arginine following washout of LNMMA. Clearly, the inhibitory effect of LNMMA on the repriming process is reversible. Responses obtained in L-arginine are not significantly larger than control responses, suggesting that L-arginine levels in the tissue are sufficient to sustain normal basal release of NO under the conditions of this experiment.

(iii) Effect of Hb on photorelaxation.

Figure 7.7 (filled triangles) shows the inhibitory effect of 5 μM Hb, introduced into the internal perfusate immediately after a DR, on photorelaxations elicited after increasing TIs. The inhibition is comparable to that caused by LNMMA. Again, the results are consistant with the existence of a NO-independent vasodilator component, superimposed on a transient vasodilator component which is due to NO activation of guanylate cyclase.

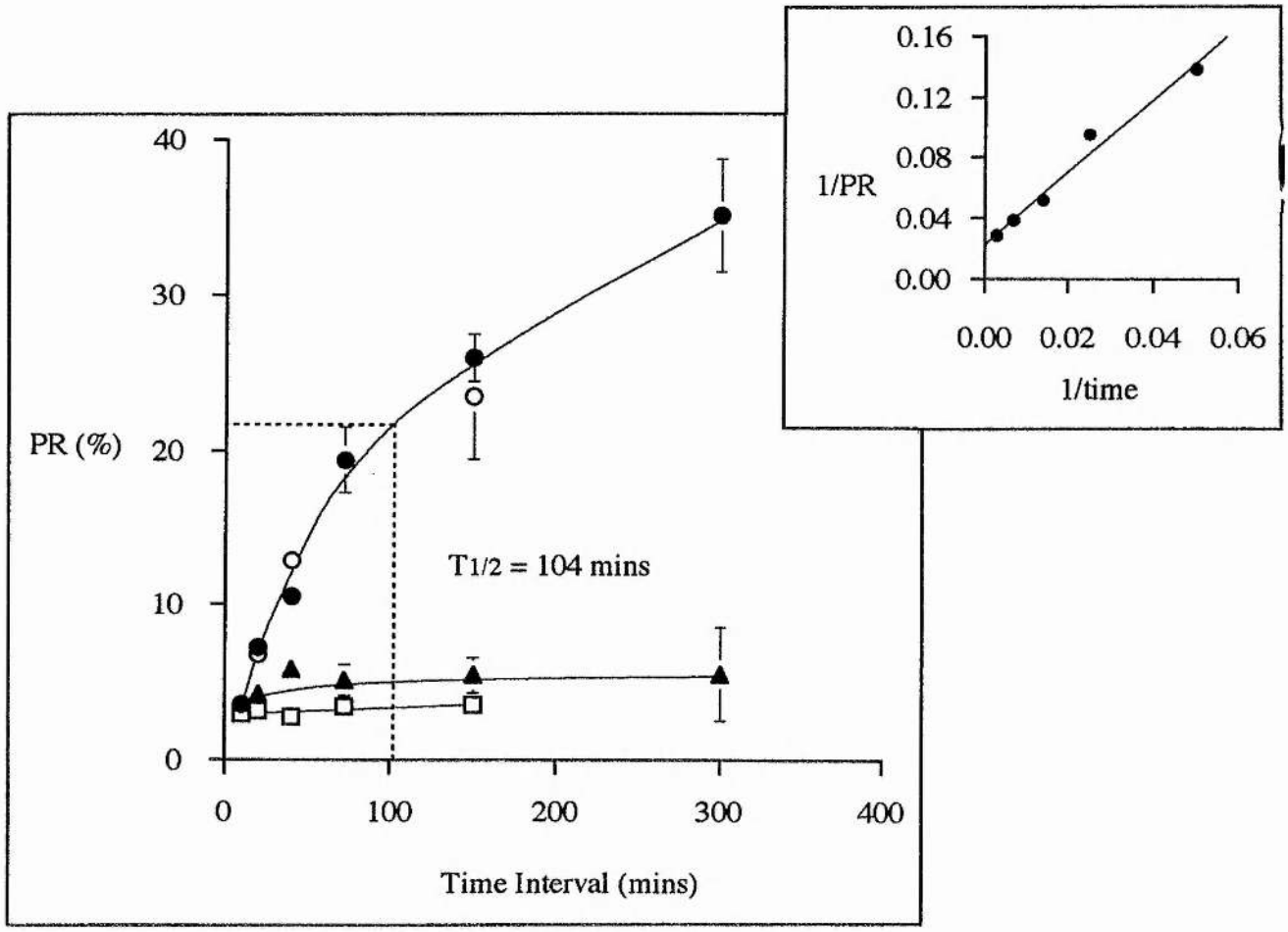


Figure 7.7. *Effect of 100 μM LNMMA (open squares; n =3 - 5) or 5 μM Hb (filled triangles; n = 3 - 4) on repriming of photorelaxation in the absence of inhibitors (filled circles; n = 3 - 5). The effect of LNMMA can be fully reversed by perfusion with 1 mM L-arginine (open circles; n = 3). A double reciprocal plot of response amplitude of control responses against time (inset) shows the estimated maximal response at TI = infinity to be ca. 45% (1/ y axis intercept).*

The results illustrated in figure 7.7 show that in the presence of either LNMMA or Hb, response amplitude is severely inhibited. However, a small component (3-6%) of the response is resistant to treatment with either inhibitor and is therefore NO-independent.

(iv) Effect of EA on photorelaxation

Perfusion of preparations with EA (100μM) had little effect on perfusion pressure. EA is a recognised inhibitor of guanylate cyclase and as such would be expected to cause an increase in pressure similar to that seen with MB. Some preparations actually showed a slow but detectable **drop** in pressure (< 15%) during EA perfusion, which failed to recover on washout.

Figure 7.8 is a histogram illustrating the inhibitory effect of EA treatment on photorelaxation after increasing TIs. Photorelaxations are shown to be eliminated in all but the longest TI (150 mins), where a 3% response was recorded, as compared to a control response of ca. 26%. This level of inhibition is more severe than either that of LNMMA or Hb. Significantly, the NO-independent component of the response, which was still present in LNMMA or Hb treated vessels, was inhibited by EA treatment.

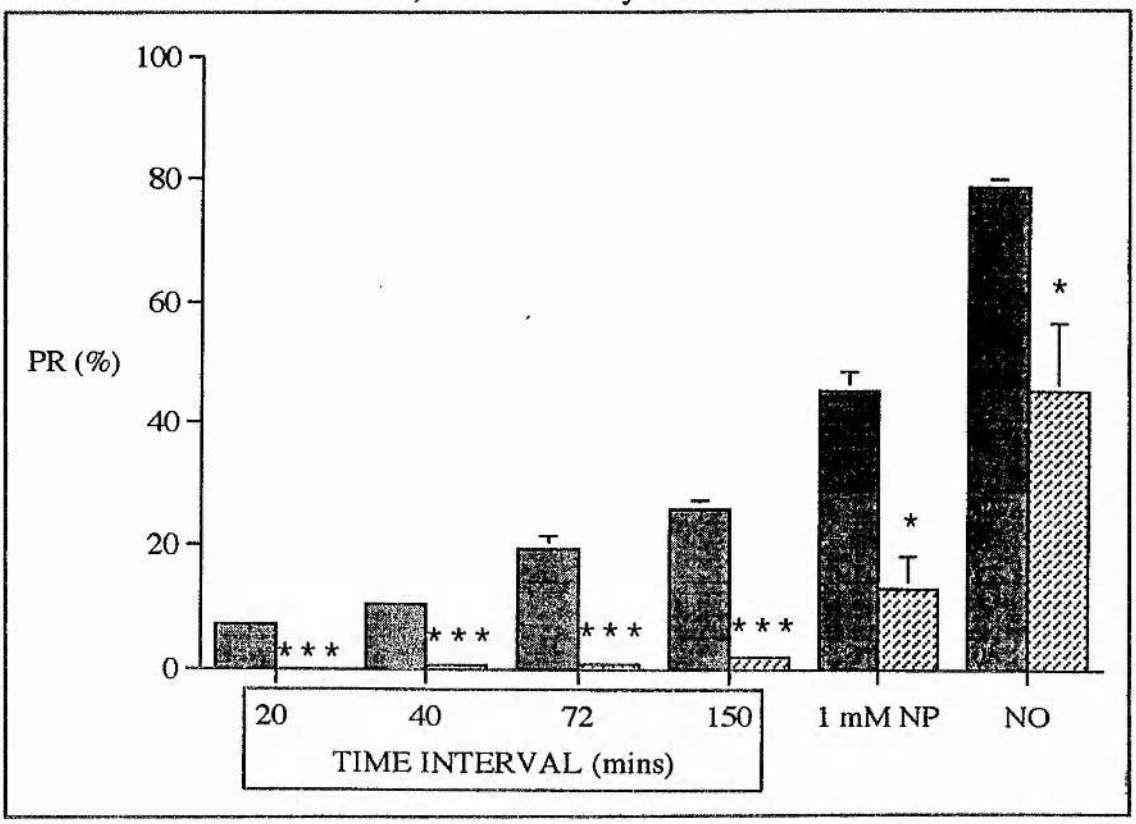


Figure 7.8. *Histogram illustrating the effect of EA pretreatment on photorelaxation after increasing TIs and on vasodilator responses due to 10µl injections of 1mM NP (n = 3) or 1.8mM NO (n = 5). *** indicates a significant difference at the P = 0.001 confidence level. * indicates a significant difference at the P = 0.05 confidence level.*

Figure 7.8 also shows that responses to 10^{-3}M NP were severely attenuated by EA, reinforcing the hypothesis that tissue thiol groups are a compulsory element in degradation of NP in the dark and/or transfer of the resultant NO to guanylate cyclase (chapter 1).

In order to ascertain the viability of guanylate cyclase after EA treatment (in the knowledge that thiol groups are involved in regulation of the enzyme), saturated solutions of authentic NO were prepared in sealed vials. The precise concentration of NO present remains unknown and is the subject of great debate (estimates varying from 1.8×10^{-3} M (Feelisch, 1992) - $<10^{-3}$ M (Martin, personal communication). In this case, a sufficiently high concentration of NO was present in microinjections to give maximal responses before EA treatment. Evidently, the response is diminished, suggesting that guanylate cyclase may be less responsive to NO stimulation following EA treatment. This is especially true since the saturated solution of NO used is supermaximal.

(v) Effect of bolus injections of NO, SNAP and NP on reprimimg.

10μl bolus injections of SNAP and NP (10^{-7} - 10^{-2}M) into the internal perfusate produce transient vasodilator responses which fully recover (within ca. 5 mins for SNAP and ca. 10 mins for NP - see chapter 3). Similar injections of authentic NO (10^{-8} - 10^{-3}M)were found to show comparable response profiles to those of SNAP but with a faster recovery rate (full recovery within ca. 3 mins). These results suggest that all three compounds clear the lumen rapidly and do not accumulate in the tissue, as was found with RBS and CUB (chapter 4).

Figure 7.9 shows pressure recordings obtained from experiments where 10μl bolus injections of SNAP (trace A) were interposed betwen TI = 20 mins responses (trace B). Responses to all SNAP doses show full recovery before the onset of the TI = 20 mins photorelaxations. Clearly, however, photorelaxation is enhanced in a dose-dependent manner by prior injection of SNAP. Similar results were obtained following NO injections. However, only the highest dose of NP used (10^{-2} M) caused a significant enhancement of the subsequent photorelaxation.

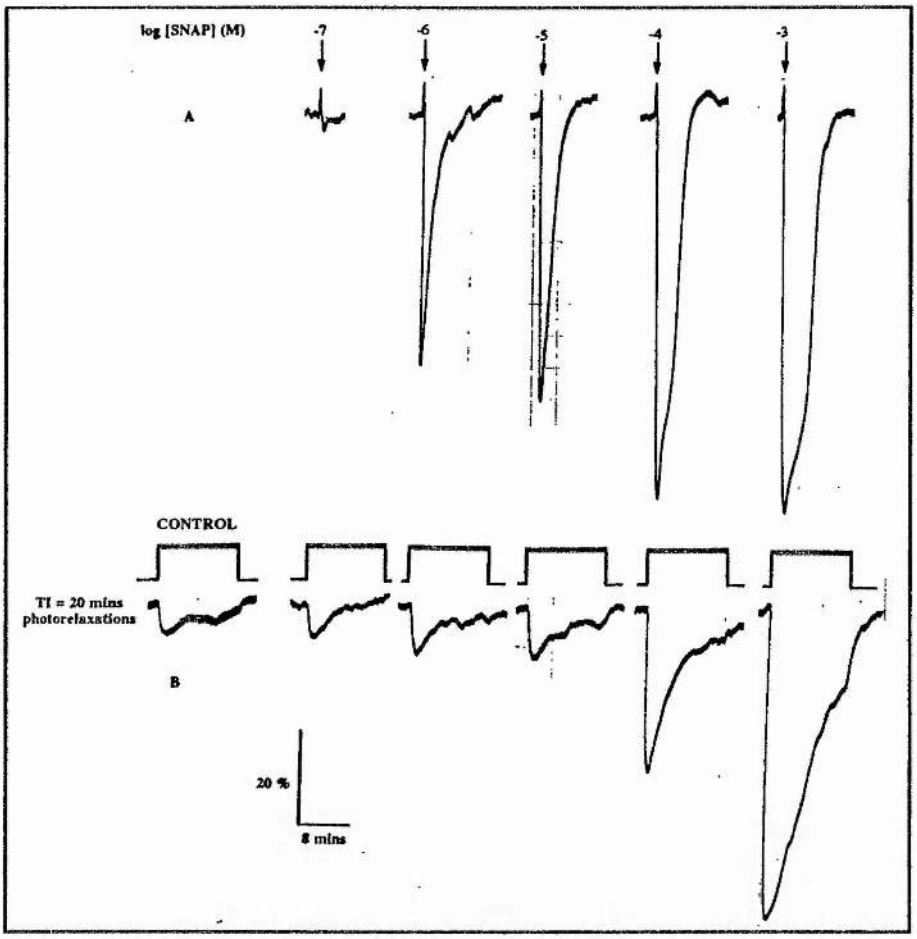


Figure 7.9. *Pressure recordings obtained for vasodilator responses to 10μl injections of SNAP (trace A). Photorelaxant responses obtained after TI = 20 mins following injections are shown in trace B. Expt. Nº 930226. 297g male Wistar rat; 4μM PE produced 137 mmHg active pressure. Exposures 514.5nm laser light (6.4mW. cm^{-2}): injections 10 μl.*

The effect of 10μl bolus injections of (a) NO, (b) SNAP or (c) NP, administered immediately following a depletion response, on photorelaxations elicited at TI = 20mins is shown in figures 7.10. The plot shows vasodilator responses to NO, SNAP and NP plotted as a function of log dose and the ***enhanced*** photorelaxation responses (= potentiated responses - control response) produced at TI = 20 mins after each dose.

Figure 7.10 shows the results obtained for (a) authentic NO, (b) SNAP and (c) NP. Vasodilator response amplitude (VR (%)) is indicated by filled symbols whilst the ***enhanced*** photorelaxation following injection is indicated by open symbols.

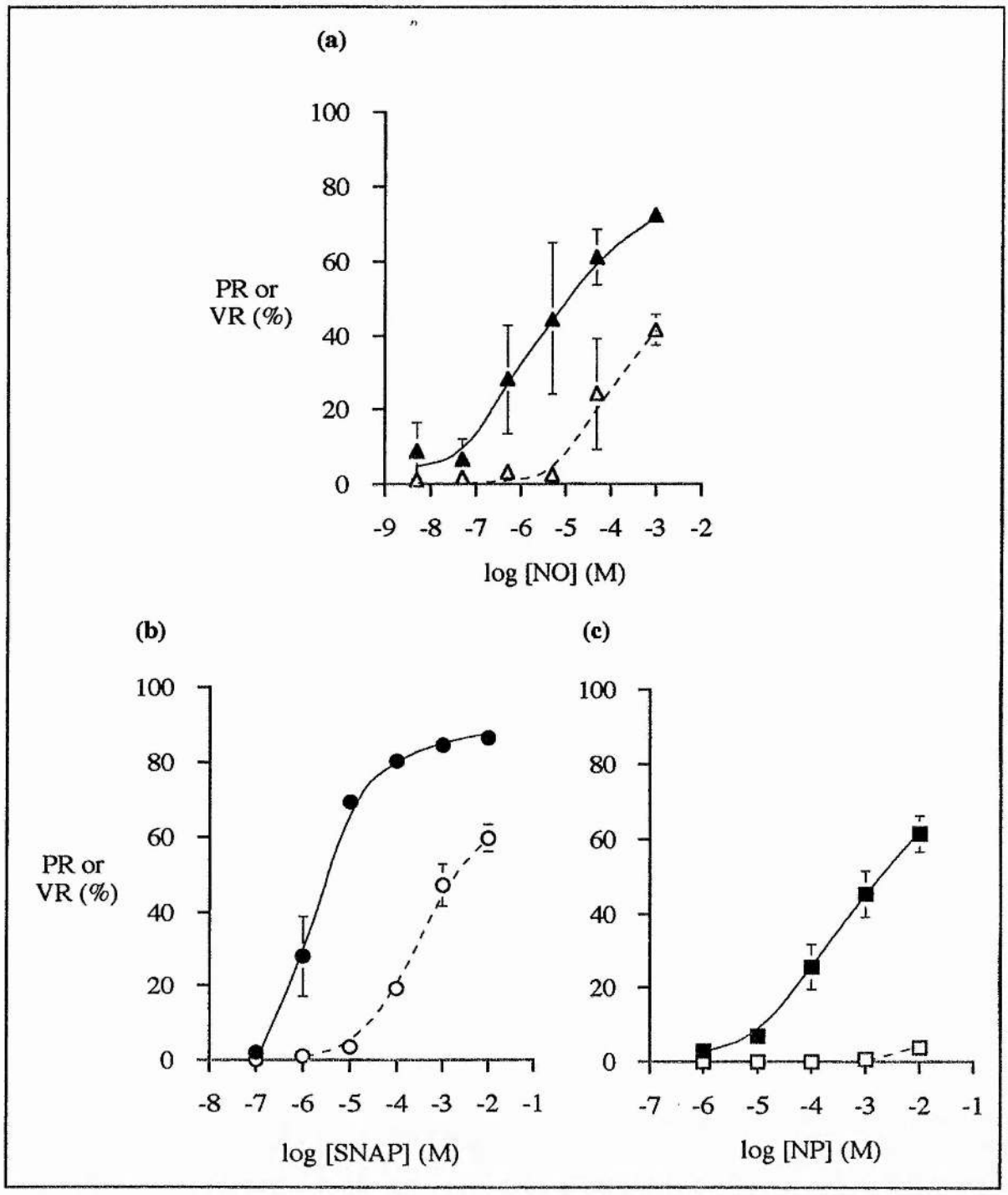


Figure 7.10. *vasodilator responses (VR (%)) to bolus injections of (a) NO (filled triangles; n = 3), (b) SNAP (filled circles; n = 5) or (c) NP (filled squares; n = 5) and subsequent photorelaxant resposes (PR (%)) evoked after TI = 20 mins (open triangles after NO, open circles after SNAP and open squares after NP).*

Figure 7.10 (a) shows the results obtained with authentic NO. The precise concentrations of NO dilutions injected are unknown. Theoretically, the maximum dose administered, a

saturated solution of NO, should be 1.8 mM at room temperature (Feelisch, 1992). In reality, the concentration may be up to a factor of 10 x less than this, depending on the extent to which it reacts with O_2 in solution (Martin, personal communication). The maximum concentration of a saturated solution of NO estimated by Martin *et al* is ca. $9x10^{-4}$M. The doses plotted in figure 7.10 (a), therefore, are the maximum likely values. In reality, the log dose-response curve may fall to the left of that shown.

What is certain, however, is that a bolus injection of NO administered prior to a TI = 20 min photorelaxation enhances the latter in a dose-dependent manner. Maximum enhancement caused by the saturated NO solution (ca. 72% vasodilation) was ca. 42% (total photorelaxation = 49 %). As discussed earlier, the rapid recovery of responses to bolus injections of NO suggests that it clears the lumen long before the subsequent photorelaxation is evoked. In addition, NO that enters the tissue as the bolus passes through the vessel is unlikely to exist as a free entity for more than a few seconds since the 'biological half-life' of NO is estimated to be between 3 and 30 secs (Griffith *et al*,1984 & Cocks *et al*,1985).

As shown in figure 7.9, responses to SNAP also recovered fully before the onset of the TI = 20 mins photorelaxations. Figure 7.10 (b) shows a steep log-dose response curve to injections of SNAP, reaching a maximum of ca. 85% relaxation at a concentration of 10^{-2} M. Subsequent TI = 20 mins responses show significant enhancement after injections of $<10^{-5}$ M. The degree of enhancement is dose dependent, reaching a maximum of ca. 60% (total photorelaxation = 67%).

Vasodilator responses to NP likewise recovered fully before irradiation began, although at a slower rate than the two drugs described previously. Figure 7.10 (c) shows that responses to doses of NP were smaller than those for corresponding doses of SNAP as expected from earlier experiments (see chapter 1). In accordance with this, only the highest dose of NP (10^{-2} M) significantly enhanced subsequent TI = 20 mins photorelaxations (enhanced by 5 %; total photorelaxation = 12%).

The results suggest that a threshold concentration of each compound is required to enhance the peak amplitude of the following photorelaxant response. Figure 7.11 is a plot of vasodilator response amplitude against enhanced photorelaxation amplitude for all three compounds.

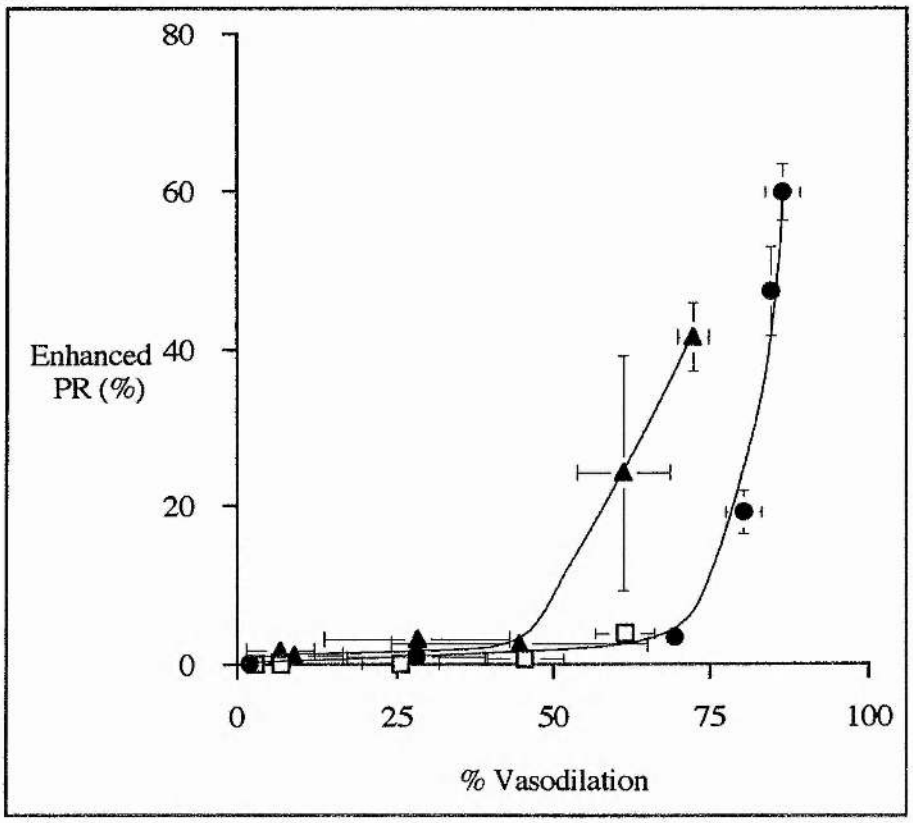


Figure 7.11. *Plot of vasodilator response amplitude (VR %) against enhanced photorelaxation amplitude (PR %) for NO (filled triangles), SNAP (filled circles) and NP (open squares).*

This shows that the threshold vasodilator response amplitude for significant photorelaxation enhancement is lower for NO microinjections (ca. 45%) than for SNAP or NP (ca. 60%). It would appear, therefore, that authentic NO is more efficient at accelerating repriming than SNAP or NP where photorelaxations must be preceded by a near-maximal vasodilator response to be enhanced.

Control experiments showed that neither perfusion with 10^{-4}M 8-bromo cGMP for 1hr nor injection of up to 20μl, 10^{-2}M isoprenaline (a cAMP-mediated β- agonist) were capable of

enhancing subsequent photorelaxant responses despite giving substantial vasodilator responses (67.9% for 8-bromo cGMP and 44.2% for isoprenaline). In this knowledge, it is apparent that vasodilation *per se* is not responsible for the phenomenon. Rather, it is essential to deliver a high dose of authentic or drug-derived NO to acheive photorelaxation enhancement.

Figure 7.12 shows pressure recordings made by a vasodilator responses to a 10μl, 10^{-4} M bolus injection of SNAP in the presence of 100 μM LNMMA followed by a TI = 20min response. Also shown are the control TI = 20 min response elicited before the introduction of LNMMA (note that irradiation continues until after the time when LNMMA first reaches the tissue in order to prevent repriming by endothelium-derived NO) and the first TI =20min photorelaxation in the presence of LNMMA.

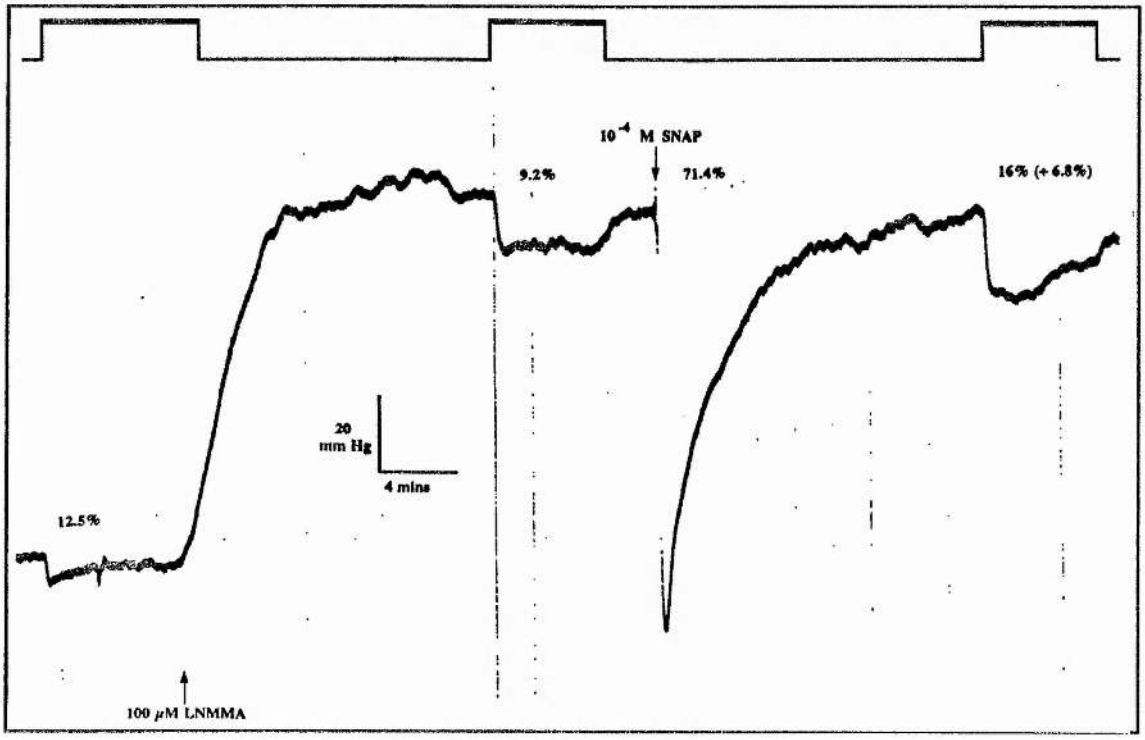


Figure 7.12. *Pressure recordings showing the effect of LNMMA inhibition of NO synthase on SNAP-induced enhancement of photorelaxation. Expt. Nº 930305: 249g male Wistar rat; 8μM PE produced 74 mmHg active pressure. All irradiations 514.5nm; 6.4mW.cm^{-2}. SNAP injection volume - 10μl.*

The photorelaxation evoked following the SNAP injection in the presence of LNMMA was 6.8% enhanced as compared to the control response evoked before the injection. Although this is not as large an effect as in the absence of LNMMA (ca. 19%), the 10^{-4} M SNAP injection produced a surprisingly small vasodilator response of 71.4% (c.f. 80.3 %). Typically, responses to NO donor drugs are enhanced in the presence of LNMMA. The importance of this experiment is that it shows SNAP-induced accelerated repriming of photorelaxation is independent of NO synthase activity.

7.4 DISCUSSION

Repriming of photorelaxation

The experiments described suggest that photorelaxation is ultimately due to NO stimulation of guanylate cyclase, and consequently is abolished by Hb (Figure 7.6 and 7.7). A minor component of light-induced responses appears to be NO-independent but may involve direct stimulation of guanylate cyclase.

The results suggest that the source of NO involved is a finite molecular 'store' within the tissue. The store can be depleted by moderately high intensity light, resulting in responses which recover during the period of illumination (figures 7.3 and 7.4). Following depletion, the store is slowly reprimed by endothelium-derived NO and the peak amplitude of subsequent responses is determined by the duration in the dark over which repriming can occur (i.e. the time interval between exposures; figures 7.3, 7.4 and 7.7).

Stereospecific inhibition of endothelium-derived NO synthesis by LNMMA deprives the store of its source of NO and prevents repriming. If LNMMA treatment commences *immediately* following depletion of the store, responses are severely attenuated and their amplitude is independent of the time interval between successive exposures (figures 7.3 and 7.7). However, when inhibition is delayed by 20 mins following a depletion exposure, repriming can occur for the specified time. The first exposure obtained in the presence of LNMMA (TI = 40mins) is then significantly larger than the control response for TI = 40mins (figure 7.5 and 7.6). It seems likely that this reflects the supersensitivity towards NO shown by guanylate cyclase when deprived of it for a short length of time (Moncada *et al* , 1991). The first exposure in the presence of LNMMA in vessels treated in this way results in the release of the NO which has accumulated in the store during the interval between depletion and inhibition of NO synthesis. Subsequent exposures, however, do not elicit responses since no repriming can occur following depletion. Normal repriming is resumed following washout of LNMMA by 10^{-3}M L-arginine. Interestingly, photorelaxations were not significantly enhanced by L-arginine, suggesting that L-arginine

levels within endothelial cells are sufficient to maintain a supply to NO synthase for basal synthesis of NO, despite the constant flow of L-arginine-free Krebs past the endothelial cells.

Endothelium-denuded preparations have been shown to be capable of photorelaxation (Furchgott, 1984). This fact does not contradict the results reported here since the illumination conditions used by Furchgott *et al* were insufficient to deplete the NO store. In addition, the store would presumably be at least partly full before the endothelium was removed. Therefore, NO was still available for photorelaxation despite the removal of the endothelium. The results reported by Furchgott *et al* show that the store is located in cells other than endothelial cells and refute the suggestion that photorelaxation is solely due to activation of endothelial NO synthase as proposed by Deliconstantinos *et al* (1992). Further evidence suggesting that the source of NO is not endothelial NO synthase comes from the fact that preparations treated with LNMMA were still capable of photorelaxation when NO is provided from an exogenous source (e.g. SNAP or NO microinjections; figure 7.12).

The involvement of NO in the photorelaxant process is therefore supported by the following:

1. Inhibition by Hb.
2. Inhibition of repriming by LNMMA (fully reversible with L-arginine).
3. Development of supersensitivity to photorelaxation in guanylate cyclase when deprived of NO for a short period of time, as described by Moncada *et al* (1991) for NO donor drugs.

Accelerated repriming following microinjections of NO or SNAP.

Microinjections of NO or SNAP, delivered immediately following a DR, produced vasodilator responses which showed rapid recovery to the pre-injection level. This indicates that cGMP levels within the tissue have returned to their pre-injection level and suggests that none of the injected dose of either NO or SNAP remains within the tissue (chapter 4). Photorelaxations elicited at TI = 20 mins were unaffected by prior microinjections of low

concentrations (< ca. 5 x 10^{-6} M of NO; < ca. 10^{-5} M of SNAP). Doses above these 'thresholds' caused enhanced photorelaxations. The amplitude of vasodilator response necessary to cause significant enhancement of the subsequent photorelaxation was lower for authentic NO than for SNAP (figure 7.11). This suggests that authentic NO is more efficient at causing accelerated repriming than NO donor drugs.

Evidently, NO derived from suitably high doses of either SNAP or injected authentic NO accelerates the repriming process, leading to enhanced photorelaxant responses. The precise concentrations of NO injected were unknown, largely as a result of practical problems involved in the exclusion of oxygen from the solution. The true log dose-response curve may well lie significantly further to the left than that shown.

Experiments involving microinjections of NP were less conclusive than those using NO and SNAP. Only the highest dose used (10^{-2}M) was capable of enhancing the subsequent photorelaxation. It is possible that NP is unable to enhance photorelaxation in the manner seen with SNAP as a result of its unique NO delivery mechanism (chapter 3) but the most likely explanation for these results is that 10^{-2}M NP was the only dose used which released sufficient quantities of NO to accelerate repriming. NP has already been shown to be a less potent vasodilator than SNAP (chapter 3). Figure 7.11 indicates that vasodilation caused by SNAP or, it appears, NP, must exceed approximately 60% in order to cause enhancement of the subsequent photorelaxation. Ideally, 10^{-1}M NP should have been injected to test this hypothesis but vasodilator responses to this concentration of NP would have required more than 20 mins to recover fully, resulting in the photorelaxation starting on a diminshed baseline.

Nature of the NO store within vascular tissue

The intracellular environment is host to a wide variety of molecules capable of binding NO and releasing it on exposure to light. Experiments were carried out to investigate the possibility that tissue thiols (both free and protein-bound) are responsible for binding NO.

The results suggest that nitrosothiols may constitute at least part of the store since alkylation using EA abolishes photorelaxation. The results concerning EA were marred, however, by the fact that responses to microinjections of authentic NO were also inhibited, although to a lesser extent than photorelaxation. EA may well, therefore, inhibit guanylate cyclase by alkylating thiol groups on the enzyme which are involved in regulating its activity (Waldman & Murad, 1987). It may even be the case that these thiol groups constitute part of the store when nitrosylated.

Therapeutic implications of a photosensitive source of NO in blood vessels

Once the chemical identity of the NO store is established, it may be possible to elicit its release *in vivo* locally using fibre-optic probes to treat conditions such as vasospasm. Alternatively, a generalised release may be acheived by infusing compounds known to undergo cross-nitrosation and then decompose thermally under intracellular conditions (Butler *et al*, 1993). This would result in a more uniform release of small amounts of NO, resulting in a generalised vasodilator response.

CHAPTER 8

CONCLUSIONS AND

SCOPE FOR FURTHER STUDY

8.1. MECHANISMS INVOLVED IN VASODILATOR RESPONSES TO BOLUS INJECTIONS OF SNAP AND NP IN THE DARK.

Preliminary experiments performed using bolus injections of test doses of NP, RBS and CUB had shown that all were capable of causing vasodilation in isolated segments of rat tail artery in the dark(figure 3.1 (a)). Perfusion with the stereospecific NO synthase inhibitor, LNMMA did not attenuate responses (figure 3.1 (c)), whilst recognised inhibitors of the NO: guanylate cyclase pathway, MB and Hb (figure 3.1 (b) and (d)) abolished responses to RBS and CUB but did not affect those to NP. These results suggested that vasodilation caused by RBS and CUB was NO-mediated but did not involve activation of NO synthase, whilst the actions of NP were apparently independent of either NO or guanylate cyclase.

The results of chapter 3 suggested that different mechanisms were involved in vasodilation evoked by each of these compounds.

A. SNAP

Vasdilations induced by bolus injections of SNAP were rapid, both in onset and recovery, perfusion pressure returning to its pre-injection level within ca. 3 mins. The ED_{50} value for SNAP was estimated as 6μM. Responses to intermediate doses of SNAP were significantly inhibited by Hb (reflected in a 7-fold increase in ED_{50} value), though not abolished entirely. It is possible, therefore, that only part of SNAP-induced vasodilation is due to NO released in solution in the lumen. The Hb-resistant element of vasodilator responses to SNAP may either be due to an NO-independent mechanism, or due to 'cross-nitrosation' of SNAP with membrane-bound or intracellular thiols (Butler *et al*, 1993). In the latter case, free NO would not exist as a single entity outside the tissue and would be resistant to Hb inactivation. (see figure 8.1 for summary).

B. NP

Vasodilator responses evoked by bolus injections of NP, though rapid in onset, recovered at a much slower rate than SNAP-induced responses. Full recovery of responses produced by 10^{-2}M NP typically took in excess of 10 mins. NP proved to be a less potent vasodilator than SNAP, with an ED_{50} of 52μM. Hb inhibition, though statistically significant for intermediate doses, was less effective for responses to NP than those to SNAP (2-fold increase in ED_{50} value). This result was less surprising in view of the results reported by Flitney & Kennovin (1988) which showed that NP does not generate NO in the dark. Furthermore, these authors concluded that mammalian vascular smooth muscle, which responds to NP in the dark, has a mechanism for degrading NP which is not present in frog ventricle. Recently, a 4 KDa, membrane-bound protein has been isolated which releases NO from NP in mammalian tissue (Kowaluk *et al*, 1992). The apparent resistance of 'dark NP' could be explained by the fact that the NO generated may never experience the intra-luminal environment; indeed it may never exist in an unbound form throughout its transition from endothelial membrane to smooth muscle guanylate cyclase (see figure 8.1 for summary).

Another possible explanation for the actions of NP is that it is capable of causing vasodilation through several mechanisms, at least one of which is cGMP independent. This possibility is all the more plausible in the knowledge that responses to bolus injections of NP are not inhibited by MB, a recognised inhibitor of guanylate cyclase (figure 3.1(b)). Reports by Diamond & Chu, 1983, Otsuka *et al*, 1988 and Feelisch, 1991 show that NP behaves differently when compared to other NO donor drugs with respect to cGMP elevation. Apparently, NP-induced vasodilation increases disproportionately to increases in cGMP levels.

Despite its extensive experimental use as a classic 'nitrovasodilator', there is considerable evidence to suggest that NP does not act solely through release of NO. Instead, it appears that NO stripped from NP by membrane bound proteins is one of perhaps several mechanisms by which it exerts its vasodilatory effect.

8.2 UNUSUALLY PROTRACTED RESPONSES TO BOLUS INJECTIONS OF IRON-SULPHUR CLUSTER NITROSYLS ARE DUE TO THEIR ACCUMULATION IN ENDOTHELIAL CELLS AND SLOW RELEASE OF NO FROM WITHIN.

Vasodilator responses evoked by bolus injections of low concentrations (< Dt; 1-10μM for RBS and 20 -100μM for CUB) of RBS and CUB produced transient (T-type) responses similar in profile to those elicited by SNAP and NP. However, doses > Dt produced responses which failed to fully recover (figures 3.4, 4.2 and 4.3). Recovery became progressively less pronounced with increasing dose, failing altogether following injections of 5mM RBS for up to 6 hrs. Perfusion of vessels with Hb or MB during sustained responses caused the pressure to rise to a level well above the-pre-injection pressure (figure 4.4), indicating two points: first, pressure in sustained responses is suppressed by continuous release of NO; and second, endothelial NO was still functioning in vessels experiencing sustained responses, since the pressure rose to a level above the pre-injection value.

Circumstantial evidence suggesting that both RBS and CUB are likely to be absorbed by the tissue, particularly in view of their lipid solubility, was confirmed by histochemical and X-ray microprobe analysis of vessels perfused for a short period of time with either compound (chapter 5). Both techniques showed that cluster nitrosyls were taken up by the endothelium and microprobe analysis revealed that cells immediately adjacent to the endothelium were unaffected.

It appears, therefore, that sustained responses evoked by bolus injections of RBS and CUB are compound in nature, comprising a transient component, due to spontaneous release of NO within the lumen, superimposed on a prolonged response, due to slow release of NO from cluster nitrosyls trapped within the endothelium. A computer simulation (figure 4.5) permitted an analysis of the kinetics of the two processes to be made (figure 4.6). From these results, it was concluded that CUB degraded 3.5 x faster than RBS in physiological

solution, reflected in a larger transient component to the response. In calculating the relative spontaneous breakdown rates, it was assumed that both compounds release their full complement of NOs on decomposition, as predicted by their 'electron precise' structures (Butler *et al*, 1985). In contrast, RBS uptake into and/or breakdown within the tissue was apparently greater for RBS than for CUB, as reflected in a lower Dt for development of S-type responses and the greater loss of pressure during the plateau phase.

Evidently, once in the tissue, breakdown of cluster nitrosyls is slow, with responses to 5mM RBS and CUB showing little or no recovery for up to 6 hrs.

The complex nature of responses to cluster nitrosyls made direct comparisons with SNAP and NP impossible. It appeared, however, that neither was as potent a vasodilator as NP, in terms of peak amplitude evoked by bolus injections. The severe attenuation of responses to RBS and CUB (figure3.5) suggests that both compounds exert their effects almost entirely through NO generated by spontaneous decomposition of the compounds within the lumen of the vessel.

Therapeutic Potential

The unique properties of RBS in particular, increase its potential as clinically viable NO donor drug. If harnessed properly, long-term vasodilation from short-term infusion of RBS is theoretically possible. However, studies in this laboratory have shown that RBS is less effective *in vivo* than results reported here would suggest. It appears that the problem lies in the avid binding of RBS to albumin, illustrated in pilot experiments using sephadex columns. Binding to albumin *in vivo* would prevent accumulation in the endothelium and impair the resulting vasodilation. Preliminary microinjection experiments suggest that RBS and CUB will only produce T-type responses in the presence of bovine serum albumin

8.3 RBS AND CUB ARE PHOTOSENSITIVE

Exposure to ultraviolet and visible light causes pre-contracted vessels to relax (Furchgott *et al*, 1961; Furchgott, 1971; Furchgott, 1984; Matsunaga & Furchgott, 1989 - see section 1.11). Photorelaxation is though to be due to release of NO from an unknown source.

Exposure of vessels to low intensity, 514.5nm laser light for 1 min evokes small amplitude responses which fully recover once irradiation ceases. Similar exposures elicited during sustained responses due to bolus injections of moderate doses of RBS or CUB (figure 6.2), caused enhancement of photorelaxation (in terms of amplitude and rate of drop in pressure) and reduced the latent period between irradiation and onset of response. Vessels continued to produce enhanced photorelaxations for up to 5 hrs following a single microinjection of RBS or CUB. Further enhancement was shown during SOD perfusion (figure 6.2 (c)), suggesting the involvement of NO in the process. It seemed probable that the phenomenon was due to light-induced acceleration of release of NO from clusters trapped wihin endothelial cells. The phenomenon therefore lends weight to the hypothesis that both cluster nitrosyls accumulate in the tissue and remain there long after the bolus injection has cleared the lumen.

Separate experiments showed that laser irradiation of low concentrations of RBS or CUB *en route* to the vessel (see figure 6.1) produced intensity- and wavelength-dependent vasodilator responses over and above the steady-state response seen in the dark (figures 6.4 and 6.5). Responses to exposed RBS were found to be inhibited by Hb and potentiated by SOD (figure 6.7), suggesting NO involvement. Increasing the delay time between perfusate containing RBS leaving the exposure tube and reaching the vessel in the absence of SOD had the effect of diminishing the amplitude of light-induced vasodilator responses, presumably as a result of NO inactivation between generation in the exposure tube and detection in the vessel (figures 6.8 (a)). Inclusion of SOD in the perfusate eliminated NO inactivation between generation and detection (figure 6.8(b)). Spectophotometric studies revealed that laser light caused intensity- and wavelength-dependent decomposition of RBS

(figure 6.9). From these results, concentrations of NO reaching the preparation, under different illumination conditions, were calculated and an ED_{50} value for NO estimated at ca. 15nM.

The results of these experiments showed that breakdown of both RBS and CUB is accelerated when exposed to light, resulting in an increased liberation of NO, and an increase in relaxation in the tissue. The effect occurs when cluster nitrosyls trapped within the tissue are exposed to light and when perfusate containing either compound is irradiated *en route* to the preparation.

RBS proved more sensitive to light than CUB and the resultant vasodilator response was dependent on the concentration of drug present. The shorter wavelength of light (457.9mW) proved more effective at causing accelerated breakdown of either RBS or CUB than 514.5nm laser light.

Results for CUB were complicated by the fact that DMSO, used as a solvent prior to dilution, was found to cause fragmentation of CUB, which subsequently re-assembled as RBS, the more stable of the two compounds (Glidewell and Glidewell, unpublished data).

Therapeutic potential

The photosensitivity of RBS and CUB adds an important dimension to their possible clinical use, especially in view of their ability to penetrate and remain in endothelial cells for considerable lengths of time. Local vasodilation could be induced with the use of light guides on vessels pretreated with RBS.

8.4 PHOTORELAXATION IS DUE TO THE RELEASE OF NO FROM A PHOTOLABILE STORE WITHIN VASCULAR SMOOTH MUSCLE.

As mentioned in 8.2 above, photorelaxation is a phenomenon which has been known about for over 30 years but whose mechanism is still poorly understood. Experimental evidence (Furchgott *et al*, 1961; Furchgott, 1971; Furchgott *et al*, 1984; Matsunaga *et al*, 1989) strongly suggests the involvement of NO from a source other than the endothelium. Recent papers, however, suggest that photorelaxation is due to stimulation of EDRF synthesis by endothelial NO synthase (Deliconstantinos *et al*, 1992). Karlsson *et al* (1984, 1985), on the other hand, believe that photorelaxation is due to direct stimulation of guanylate cyclase in the smooth muscle.

Experiments performed here showed that photorelaxation is due to the release of NO from a finite molecular store within the tissue (figure 8.1). Once depleted, repriming of the store could be acheived by NO derived from the endothelium (blocked by LNMMA; figures 7.3, 7.6 and 7.7) or by NO from an exogenous source (SNAP, authentic NO or to a lesser extent, NP; figure 7.10).

Photorelaxation is abolished by Hb but enhanced in vessels where guanylate cyclase has been deprived of basal release of NO for a short period of time, leading to its becoming supersensitised as described by Moncada *et al*, 1991 (figures 7.5 and 7.6). These findings together strongly suggest the involvement of NO in photorelaxation.

Photorelaxation is also abolished in vessels pre-treated with ethacrynic acid, an agent which irreversibly alkylates thiol groups (figure 7.8). This result suggests that nitrosothiols constitute at least part of the store. However, the fact that responses to authentic NO were inhibited, although to a lesser extent than photorelaxant responses, suggests that guanylate cyclase may be partially inhibited by EA, due to alkylation of essential regulatory thiol groups on the enzyme (Waldman & Murad, 1987).

Nevertheless, the results strongly suggest that release of NO from a photolabile molecular store within the tissue is responsible for photorelaxation. It is not considered likely that activation of NO synthase is responsible because vessels in which photorelaxation is inhibited by LNMMA are still capable of responding to light if an alternative source of NO is provided (SNAP; figure 7.12). Rather, it appears that the role of the endothelium is to provide NO for repriming of the store.

A detailed study involving comparison of the biological 'action spectrum' with light-induced decomposition of known nitrosothiols, nitrosyl-haem and iron-sulphur nitrosyl compounds would be necessary to establish the identity of the NO store involved. In addition, the possible involvement of glutathione (which accounts for ca. 90% of 'free' thiol concentration within cells) could be investigated using the glutathione synthesis inhibitor, buthionine sulphoximide.

Therapeutic potential

The value of determining the mechanism involved in photorelaxation lies in a better understanding of the physiology involved in the L-arginine:NO - guanylate cyclase pathway. However, the phenomenon has therapeutic potential, either with the use of light guides to cause local vasodilation or by exploiting cross-nitrosation reactions (Butler *et al*, 1993), whereby NO could be transferred from the store to a thiol which then degrades under physiological conditions, releasing NO in the proximity of guanylate cyclase. The feasibility of this as a therapeutic strategy has yet to be investigated.

8.5 SUMMARY

Figure 8.1 is a diagram summarising the vasodilator mechanism of SNAP, NP, RBS and CUB as suggested by the results obtained in this invesigation. In addition, the proposed mechanism involved in photorelaxation is illlustrated.

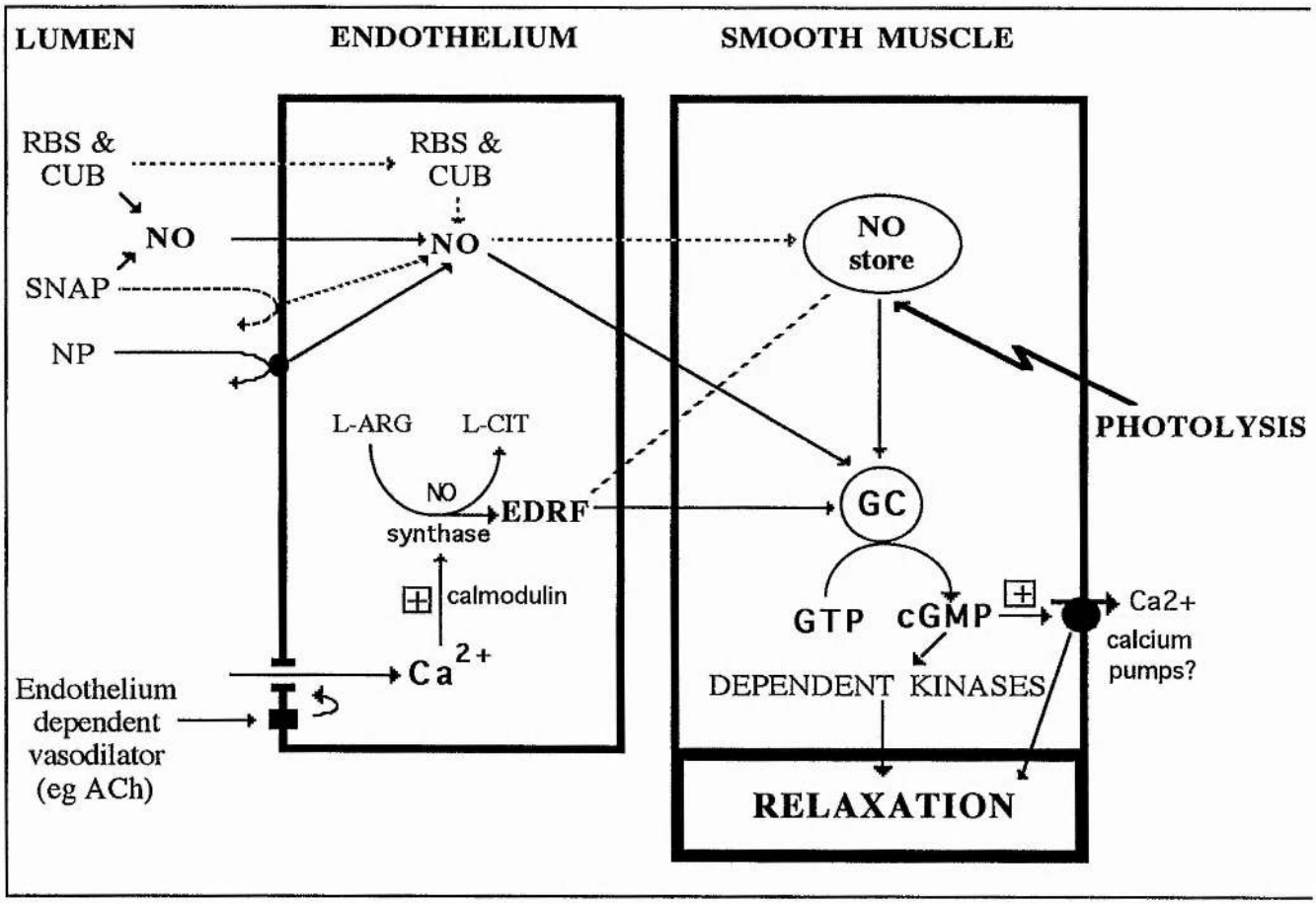


Figure 8.1. *Proposed vasodilator mechanisms of RBS, CUB, SNAP and NP as suggested by the results obtained in this investigation. The existance of a photolabile NO store within the smooth muscle is also illustrated.*

RBS and CUB release NO spontaneously either within the lumen or from molecules trapped within the endothelium. The process occurs in the dark but is accelerated by light.

SNAP releases some NO spontaneously in the lumen but also appears to be 'stripped' of NO by the tissue as it passes through the lumen. The mechanism is not yet known but may involve cross-nitrosation between SNAP and tissue thiols.

NP does not release NO spontaneously in the dark, rather it is removed by a small protein bound to the endothelial membrane.

NO, whether from an exogenous or an endogenous source, passes to the smooth muscle and stimulates guanylate cyclase and causes relaxation. However, some NO is utilised to replenish a molecular store within smooth muscle. The nature of the store is not yet known but nitrosothiols may constitute part of it. NO is released from the store on exposure to light, leading to stimulation of guanylate cyclase and photorelaxation.

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APPENDICES

APPENDICES

Mean increase in pressure caused by 15μM Hb (n=15): **55.92 +/-6.4**

Mean increase in pressure caused by 5μM Hb (n=8): **56.5 +/-4.9**

" " " " " " 100μM LNMMA (n=10): **57.1 +/-6.1**

APPENDIX TO GRAPH 3.3

Below are the peak amplitude (% Active pressure) of responses to 10μl injections of SNAP and NP.

SNAP

Expt. Nº	10^{-7} M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M
930112	98.1	95.4	56.2	43.7	25.6
930113/1	98.3	56.2	90.1	35.3	32.9
930114	97.1	92.4	35.4	20.3	10.5
930115	98.1	97.8	33.9	24.3	16.4
930113/2	90.1	87.7	44	35.3	33.9
MEAN	**96.7**	**83.03**	**49.02**	**24.28**	**18.64**
S.E.	**1.68**	**7.62**	**10.35**	**4.17**	**5.9**

SNAP In 15μM Hb

* = statistically significant from the above at P = 0.05 confidence level.

Expt. Nº	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M
930112		100	94.8	45.7
930114		79.1	66.2	33.3
930115	99	65.8	37.1	10.2
930113/2	100	96.7	68.1	41
MEAN	**99.5**	**85.4***	**55.1***	**32.6**
S.E.	**0.5**	**7.9**	**7.4**	**7.8**
P =	**0.335**	**0.044**	**0.019**	**0.349**

NP RESULTS

Expt. №	10^{-7} M	10^{-6}M	10^{-5}M	$2x10^{-5}$M	$5x10^{-5}$M	10^{-4}M	10^{-3}M	10^{-2}M
901017/1	97	98	92	84	70	62	28	31
901017/2	96	97	85	75	63	55	32	31
901022/1	89	88	76	75	62	58	44	35
901022/2	78	74	64	59	49	45	34	30
901023/1	99	98	90	83	71	67	47	38
901106	93	93	86	73	65	58	41	33
MEAN	**92**	**91**	**82**	**75**	**63**	**57.5**	**38.5**	**33**
S.E.	**3**	**4**	**4**	**4**	**3**	**3**	**3**	**1**

NP in 15μM Hb

* = significantly different from the above at P = 0.05 confidence level.

** = significantly different from the above at P = 0.01 confidence level

Expt. №	10^{-7} M	10^{-6}M	10^{-5}M	$2x10^{-5}$M	$5x10^{-5}$M	10^{-4}M	10^{-3}M	10^{-2}M
901022/1	98	99	92.5	88	73	60	37	23
901022/2	100	99	95	89	80	73	47	34
901023	100	100	94	91	82	70	50	39
901106	100	99	95	89	80	73	47	34
901016/2	100	100	97	91	89	89	68	46
901114	100	98	91	84	77	65	50	38
MEAN	**100**	**99**	**94***	**87****	**79****	**70***	**50***	**35**
S.E.	**0.3**	**0.3**	**1**	**2**	**2**	**4**	**4**	**3**
P =	**0.054**	**0.068**	**0.021**	**0.005**	**0.002**	**0.018**	**0.039**	**0.444**

APPENDIX TO FIGURE 3.5

RBS RESULTS

Expt. Nº	10^{-7} M	10^{-6}M	10^{-5}M	$2x10^{-5}$	$5x10^{-5}$	10^{-4}M	$2x10^{-4}$	$5x10^{-4}$	10^{-3}M	$5x10^{-3}$
910121/1	95	95	89	81	74	65	58	51	46	23
910123/2	98	96	94	96	88	78	71	54	44	15
910123/1	100	100	97	91	84	67	45	41	26	15
910130/2	100	100	100	100	100	90	89	82	53	38
910201/1	100	100	100	100	96	91	88	83	73	35
MEAN	**99**	**99**	**96**	**93**	**86**	**78**	**68**	**61**	**48**	**25**
S.E.	**1**	**1**	**2**	**4**	**4**	**5**	**8**	**8**	**8**	**5**

RBS in 15μM Hb

** = statistically different from the above at the P = 0.01 confidence level.

*** = statistically different from the above at the P = 0.005 confidence level.

Expt. Nº	10^{-3}M	$5x10^{-3}$M
910114/2	99	95
910115/1	100	92
910115/2	-	89
910117/1	93	52
910117/2	97	49
910118/2	97	59
MEAN	**97*** **	**72.6** **
S.E.	**1**	**8**
P =	**0.000**	**0.002**

CUB RESULTS

Expt. Nº	10^{-7} M	10^{-6}M	10^{-5}M	$3x10^{-5}$	$5x10^{-5}$	10^{-4}M	$2x10^{-4}$	$5x10^{-4}$	10^{-3}M	$5x10^{-3}$
901130/1	100	100	97		83	75	67		48	30
901203/1	97	99	95	92	88		70	58	43	29
901203/2	99	100	94	91	81	74	56	42	32	24
901207/1	97	96	90	75	61	58	51	46	42	40
901210/2	95	97	96	93	85	61	41	35	33	30
901210/1	100	98	94	90	80	72	47	33	32	25
MEAN	**98**	**98**	**94**	**88**	**80**	**68**	**55**	**43**	**38**	**30**
S.E.	**0.8**	**0.7**	**1**	**3**	**4**	**4**	**5**	**5**	**3**	**2**

CUB in 15μM Hb

*** = statistically different from the above at the P = 0.005 confidence level

Expt. Nº	10^{-3}M	$5x10^{-3}$M
901130	100	89
901210/1	100	96
901207	100	98
901203/1	100	98
901210/1	99	88
901203/2	100	96
mean	**100*** **	**94*** **
S.E.	**-**	**2**
P =	**0.000**	**0.000**

APPENDIX TO FIGURE 4.3 (a) & (b)

Below are the mean and standard error values for X/Y (% active pressure) and Z/Y (% active pressure) for 10μl injections of SNAP and NP.

	SNAP				NP			
log dose (M)	**X/Y**	**SE**	**Z/Y**	**SE**	**X/Y**	**SE**	**Z/Y**	**SE**
10^{-7}	96.7	1.7	97.1	2	92	3	100	
10^{-6}	83.0	7.6	95.6	1.43	91	4	101	
10^{-5}	49.02	10.4	96.2	2.45	82	4	100	1
$2x10^{-5}$					75	4	103	1.5
$5x10^{-5}$					63	3	103	2.5
10^{-4}	24.28	4.2	93.1	8.7	58	3	103	
10^{-3}	18.64	5.9	93.6	6.9	39	3	101	2
10^{-2}					33	1	107	4

Figure 4.3 (c) & (d)

Below are the mean and standard error values for X/Y (% active pressure) and Z/Y (% active pressure) for 10μl injections of RBS and CUB.

	RBS				CUB			
log dose (M)	**X/Y**	**SE**	**Z/Y**	**SE**	**X/Y**	**SE**	**Z/Y**	**SE**
10^{-7}	99	1	100	-	98	0.8	100	-
10^{-6}	99	1	99	0.5	98	0.7	100	-
10^{-5}	96	2	98	1	94	1	100	-
$2x10^{-5}$	93	4	97	1	88	3	100	-
$5x10^{-5}$	86	4	95	2	80	4	99	0.5
10^{-4}	78	5	92	4	68	4	98	2
$2x10^{-4}$	68	8	85	7	55	5	96	3
$5x10^{-4}$	61	8	77	9	43	5	89	7
10^{-3}	48	8	57	8	38	3	81	7
$5x10^{-3}$	25	5	25	4	30	2	45	6

APPENDIX TO FIGURE 4.6

Below are the values for V1 (transient vasodilator response) and V2 (sustained vasodilator response) as projected by computer simulation.

	RBS		CUB	
dose (M)	**V1(%)**	**V2 (%)**	**V1(%)**	**V2(%)**
10^{-7}	1	0	2	0
10^{-6}	1	0	2	0
10^{-5}	4	0	5	0
$2x10^{-5}$	5	0	12	0
$5x10^{-5}$	11	1	19	1
10^{-4}	20	0	31	1
$2x10^{-4}$	24	6	42	0
$5x10^{-4}$	25	7	52	5
10^{-3}	27	22	51	7
$5x10^{-3}$	27	32	41	39

APPENDIX TO FIGURE 6.5

The following tables summarise the maximum amplitude attained during laser-induced vasodilator responses (LIVR's; %) under the conditions specified.

(a) **0.9μm RBS, 457.9nm** laser light

Expt. №	**0.2 mW**	**0.4 mW**	**0.8 mW**	**1.6 mW**	**3.2mW**	**6.0 mW**
910820/1	13.8	22.2	38.1	42		
910820/2	19.8	25.3	35.2	42.8	61.5	62.6
910820/3	4.6	10.8	17.7	33.1	45.1	51.4
910821/1	14.3	28.6	46.7	55.5	76.9	82.4
910821/2	7.3	12.2	54.3	66.8	81.2	84.1
910821/3	13.5	25.8	43.3	55.6	60.1	69.7
MEAN	**12.3**	**20.9**	**39.2**	**49**	**65**	**70.1**
S.E.	**2.24**	**3.02**	**5.1**	**4.84**	**6.47**	**6.14**

0.9 μM RBS, 514.5nm laser light

Expt. №	**0.5mW**	**1mW**	**2mW**	**4mW**	**8mW**
910729			48.1	55	76.5
910730/1	4.8	14.3	20.1	38.6	53.4
910730/2	17.5	32.2	48	58.8	61.5
910731	32.9	38.2	42.9	50	58.2
910805	10.2	19.8	29.9	35.6	48.8
910806	6.1	14.5	28.5	40.8	41.7
MEAN	**14.3**	**23.8**	**36.1**	**48.1**	**56.9**
S.E.	**5.15**	**4.85**	**4.81**	**4.82**	**4.86**

(b) **0.3 μM RBS, 457.9nm** laser light

Expt. №	0.105mW	0.2 mW	0.4 mW	0.8 mW	1.6 mW	3.2mW	6.0 mW
920319	18.5	34.1	46.3	55.3	53.3	-	-
920320	12.8	27.8	33.3	42.6	47.4	-	-
920323	12.0	17.9	25.5	33.7	43.2	45.1	48.6
920324	10.4	22.4	27.5	37.5	39.5	41.1	50.7
920325	13.6	27.7	37.5	46.6	50.0	56.6	57.1
920326	18.9	22.4	-	36.1	-	54.2	51.2
MEAN	**14.4**	**25.4**	**32.1**	**42.0**	**45.1**	**49.2**	**51.9**
S.E.	**1.4**	**2.3**	**3.8**	**3.3**	**2.6**	**3.7**	**1.8**

0.3μM RBS, 514.5nm laser light

Expt. №	0.7mW	1.4 mW	2.8 mW	5.2 mW	10 mW
920302	9.5	17.9	27.5	37.4	52.2
920305	6.8	12.9	21.9	40.4	50.2
920309	11.4	20.1	33.3	46.7	54.3
MEAN	**9.2**	**17.0**	**27.6**	**41.5**	**52.2**
S.E.	**1.3**	**2.1**	**3.3**	**2.7**	**1.2**

(c) 1.6 μM CUB 457.9nm laser light

Expt. №	2mW	4mW	8mW	16mW	32mW
910813			2.7	12.4	27.6
910814	6	14.7	29.3	33.7	33.7
910815	3.6	17.9	52.8	56.4	56.9
910820	9.6	39.4	64.9	68.1	71.3
910819	15.2	30.4	45.6	51.9	54.4
MEAN	**6.89**	**20.5**	**39.1**	**44.5**	**48.8**
S.E.	**2.6**	**6.77**	**18.76**	**9.75**	**6**

1.6μM CUB 514.5nm laser light

Expt. №	4mW	8mW	16mW	32mW	64mW
910813			9.6	18.1	38.3
910812	9.5	17	23.8	31.3	36.1
910814	0	4.6	19.8	41.7	61.5
910815	0	5.7	18.6	32.4	41.8
910818	6.1	15.6	30	35	37.2
910819	0	4.2	13	27.1	38
MEAN	**3.2**	**8.5**	**17.7**	**28.6**	**40.3**
S.E.	**1.98**	**3.3**	**3.84**	**4.43**	**4.68**

APPENDIX TO FIGURE 6.7

EFFECT OF INCREASING DELAYS ON LIVR AMPLITUDE (%)

457.9nm/ 3.3mW	0.732 mins	1.047 mins	DELAY 1.326 mins	1.689 mins	2.54 mins
Expt. №					
920127	53.0	39.0	26.0	23.0	12.0
920129	40.0	42.0	25.0	26.0	10.0
920130	50.0	29.0	19.0	13.0	7.0
920131	50.0	37.0	19.0	15.0	9.0
920211	43.0	-	21.0	-	-
920213	57.0	-	37.5	-	-
920214	33.0	-	17.0	-	-
920217	31.0	-	-	-	-
920218	41.0	-	-	-	12.0
MEAN	**44.2**	**36.75**	**23.5**	**19.25**	**10.00**
S.E.	**3.0**	**2.8**	**2.6**	**3.1**	**0.9**

514.5nm/ 10mW	0.732 mins	1.047 mins	DELAY 1.326 mins	1.689 mins	2.54 mins	3.115 mins
Expt. №						
911206	-	27.0	19.0	-	-	-
911209	46.0	34.0	22.0	20.0	14.0	5.0
911210	42.0	-	-	-	14.0	11.0
911212	51.0	47.0	32.0	31.0	13.0	13.0
911213	53.0	40.0	28.0	20.2	9.0	5.0
MEAN	**48.0**	**37.0**	**25.25**	**23.67**	**12.5**	**8.5**
S.E.	**2.5**	**4.3**	**2.9**	**3.7**	**1.2**	**2.1**

LIVR's AT INCREASING DELAYS + SOD.

457.9nm/ 3.3mW	0.732 mins	1.047 mins	DELAY 1.326 mins	1.689 mins	2.54 mins
Expt. Nº					
920127	-	-	55.0	-	-
920211	-	-	64.0	-	-
920213	-	-	-	-	-
920214	37.0	-	-	-	-
920217	37.0	44.0	47.0	57.0	48.0
920227	47.8	35.0	34.7	40.2	41.7
MEAN	**40.6**	**39.5**	**50.18**	**48.6**	**44.85**
S.E.	**3.6**	**4.5**	**6.2**	**8.4**	**3.2**

APPENDIX TO 7.4

Mean responses (% active pressure) at times stated during photorelaxations elicited following set time intervals (TIs) following depletion exposure. S.E. values are shown in brackets.

Time (secs)	TI = 20 mins	TI = 40 mins	TI = 72 mins	TI = 150 mins	TI = 300 mins
12	95 (1.16)	92.5 (0.91)	85.6 (0.95)	81.1 (1.7)	71.2 (5.4)
24	94.17 (0.72)	89.8 (0.64)	82.9 (0.65)	75.5 (1.09)	67.4 (3.73)
48	94.6 (0.611)	91.2 (0.29)	85.3 (0.91)	77.5 (1.2)	72.4 (2.7)
72	95.33 (0.59)	92.4 (0.29)	88.7 (0.81)	82.0 (1.65)	77.1 (3.2)
96	95.87 (0.536)	93.7 (0.36)	91.6 (0.77)	85.6 (1.73)	82.5 (1.9)
120	96.6 (0.551)	94.8 (0.34)	93.4 (0.86)	88.3 (1.32)	85.6 (0.9)
144	97.6 (0.533)	95.7 (0.37)	94.4 (0.76)	90.1 (1.35)	88.2 (0.38)
168	97.7 (0.463)	96.4 (0.5)	95.2 (0.71)	91.8 (1.14)	89.4 (0.1)
192	97.8 (0.361)	96.9 (0.5)	95.9 (0.85)	92.8 (1.18)	90.8 (0.33)
216	97.97 (0.35)	97.34 (0.51)	96.4 (0.96)	93.7 (1.51)	91.6 (0.76)
240	97.93 (0.49)	97.42 (0.56)	96.4 (1.08)	93.9 (1.55)	91.8 (0.55)
264	98.37 (0.43)	97.58 (0.56)	96.6 (1.04)	94.3 (1.48)	92.6 (0.5)
288	98.7 (0.437)	97.66 (0.44)	96.95 (1.00)	94.9 (1.41)	92.8 (0.52)
312	98.93 (0.41)	97.88 (0.35)	97.62 (1.02)	95.2 (1.27)	93.3 (0.72)
336	98.63 (0.30)	97.82 (0.38)	97.6 (1.15)	95.7 (1.16)	93.3 (0.75)
360	98.53 (0.20)	97.72 (0.32)	97.5 (1.23)	95.9 (1.23)	93.3 (0.75)
384	98.87 (0.09)	98.34 (0.29)	98.4 (0.87)	96.7 (1.3)	95.4 (0.82)
408	99.53 (0.26)	98.84 (0.30)	98.8 (0.70)	97.4 (1.21)	96.7 (0.66)
432	99.37 (0.63)	99.16 (0.22)	99.1 (0.52)	98.4 (0.98)	97 (0.74)
456	99.5 (0.5)	99.38 (0.19)	99.3 (0.404)	99.0 (0.71)	97.3 (0.66)
480	99.7 (0.33)	99.56 (0.12)	99.5 (0.33)	99.1 (0.71)	97.7 (0.98)

APPENDIX TO 7.5

EFFECT OF DELAYED LNMMA INFUSION FOLLOWING A DEPLETION EXPOSURE, ON THE FIRST TI = 40 RESPONSE IN ITS PRESENCE.

** = statistically different from control TI = 40 mins response at the P = 0.01 confidence level.

*** = statistically different from control TI = 40 mins response at the P = 0.001 confidence level.

	CONTROL TI = 40 mins (%)	+ LNMMA (immed after depl) TI = 40 mins (%)	+ LNMMA (20 min delay) TI = 40 mins (%)
MEAN	**10.85**	**2.75*** **	**15.6** **
S.E.	**0.56**	**0.433**	**1.05**
P = (unpaired T-test)		**0.000**	**0.008**

APPENDIX TO FIGURE 7.7

EFFECT OF INCREASING INTERVALS BETWEEN EXPOSURES ON THE PEAK AMPLITUDE OF PHOTORELAXANT RESPONSES.

Expt. №	10 mins	20 mins	40 mins	72 mins	150 mins	300 mins
930128	2.8	5.8	9.4	17.0	-	-
930129	5.2	9.5	11.6	27.8	-	-
930201		9.7	-	-	30.7	-
930202	2.6	6.3	10.3	17.1	26.7	-
930203	3	7.1	12.1	18.9	27.1	-
930504	-	-	-	-	-	37.8
930505	-	-	-	-	-	37.7
930506	-	-	-	-	-	30.0
MEAN	**3.55**	**7.21**	**10.85**	**19.4**	**25.98**	**35.17**
SE	**0.4**	**0.71**	**0.56**	**2.15**	**1.51**	**3.62**

PHOTORELAXATION IN 100μM LNMMA (AND FOLLOWING WASHOUT WITH 1mM L-ARGININE).

A TI = 20 mins response was obtained before LNMMA infusion to evaluate the sensitivity of the preparation. This value was compared to the mean TI = 20 mins photorelaxant response (7.21 %) and all subsequent responses scaled accordingly.

	IN 100μM LNMMA					/ IN 1mM L-ARG		
Expt. №	**10 mins**	**20 mins**	**40 mins**	**72 mins**	**150 mins**	**20 mins**	**40 mins**	**150 mins**
930201		2.7	1.77	2.9	-	6.13	-	19.4
930211	3.42	3.34	4.22	-	-	-	-	-
930212	1.86	2.3	2.3	-	-	-	-	-
930216		2.2	3.1	-	4.8	-	-	-
930218	2.1	2.6	2.36	3.9	2.3	-	-	-
930302	4.4	4.8	-	-	-	-	-	-
930305	-	4.86	-	-	-	5.5	-	-
930303	-	2.3	-	-	-	8.6	12.8	27.5
MEAN	**2.95**	**3.13**	**2.75**	**3.4**	**3.55**	**6.75**	**12.8**	**23.5**
S.E.	**0.338**	**0.351**	**0.433**	**0.5**	**1.25**	**0.95**	-	**4.0**

PHOTORELAXATION IN 5μM Hb

Responses in each experiment have been scaled by the factor that the control TI = 20 mins response recorded before the introduction of Hb is scaled by to equal the mean TI =20 mins response above (7.21%).

Expt. №	**20 mins**	**40 mins**	**72 mins**	**150 mins**	**300 mins**
930331	4.73	6.82		8.78	-
930401	3.56	3.02	3.57	2.51	-
930402	4.35	7.53	6.72	5.02	-
930507	-	-	-	-	6.1
930510	-	-	-	-	0
930512	-	-	-	-	10.4
MEAN	**4.21**	**5.79**	**5.11**	**5.44**	**5.5**
SE	**0.381**	**0.915**	**1.0**	**1.15**	**3.02**

APPENDIX TO FIGURE 7.8

Below is a summary table for the effect of 100 μM ethacrynic acid (EA) (n =3 -5) on photorelaxations and responses to 10 μl bolus injections of NP and NO.

	CONTROL	**S.E.**	**+ E.A.**	**S.E.**
TI = 20 mins	7.21	0.71	0	-
TI = 40 mins	10.85	0.56	0.8	-
TI = 72 mins	19.4	2.15	1	-
TI = 150 mins	26.0	1.5	2.1	-
1mM NP	45.4	6.2	15.9	1.8
NO	79	1.3	45.5	11.5

APPENDIX TO FIGURE 7.10 and 7.11

Below are summary tables showing peak amplitudes of responses to increasing doses of NO, SNAP or NP and the **enhanced** photorelaxations following (i.e. after subtraction of the TI = 20 mins response before drug injection). All photorelaxations were elicited at a TI of 20 mins.

mean of 3 NO experiments:

log [NO] (M)	Response (%)	S.E.	TI = 20 PR (%)	S.E.
-3	72.3	2.5	41.6	4.3
-4.3	61.2	7.4	24.2	15
-5.3	44.5	20.5	2.55	1.65
-6.3	28.2	14.7	3.15	0.45
-7.3	6.7	5.3	1.7	0
-8.3	8.9	7.5	1.1	1.1

mean of 5 SNAP experiments:

log [SNAP] (M)	Response (%)	S.E.	TI = 20 PR (%)	S.E.
-2	86.5	2.73	59.78	3.57
-3	84.6	1.87	47.35	5.6
-4	80.3	2.83	19.2	2.73
-5	69.3	1.67	3.43	0.347
-6	28	10.9	0.95	0.366
-7	1.95	0.52	0.075	0.075

mean of 5 NP experiments:

log [NP] (M)	Response (%)	S.E.	TI = 20 PR (%)	S.E.
-3	61.43	4.7	3.83	1.52
-4	45.44	6.22	0.66	0.41
-5	25.52	6.1	0	0
-6	6.83	1.68	0.02	0.02
-7	2.85	0.47	0	0

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Journal of Physiology, **430**, 42*P*, 1990]

Nitrosylated iron–sulphur clusters, a novel class of nitrovasodilator: studies on the rat isolated tail artery

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Two iron–sulphur–nitrosyl complexes (A, heptanitrosyltri-μ_3-thioxotetraferrate(−1) and B, tetranitrosyl-tetra-μ_3-sulphido-tetrahedro-tetrairon) have been investigated as potential antihypertensive agents. Compounds A and B contain seven and four nitrosyl groups respectively, linked to an iron–sulphur framework (Fig. 1*A*, *B*; see Butler *et al.* 1988). Rat isolated tail arteries (10–15 mm long) were internally perfused (2 ml min^{-1}) with Krebs solution containing phenylephrine (1–5 μM). A (0·5 mM) and B (0·1 mM) were injected (10 μl) into the perfusate and responses were monitored by a pressure transducer. Both compounds evoked vasodilator responses (Fig. 1*C*). Haemoglobin (15 μM), haematoporphyrin (5 μM) and methylene blue (75 μM) suppressed responses to A and B, but protoporphyrin IX (5 μM), the demetallated precursor of haematoporphyrin, and L-N^G-monomethyl-arginine (100 μM), an inhibitor of endothelial nitric oxide (NO$^\cdot$) synthetase, did not do so.

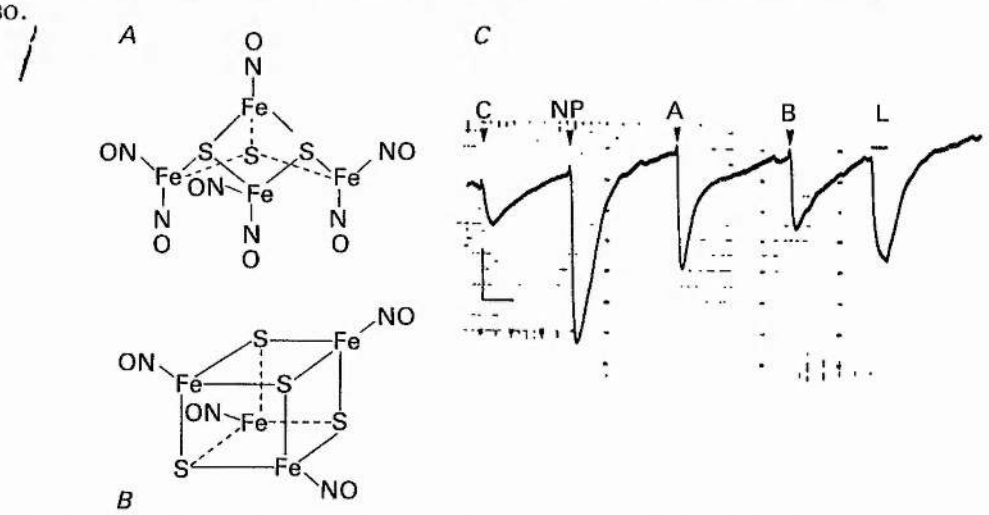

Fig. 1. *A*, *B*. Three-dimensional structures of iron–sulphur–nitrosyl complexes. *C*, vasodilator responses to A and B, compared to those evoked by 10 μl injections of carbachol (C, 10^{-2} M) and nitroprusside (NP, 250 μM) and by exposure to laser light (L; photorelaxant response: wavelength 514·5 nm, intensity 1 mW for 2 min, indicated by horizontal bar). Artery pre-contracted (173 Torr) with 5 μM-phenylephrine. Temp: 33 °C. Vertical scale: 20% agonist-induced tone; horizontal scale = 4 min. Expt: 900314.

The results suggest that A and B cause vasodilatation by liberating NO$^\cdot$ and stimulating guanylate cyclase, thereby mimicking the effect of endogenous (endothelial) NO$^\cdot$ (Palmer *et al.* 1987).

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Br. J. Pharmacol. (1992), **107**, 842–848

Iron-sulphur cluster nitrosyls, a novel class of nitric oxide generator: mechanism of vasodilator action on rat isolated tail artery

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1 Two iron-sulphur cluster nitrosyls have been investigated as potential nitric oxide (NO·) donor drugs (A: tetranitrosyltetra-μ3-sulphidotetrahedro-tetrairon; and B: heptanitrosyltri-μ3-thioxotetraferrate(1-)). Both compounds are shown to dilate precontracted, internally-perfused rat tail arteries.

2 Bolus injections (10 μl) of compound A or B generate two kinds of vasodilator response. Doses below a critical threshold concentration (D_T) evoke transient (or *T*-type) responses, which resemble those seen with conventional nitrovasodilators. Doses $> D_T$ produce sustained (or *S*-type) responses, comprising an initial, rapid drop of pressure, followed by incomplete recovery, resulting in a plateau of reduced tone which can persist for several hours.

3 *T*- and *S*-type responses are attenuated by ferrohaemoglobin (Hb) and by methylene blue (MB), but not by inhibitors of endothelial NO· synthase. Addition of either Hb or MB to the internal perfusate can restore agonist-induced tone when administered during the plateau phase of an *S*-type response. Moreover, subsequent removal of Hb causes the artery to re-dilate fully.

4 We conclude that *T*- and *S*-type responses are both mediated by NO·. It is postulated that *S*-type responses represent the sum of two vasodilator components: a reversible component, superimposed upon a non-recoverable component. The former is attributed to free NO·, preformed in solution at the time of injection; and the latter to NO· generated by gradual decomposition of a 'store' of iron-sulphur-nitrosyl complexes within the tissue. This hypothesis is supported by histochemical studies which show that both clusters accumulate in endothelial cells.

Keywords: Nitric oxide donors; iron-sulphur-nitrosyls; vasodilator responses; nitrovasodilators; endothelium

Introduction

Endothelial cells release a labile factor which relaxes vascular smooth muscle (Furchgott & Zawadski, 1980). Endothelium-derived relaxing factor (or EDRF) has recently been identified as either nitric oxide (NO·; Palmer *et al.*, 1987; Ignarro *et al.*, 1988); a labile nitrosothiol, possibly S-nitroso-cysteine (Myers *et al.*, 1990); or a nitrosyl-iron complex with thiol ligands (Vanin, 1991). NO· is formed from the terminal guanidino nitrogen atom of L-arginine by a citrulline-forming enzyme, referred to as NO synthase (Palmer *et al.*, 1988a,b). Endothelial NO synthase (NOS) is NADPH- (Palmer & Moncada, 1989) and calmodulin-dependent (Bredt & Snyder, 1990). It can be inhibited by several L- (but not D-) analogues of arginine (Rees *et al.*, 1990).

Haemodynamic studies using stereospecific NOS inhibitors have firmly established the importance of NO· in controlling peripheral resistance *in vivo* (Vallance *et al.*, 1989; Aisaka *et al.*, 1989; Rees *et al.*, 1989; Gardiner *et al.*, 1990; Chu *et al.*, 1991). Endothelium-dependent relaxations of vascular smooth muscle are attenuated in some diseased states, notably in hypertension (Winquist *et al.*, 1984; Luscher & Vanhoutte, 1986; Otsuka *et al.*, 1988; Tesfamariam & Halpern, 1988; Sunano *et al.*, 1989) and in atherosclerosis (Harrison *et al.*, 1987; Chappell *et al.*, 1987; Henry *et al.*, 1987; Forstermann *et al.*, 1988, Guerra *et al.*, 1989), though it remains unclear whether these conditions are necessarily associated with impaired synthesis and/or release of NO· (Van der Voorde & Leusen, 1986; Hoeffner & Vanhoutte, 1989; Chester *et al.*, 1990; Jacobs *et al.*, 1990; Fozard & Part, 1991).

Clinical 'nitrovasodilators', including amyl nitrite, glyceryl trinitrate, nitroprusside (NP) and molsidomine, act independently of the endothelium, augmenting its function temporarily by releasing NO· *in vivo* (Waldman & Murad, 1984; Feelisch & Noak, 1987). Their hypotensive actions are generally of short duration and continuous drug infusions are necessary to effect sustained responses (Kreye, 1980). Here we show that two tetrairon-sulphur cluster nitrosyls (compound A: tetranitrosyl-tetra-μ3-sulphidotetrahedro-tetrairon; and compound B: heptanitrosyl-tri-μ3-thioxotetraferrate(1-), also known as Roussin's Black Salt; Roussin, 1858) are able to generate unusually protracted vasodilator responses when tested on rat isolated, internally-perfused tail artery preparations. The structures of both clusters, based on X-ray diffraction studies (Chu & Dahl, 1977; Chu *et al.*, 1982), are shown in Figure 1a,b. The vasodilator actions of A and B are blocked by haemoglobin and also by methylene blue, but not by agents which suppress NOS. Histochemical studies reveal that both clusters are able to penetrate cell membranes with extraordinary ease and that they accumulate inside the endothelium, forming a molecular 'store' of NO·. Our results suggest that their gradual decomposition from sites within the endothelium generates free NO· and that its slow release serves to sustain their vasodilator actions.

Preliminary accounts of some aspects of this work have appeared elsewhere (Flitney *et al.*, 1990; 1992)

Methods

Preparation

Experiments were performed on segments of tail artery taken from normotensive adult Wistar rats (250–460 g). Animals

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were killed by cervical dislocation. A length of artery (0.8–1.5 cm) was dissected free, cannulated (Portex cannula) and transferred to a Perspex bath.

Apparatus

The apparatus is shown in Figure 1. The cannula (C) formed part of a constant flow perfusion circuit, driven by a peristaltic pump (P1; Gilson Minipuls). The vessel (V) was perfused internally (flow rate: 2 ml min^{-1}) with solution pre-warmed by passage through a heat exchanger. Drugs were introduced into the lumen of the artery by bolus injection through a side tube (I). The outer surface of the vessel was superfused continuously with solution driven by a second peristaltic pump (P2). The temperature of the chamber was held at 30–35°C by adjustment of the flow rate in the external circuit (ca. 8 ml min^{-1}).

Light from an Argon ion laser (L; Spectra Physics Ltd., type 168-09) could be made to irradiate the artery directly, so as to induce photorelaxation of vascular smooth muscle (Furchgott *et al.*, 1961). The beam ($\lambda = 514.5$ nm; output intensity 1 mW) was reflected onto the preparation by means of a front-silvered mirror (M; beam diam. at preparation: approx 2 cm).

A differential pressure transducer (T: Sensym type SCX 150NC; Farnell Electronic Components, Leeds) detected changes in back pressure due to changes in arterial tone. Responses were displayed on a chart recorder.

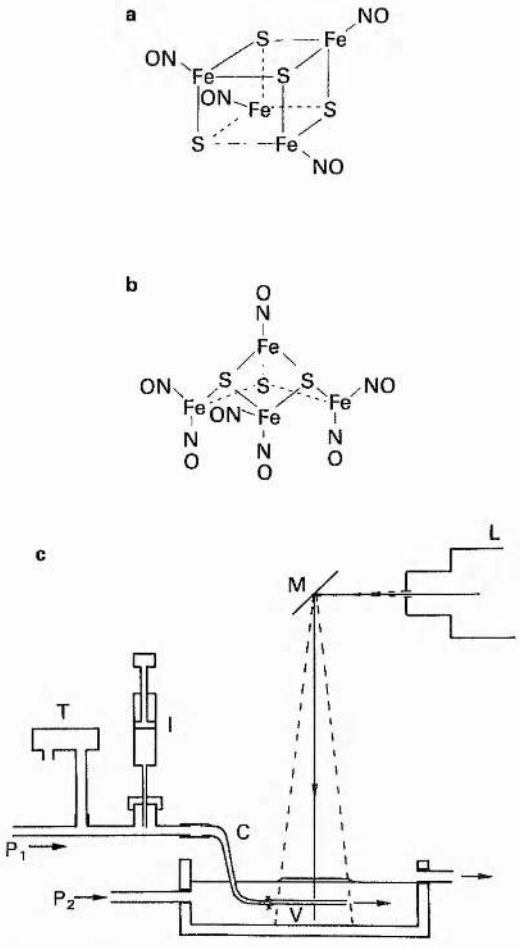


Figure 1 (a, b) Molecular structures of clusters A and B, as determined by X-ray diffraction studies. Compound A contains four and B seven ligated NO· groups per molecule. (c) Apparatus used for perfusing isolated segments of rat tail artery and for irradiating preparations with laser light. See text for full description and explanation of lettering.

Experimental protocol

Arteries were perfused internally with oxygenated Krebs solution (composition, mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.15, $CaCl_2$ 2.5, $MgCl_2$ 1.1, glucose 5.6, gassed with 95% O_2/5% CO_2 to maintain pH 7.4), initially at a low flow rate which was increased gradually over the next 10–20 min to reach a final value of 2 ml min^{-1}. The preparation was allowed to stabilize for 20–30 min, after which it was precontracted with Krebs plus phenylephrine HCl (= Krebs-+PE: mean (± s.e.) [PE] = 6.5 ± 0.5 μM. Mean (± s.e.) agonist-induced perfusion pressure = 101 ± 3.5mmHg).

Experiments were made to establish the mode of action of iron-sulphur cluster nitrosyls. Responses to bolus injections (10 μl) of A and B were compared with those evoked by (i) carbachol (CCh), an endothelium-dependent vasodilator; and (ii) either nitroprusside (NP), S-nitroso-N-acetylpenicillamine (SNAP) or exposure to laser light (L) all of which relax vascular smooth muscle independently of the endothelium.

The effects of adding the following to the internal perfusate were examined: (i) ferro-haemoglobin (Hb) a nitric oxide 'scavenger'; (ii) methylene blue (MB); and (iii) either N^G-monomethyl-L-arginine (L-NMMA) or N-nitro-L-arginine methyl ester (L-NAME), both stereospecific inhibitors of NOS. Comparisons were made with responses evoked by NP or SNAP.

Drugs: commercial sources and synthetic procedures

L-phenylephrine HCl (mol. wt. 203.7; Sigma Chemicals) was used at concentrations ranging from $2–12 \times 10^{-6}$M. Stock solutions (10^{-2}M) of NP (mol. wt. 298; BDH Ltd., 'Analar' grade) were made and the dose required adjusted by serial dilution immediately prior to use. SNAP (mol. wt. 220) was synthesized by reacting N-acetylpenicillamine with sodium nitrite (Field *et al.*, 1978). Compound B was prepared by reacting iron(II) sulphate heptahydrate, sodium nitrite and sodium sulphide in hot aqeous solution under nitrogen gas (Brauer, 1960). Compound A was prepared by addition of elemental sulphur to a solution of B dissolved in re-distilled toluene and refluxed overnight (16 h) under dry nitrogen gas (Gall *et al.*, 1974).

Purities of iron sulphur nitrosyl compounds were checked by (a) infra red spectroscopy (A had a single IR absorption maximum at wave number 1790 cm^{-1}; B had three maxima, at 1795 cm^{-1}, 1747 cm^{-1} and at 1707 cm^{-1}) and (b) ^{14}N-nuclear magnetic resonance (NMR) spectroscopy.

Solutions of B (mol. wt. 553) were made up in Krebs solution, immediately before use. Compound A (mol. wt. 472) is not soluble in water and so working solutions were made by serial dilution of 5 mg A dissolved in 1 ml dimethyl sulphoxide (ca. 10^{-2}M).

An important note concerning experimental procedures

All experiments were conducted in a darkened laboratory with a red safelight (60W) as the sole means of illumination. There were two reasons for this. First, the ambient lighting under normal laboratory conditions is sufficient to induce photorelaxation of precontracted vascular smooth muscle, causing a progressive loss of vessel tone. Second, NP, A and B are photosensitive and decompose when exposed to light, releasing free NO· (Wolfe & Swinehart, 1975; Flitney & Kennovin, 1988; Flitney *et al.*, unpublished results). Drug solutions were therefore made up in the dark and stored in glass vials covered with aluminium foil prior to use.

Results

Bolus injections of either NP or SNAP invariably produce fully-reversible (transient or *T*-type) vasodilator responses: an

initial, rapid drop of pressure, followed by complete recovery, often with some positive 'overshoot'. Compounds A and B produced *T*-type responses only at doses below a critical threshold concentration (D_T). Doses$> D_T$ generate long-lasting (sustained or *S*-type) responses, comprising an initial, rapid decrease of pressure, followed either by partial recovery only, or no recovery at all. The hallmark of the *S*-type response is a remarkably stable plateau of reduced vessel tone which can persist for several hours.

Mediation of T*-type responses by NO·*

Figure 2 compares vasodilator responses to carbachol (CCh), nitroprusside (NP) and laser light (L; = 514.5 nm; 1 mW intensity) with *T*-type responses produced by A and B. Control responses are shown in Figure 2a. The responses to A and B were blocked by either methylene blue (b; 75 μM) or by ferro-haemoglobin (c; 15 μM), a NO· scavenger. However, neither L-NMMA (d; 100 μM) nor L-NAME (not illustrated), both of which suppress endothelial NOS (Rees *et al.*, 1990), inhibited responses to A or B. Indeed, it was usual to find that treatment with either L-NMMA or L-NAME increased the responsiveness of arteries to A and B.

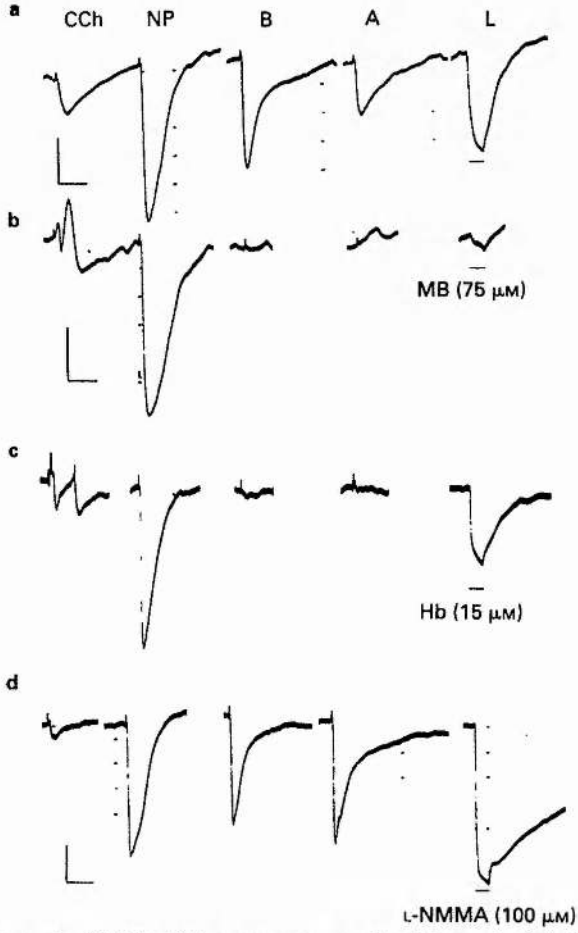


Figure 2 (a) Vasodilator responses produced by microinjection (10 μl) of test doses of carbachol (CCh: 10^{-2} M) and nitroprusside (NP; 2.5×10^{-4} M) and by irradiation with laser light (L; λ = 514.5 nm; 1 mW; 2 min), compared with *T*-type responses produced by clusters A (10^{-4} M) and B (5×10^{-4} M). Artery pre-contracted with 5×10^{-6} M phenylephrine (agonist-induced pressure = 135 mmHg). Female rat, 274 g. Temp., 32°C. (b) Suppression of responses to A and B and to laser light by treatment with methylene blue (MB 7.5×10^{-5} M). Details as above. (c) Inhibition of responses to compound A and B by haemoglobin (Hb, 15×10^{-6} M). Hb increased agonist-induced pressure to 1.8x control level (after ca. 6 min). Artery pre-contracted with 4×10^{-6} M phenylephrine (agonist-induced pressure = 114 mmHg). Female rat, 373 g. Temp. 32°C. (d) The NO synthase (NOS) inhibitor, N^G-monomethyl-L-arginine (L-NMMA, 1×10^{-4} M) increased agonist-induced tone to around 1.28x the control level. Drugs tested 20 min after beginning perfusion with L-NMMA. Responses to carbachol attenuated by L-NMMA, but not those produced by nitroprusside (NP), A, B or laser light. Details as for (c) above. Calibration bars (all recordings): vertical, 20% agonist-induced tone (0.2Y; see Figure 3a); horizontal, 4 min.

S*-type responses produced by sequential injections of increasing doses of A or B*

Pressure recordings made by injection of increasing doses of NP (Figure 3c) or SNAP (not illustrated) confirm that both compounds produce *T*-type responses only. Similar experiments with A or B show that *T*-type responses give way to *S*-type responses when the injected dose exceeds D_T: both the rate and extent of the recovery following successive injections become progressively reduced, resulting in a stepwise and persistent loss of vessel tone (Figure 3a,b).

Three parameters have been measured (X, Y and Z; see Figure 3a) to enable quantitative comparisons to be drawn between NP and SNAP and either A or B. Figure 4 shows perfusion pressure minima (filled circles) immediately following injection of each drug (X) and the steady-state pressures attained after recovery (Z; open circles), both expressed as a fraction of the initial agonist-induced pressure (Y) and plotted as a function of $\log_{10}$ injected dose. The concentrations of SNAP, NP, A and B (μM) required to produce half-maximal

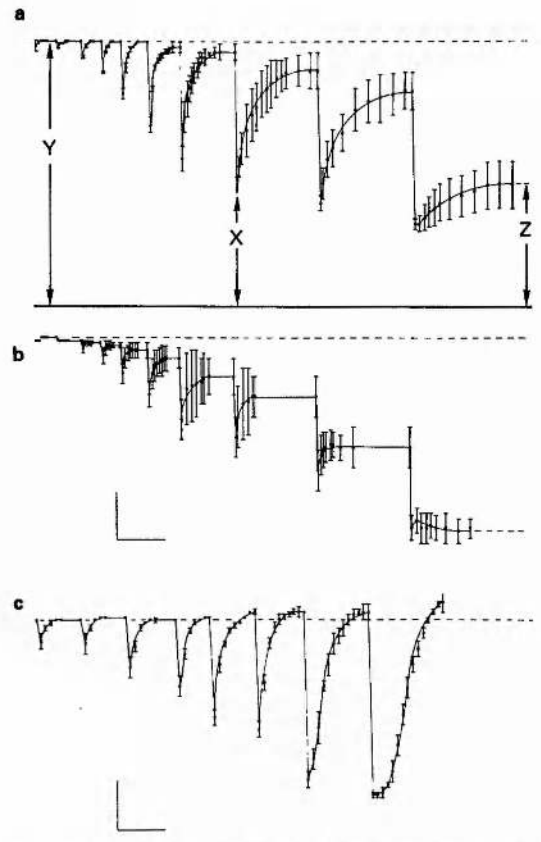
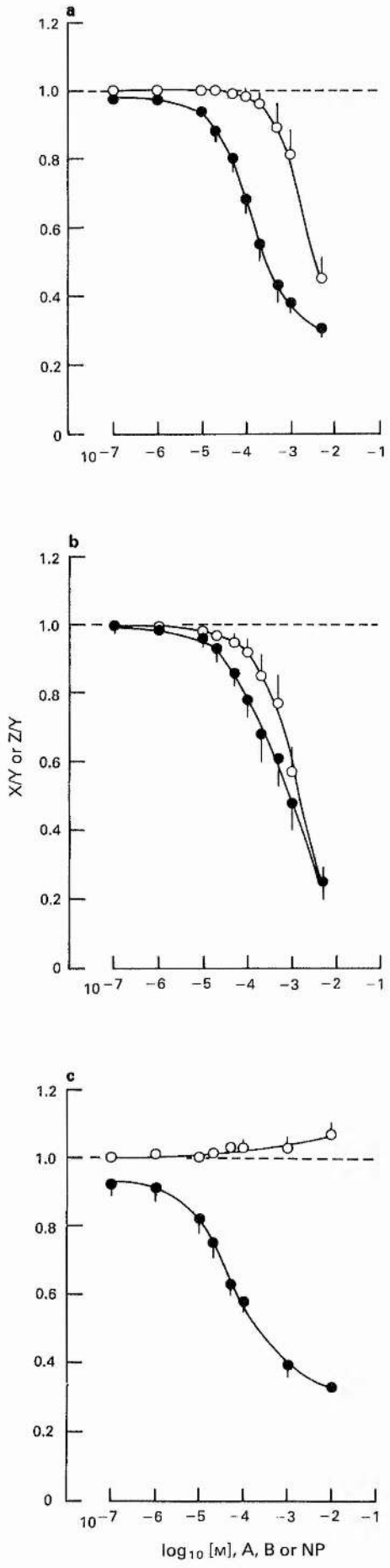


Figure 3 Averaged pressure recordings obtained by successive injections of increasing doses of compound A, B or nitroprusside (NP) (graphs a, b and c, respectively). Mean values (± s.e., vertical bars) are plotted (n = 6 preparations for a, and n = 5 for both b and c). Note that injection doses of A or B which exceed D_T (see text) produce a sustained drop in vessel tone (*S*-type response), whereas responses to NP are fully reversible (*T*-type response). In (a), Y = agonist-induced pressure; X = pressure minimum following each injection; and Z = steady-state (plateau) pressure attained after recovery (see also Figure 4). Calibration bars: vertical = 0.2Y; horizontal = 20 min (A and B) or 10 min (C).



decreases of X/Y (EC_{50} values) are 36, 43, 126 and 399, respectively. The corresponding curves for Z/Y reflect the tendency for A and B (but not NP) to generate S-type responses. The effect is more pronounced for B than for A, as shown by the greater proximity of the X/Y and Z/Y curves. D_T is estimated to be around 1–10 μM for B and 20–100 μM for A.

Abolition of the 'plateau' phase of the S-type response by Hb or by MB

The *S*-type response was remarkable in that even prolonged perfusion ('wash-out') with Krebs+PE solution alone (up to 5 h) failed to induce any further vasoconstriction, once the plateau phase was established. However, the addition of either Hb (15 μM) or MB (> 10 μM; not illustrated) to the internal perfusate initiated a prompt and complete restoration of all agonist-induced tone; indeed, as Figure 5 clearly shows, the perfusion pressure rises well above the control

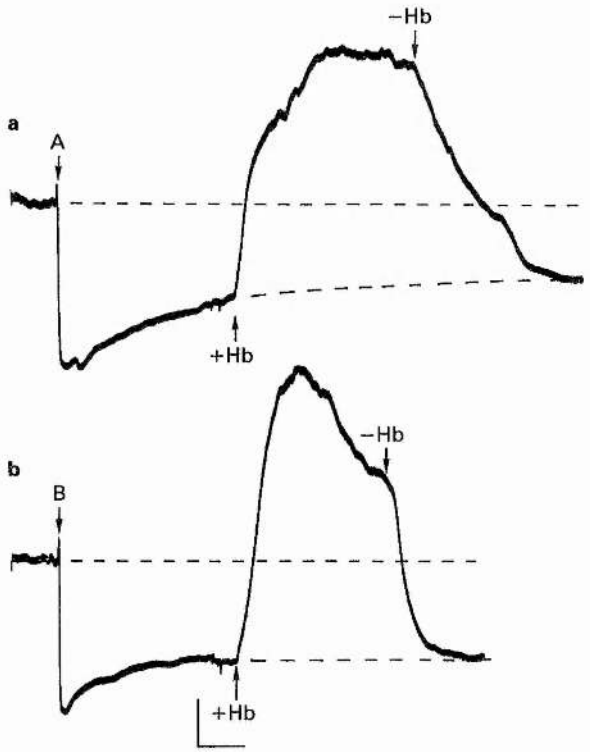


Figure 5(a, b) Pressure recordings from two preparations showing responses to ferro-haemoglobin (Hb, 31.5×10^{-5} M) added to, and then later removed from, the internal perfusate during the plateau phase of an *S*-type response. *S*-type responses were produced by a single bolus injection of 5×10^{-3} M A or B (upper and lower recordings respectively). The addition of Hb (upward arrows) elicits a prompt vasoconstriction which causes the perfusion pressure to rise above the control (pre-injection) value. Removal of Hb produces a vasodilatation which returns the pressure to the plateau value (curved dashed lines). Similar responses to these have been recorded when the time delay between the injection of A or B and treatment with Hb was increased to 6 h. Calibration bars: vertical = 0.2Y; horizontal = 4 min.

Figure 4 Mean (+ or − s.e., vertical lines values for X/Y (●) and Z/Y (○; see Figure 3a) plotted as a function of $\log_{10}$ injected dose for A (a), B (b) and nitroprusside (NP) (c). Responses to NP show complete recovery, with some pressure overshoot at the higher doses (Z/Y > 1.0). S-nitroso-N-acetylpenicillamine (SNAP) (data not shown) gives results which are qualitatively similar to those seen with NP. The values of Z/Y for both clusters (measured 20 min after each injection) are < 1.0 at doses which exceed D_T (by definition; see text), reflecting the tendency for A and B to evoke *S*-type responses. Data obtained from same pressure recordings used to construct Figure 3 (a-c).

level (Y), here to around 1.6Y (A) and 1.8Y (B). Even more striking, the subsequent removal of Hb (but not of MB) causes arteries to re-dilate fully (Figure 5a,b). These observations lead us to conclude that extremely brief exposure of arteries to A or B (the 'transit' time for a 10 μl bolus passing through the vessel is ca. 0.3 s) can suffice to establish a durable source of NO· within the tissue.

Histochemical studies showing that iron sulphur cluster nitrosyls penetrate endothelial cells and accumulate there

The existence of a long-lasting source (store) of NO· in vessels exposed to either cluster is supported by microscopic studies of treated arteries. Solutions of A and B are intensely black and visual inspection of segments of artery subjected to repeated bolus injections (experiments of the type used to construct Figure 3) revealed some discolouration of the vessel lining. Microscopic examination of freshly-frozen, unfixed sections of artery exposed to cluster B (5×10^{-3}M, 5 min continuous perfusion, followed by 15 min washout with Krebs + PE to remove excess drug from the lumen of the artery) showed that the discolouration was confined to the endothelium (Figure 6a). Unfixed, frozen sections which had been stained with either bathophenanthroline (Figure 6b) or ferrocyanide (not shown), to detect iron(II) derived from the clusters (Pearse, 1972), showed a similar distribution of reaction product, located predominantly in endothelial cells, but sometimes with some faint staining of adjacent smooth muscle cells also. Control (untreated) arteries did not react positively for iron.

Discussion

This paper establishes iron-sulphur cluster nitrosyls as a novel class of NO· donor drug with unusual vasodilator properties. The compounds investigated here are able to generate two distinct kinds of response, designated *T*-and *S*-type responses. In this respect they differ from either NP or SNAP, both of which give fully-reversible responses only (Figures 3 and 4). The ability of Hb and of MB, but not NOS inhibitors, to block *T*- and *S*-type responses (Figure 2) shows that they are mediated by NO· derived from each cluster, and not by enhanced EDRF production through stimulation of the endothelial L-arginine:NO· pathway.

Composite nature of the S-type response

Our results lead us to postulate that the S-type response represents the sum of two, distinct vasodilator components: a reversible component, attributable to free NO· generated by the spontaneous decomposition of clusters in solution and present at the time of injection; superimposed on a 'non-recoverable' component, caused by the delayed release of NO· from clusters which have become trapped within the endothelium. This hypothesis is supported by histochemical studies (Figure 6), which revealed the presence of Fe(II) within endothelial cells of treated (but not control) arteries, and by our observation that the plateau phase of the *S*-type response can be abolished in a reversible manner by perfusing preparations with Hb (Figure 5).

Vanin and co-workers (Vanin, 1991) have shown that iron-dinitrosyls ($Fe(NO)_2$) complexed with low molecular weight thiols (e.g. cysteine or glutathione) can vasodilate isolated blood vessels and also inhibit platelet aggregation. Interestingly, the vasodilator action exhibits two-phase kinetics when tested on intact animals, resembling the *S*-type response described in the present study: that is, an initial, rapid decrease of arterial pressure, followed by a persistent (several hours) hypotensive action. The rapid and sustained components of the response are attributed to NO· released from (i) iron-dinitrosyl low-molecular thiol complexes (= rapid phase) and

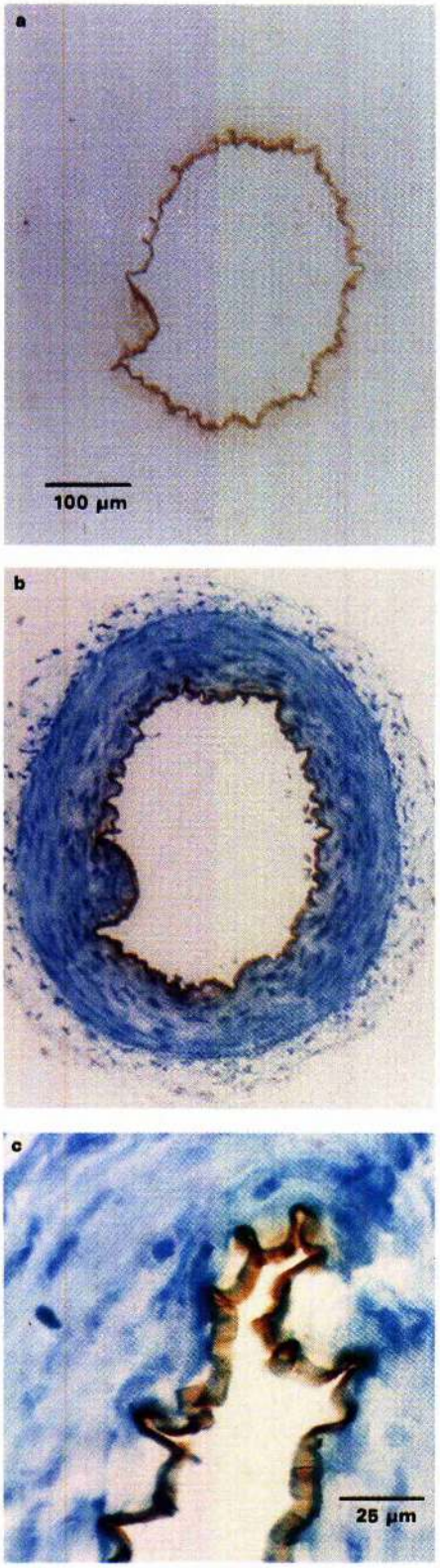


Figure 6 Transverse sections (20 μm) of a freshly-frozen artery perfused with B (5 mM, 2 ml min^{-1}) for 5 min, followed by 15 min perfusion (wash-out) with Krebs + PE solution only. (a) Unstained, unfixed section showing discolouration of the vessel lining. (b) Freshly-frozen, unfixed section after staining for 7 h with bathophenanthroline, followed by brief (4 min) counterstaining with methylene blue (scale as in (a)). Endothelial cells stain positively (red-brown colouration) for iron(ii) derived from the cluster. (c) Unfixed section stained for 4 min with methylene blue to show up smooth muscle cells. Note that brown colouration is confined to the endothelial lining of the vessel.

(ii) from iron-dinitrosyl protein-thiol complexes (= sustained phase) formed within the tissue. The latter are thought to result from the transfer of $Fe(NO)_2$ groups from low molecular weight to protein-borne ligands, forming a long-lasting, molecular 'store' of NO· in the vascular bed.

Suppression of endogenous EDRF production did not occur during the plateau phase of S*-type responses*

The effects of Hb administered during established *S*-type responses lead us to conclude that the formation of endogenous NO· from L-arginine is not suppressed by either cluster and that its continued release from endothelial cells helps to sustain the plateau phase. Thus, perfusing vessels with Hb does not merely re-establish the control (pre-injection) perfusion pressure, but instead drives it to a level substantially greater than this (Figure 5). The inference to be drawn is that Hb scavenges NO· from both exogenous (cluster-derived) and endothelial sources.

The nature of the intracellular mechanism(s) underlying the vasodilator actions of iron-sulphur nitrosyls remains to be established before meaningful comparisons can be made with other nitrovasodilators. The fact that Hb and MB can block responses to either cluster (and also to SNAP) but not those evoked by NP (Figure 2) clearly implies different cellular mechanisms of action. This has not been investigated in the present study. However, there is evidence to suggest that NP may relax vascular smooth muscle through both guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-dependent and cyclic GMP-independent pathways (Otsuka *et al.*, 1988). A component of the relaxant effect of glyceryltrinitrate may also be mediated by a mechanism which does not involve elevated cyclic GMP levels (Diamond & Chu, 1983).

Physicochemical properties of iron-sulphur cluster nitrosyls

The physicochemical properties of iron sulphur cluster nitrosyls (Butler *et al.*, 1988) provide some insight into their vasodilator actions. Both compounds are potentially able to transport large quantities of NO·, with 4 and 7 mol. of ligated NO· per mol. of A and B, respectively. Their selective retention and gradual decomposition from within the endothelium can evidently generate physiologically significant amounts of NO· at this most relevant of sites. The mechanism by which NO· is released from clusters trapped inside the endothelium (whether spontaneous or enzymatic) is not known. Extended Hückel molecular orbital calculations show that A and B are electron precise (Sung *et al.*, 1985): this means that addition of an electron to A, and either the addition or removal of an electron in the case of B, will weaken the cage bonding and cause the iron sulphur framework to disintegrate, releasing free NO·. The oxidative status of the endothelial cell may therefore prove to be an important factor in determining the rate of NO· release *in vivo*.

The apparent ease with which both clusters are able to penetrate the endothelial cell membrane is probably related to their high solubility in non-polar solvents (Butler *et al.*, 1988). Compound B is especially interesting in this regard because it is ionic (A is neutral) and therefore soluble in polar solvents too. Curiously, though, the marked contrast in the intensity of staining as seen between endothelial cells and overlying smooth muscle cells (see Figure 6) shows that neither compound can traverse the endothelial cell layer readily. The faint reaction product which is sometimes discernible in smooth muscle cells immediately adjacent to the endothelium is probably a diffusion artefact of the staining procedure, because neither cluster survives conventional fixation methods (including vapour fixation) and so histochemical observations were of necessity made on freshly-frozen sections. This explanation is supported by preliminary X-ray microprobe studies of rapidly-quenched arteries following treatment with A or B, which reveal elemental Fe and S within endothelial cells, but no evidence for either in nearby smooth muscle cells (unpublished observations: Elder, Pediani, Megson & Flitney).

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Iron–sulphur-cluster nitrosyls: long-acting nitric oxide-donor drugs

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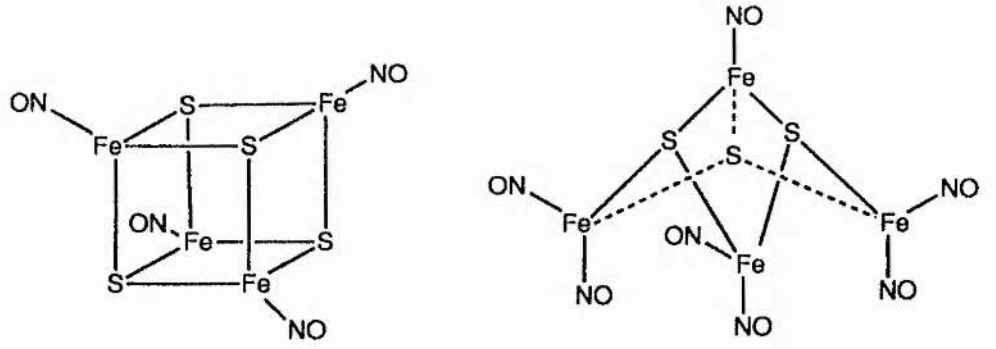
A number of clinically useful vasodilator drugs (glyceryl trinitrate, amyl nitrite and sodium nitroprusside) appear to act by release of nitric oxide (NO) *in vivo*. Other compounds containing NO as a ligand have the same potential and we have examined the vasodilator properties of two such compounds (Fig. 1): tetra-nitrosyltetra-μ_3-sulphidotetrahedro-tetrairon (Cubane) and heptanitrosyl-μ_3-thioxotetraferrate (1−) (Roussin's Black Salt). The latter is made by self-assembly from sulphide, iron (2+) and nitrite and converted into Cubane by reaction with elemental sulphur [1]. Both compounds are known to release NO readily [2].



Cubane Roussin's Black Salt

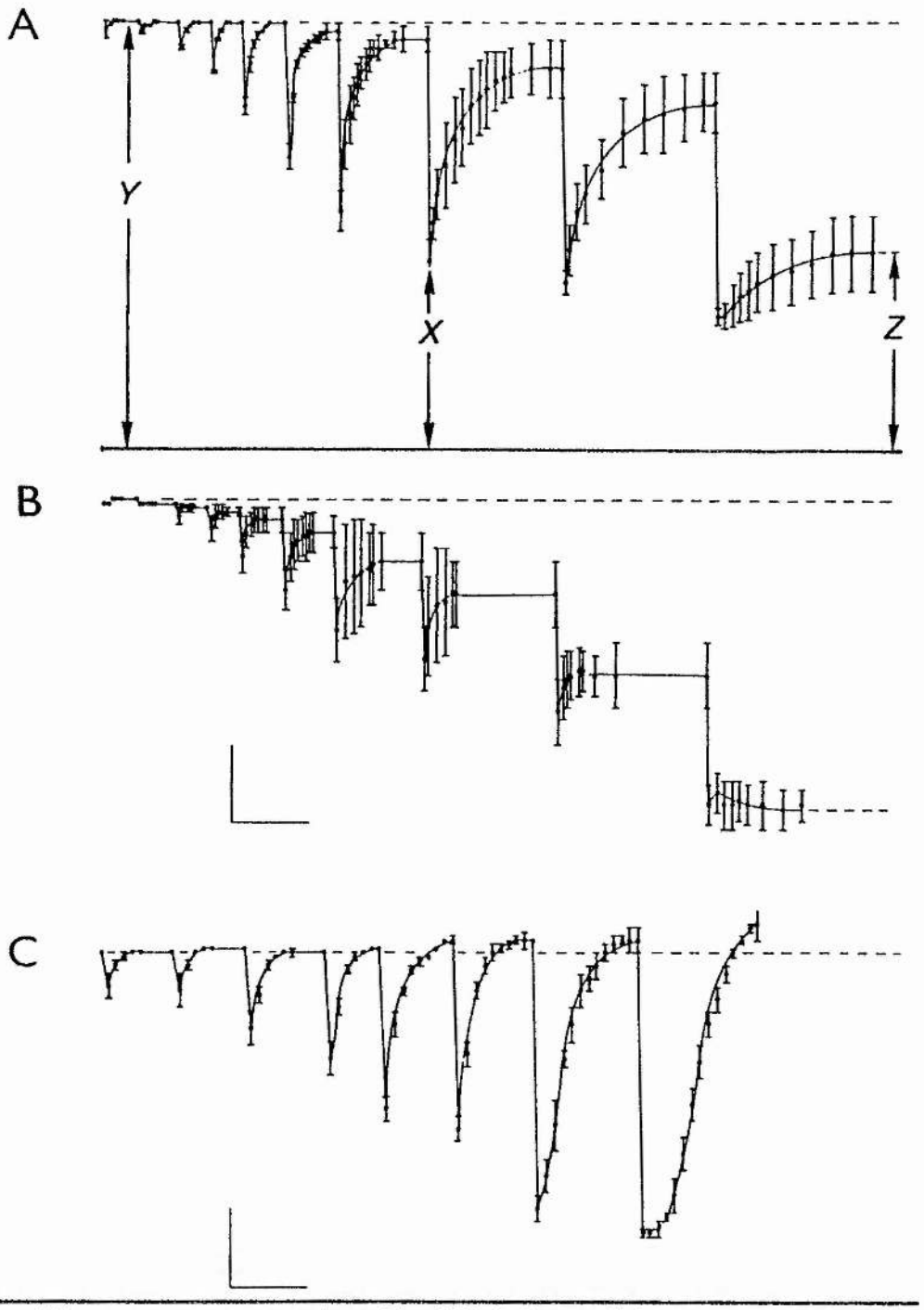
Figure 1

The physiological action of these compounds was examined by measuring their effect on pressure generated in a small segment of pre-contracted rat tail artery perfused internally at constant flow rate. Relaxation of arterial muscle is registered on a pressure transducer and visualized on a chart recorder [3].

Bolus injection (10 μl) of Cubane solution or Roussin's Black Salt solution into the internal perfusate generated two types of vasodilator response: (*a*) below a certain critical concentration the responses are transient and resemble those obtained with sodium nitroprusside (see Fig. 2, bottom trace), and (*b*) at higher concentrations there is an initial, rapid drop in pressure which is sustained and shows only partial recovery (see Fig. 2, top two traces). The vasodilator effects of both Cubane and Roussin's Black Salt are blocked by methylene blue, an inhibitor of the soluble guanylate cyclase and by ferrohaemoglobin, a NO scavenger, but not by N^G-monomethyl-L-arginine or by N^G-nitro-L-arginine methyl ester, both of which suppress NO synthase.

Solutions of both compounds are intensely black and visual inspection of perfused preparations revealed discoloration of the artery lining. Microscopic examination of freshly frozen arteries showed that the discoloration is confined to the endothelium. Unfixed, frozen solutions stained with bathophenanthraline showed a similar distribution of reaction product, located predominantly in endothelial cells but with some faint staining of adjacent smooth muscle cells.

Figure 2



Vasodilator responses produced by (A) Cubane, (B) Roussin's Black Salt, (C) sodium nitroprusside

The horizontal time scale is 20 min for A and B, and 10 min for C. The vertical scale is 20% agonist-induced pressure.

Conclusions

The results described allow a number of conclusions to be made: (*a*) The vasodilatation brought about by Cubane and by Roussin's Black Salt is caused by the release of NO. (*b*) Both compounds are selectively taken up by endothelial cells and constitute a store from which NO is slowly released. (*c*) The transient responses

observed are caused by the NO present in solution at the time of injection. (*d*) The slow release of NO from the store within the tissue is responsible for the sustained responses observed. (*e*) The ease with which the compounds are taken up by endothelial cells may be due to their substantial solubility in organic solvents; this is particularly surprising in the case of Roussin's Black Salt which is ionic. (*f*) Release of NO from both compounds occurs on oxidation and so the rate of NO release *in vivo* may be controlled by the redox environment.

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Complexes of nitric oxide with nucleophiles as agents for the controlled biological release of nitric oxide

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Introduction

We have found that complexes between nitric oxide (NO) and various nucleophiles [1] can be used for the controlled biological release of NO, and that this spontaneous, non-enzymic generation of NO provides an advantageous research tool, as well as a possible basis for drug design. Three specific applications of these NO/nucleophile adducts illustrate what we believe to be their considerable utility in biomedical investigations.

Vasorelaxation

To provide the groundwork for this first biological application, we measured the rates and extents of NO release from five structurally diverse NO/nucleophile complexes in pH 7.4 buffer at 37 °C. The specific compounds studied were those described by Maragos *et al.* [2]. All contained the anionic moiety, X-{N(O)-NO}$^-$, in which the nucleophile residue, X, was that of a secondary amine [Et_2N, as in Et_2N-{N(O)-NO}Na, **1**], a primary amine [^{i}PrHN, as in ^{i}PrHN-{N(O)-NO}Na, **2**], a polyamine, spermine [as in the zwitterion, H_2N-$(CH_2)_3$-NH_2^+-$(CH_2)_4$-N{N(O)-NO}$^-$-$(CH_2)_3$-NH_2, **3**], oxide [as in NaO-{N(O)-NO}Na, **4**], and sulphite [as in NH_4O_3S-{N(O)-NO}NH_4, **5**]. Half-lives ranged from 2.1 min for **1** to 39 min for **3**, with the amount of NO produced per molecule of NO/nucleophile adduct decomposed being as high as 1.9 (for **3**) and as low as 0 (for **5**) [2].

Because NO is widely considered to play a key role in vasodilatation [3], NO/nucleophile complexes that release the most NO should be potent relaxants

determined by lymphocyte [^{3}H]TdR uptake. Again, at high concentrations of RAW 264.7 (5 and 10×10^4 per well), lymphocyte proliferation was inhibited and this inhibition was reversed in the presence of L-NMMA (data not shown).

An immune response is characterized by cellular interactions for both initiation and modulation of the response. Engagement of the T-cell receptor and provision of an as yet incompletely defined co-stimulus by the macrophage results in T-cell signalling and production of cytokines necessary for T-lymphocyte proliferation. It is now clear that a previously undefined mechanism of macrophage modulation of the immune response exists via initiation of the NO pathway. This mechanism is operative at the post-cytokine production step, since detectable levels of cytokines able to induce T-cell proliferation are present in the culture supernatants and yet lymphocyte proliferation is inhibited when NO is produced.

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The inhibition of lymphocyte proliferation by nitric oxide-producing molecules and cells

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Introduction

Induction and amplification of a specific immunological response to antigen involves the proliferation of responding lymphocytes. As a consequence of such responses non-specific cellular effector mechanisms may be generated by the release from lymphocytes of activating cytokines as, for example, in the activation of macrophages by lymphocyte-released interferon-γ. Activated macrophages synthesize nitric oxide (NO). Here we demonstrate the effects of NO on lymphocyte proliferation and suggest a possible regulatory role for macrophage-derived NO. We also demonstrate functional mimicry of macrophage-synthesized NO by the NO-releasing compounds, streptozocin, sodium nitroprusside and Roussin's black salt.

Methods

Thymic lymphocytes and activated macrophages were obtained from CBH/Cbi strain rats. Macrophages were activated by intraperitoneal injection of 2 mg *Corynebacterium parvum* organisms 5 days before the cells were harvested by lavage. Monolayers of adherent macrophages in the wells of 96-well plates were obtained by incubating the macrophage-containing cell suspensions in the wells for approximately 1 h, after which non-adherent cells were washed off. Thymic lymphocytes were, after washing, suspended at 5×10^6 ml^{-1} in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (v/v) (FCS), 1 mM-L-arginine and 2 μg ml^{-1} concanavalin A. For cultures containing macrophages, 100 μl of lymphocyte suspension was added to 100 μl of DMEM (without concanavalin A) in the wells; to half of these cultures 500 μM-N^G-monomethyl-L-arginine (L-NMMA) was added. To test the effects of NO-releasing compounds, 100 μl of each concentration of compound (compounds were serially diluted in medium) was added to 100 μl of lymphocyte suspension. Six replicates of each concentration of macrophages or compound were made. The cultures were incubated for 48 h, after which 100 μl of the supernatant medium was removed for determination of nitrite concentration. [^{3}H]Thymidine was added (0.5 μCi well^{-1}) and the cultures incubated for a further 12 h. Cells were harvested onto glass fibre discs. Incorporated [^{3}H]thymidine, a measure of lymphocyte proliferation, was then determined by scintillation counting. [^{3}H]Thymidine incorporation by lymphocytes in the presence of macrophages or NO-releasing compounds was expressed as a percentage of the incorporation by lymphocytes alone (control

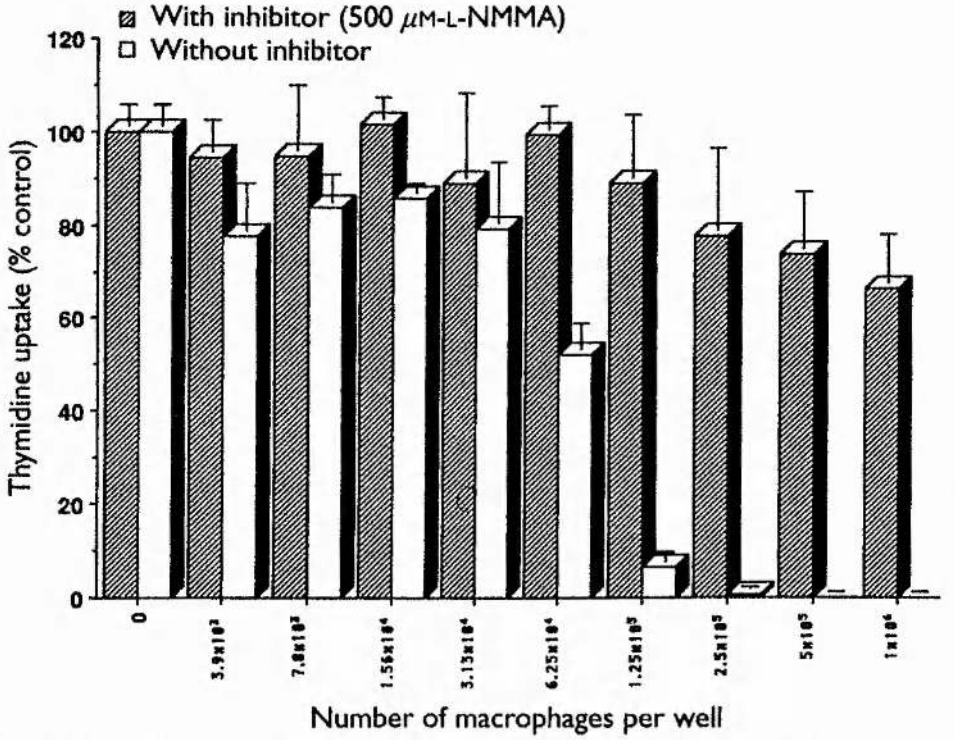


Figure 1

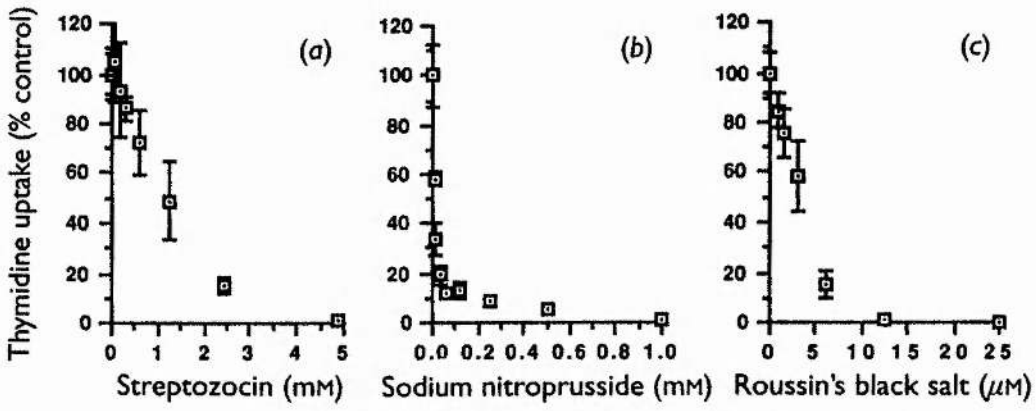
Plot showing the inhibition of lymphocyte proliferation in the presence of increasing numbers of macrophages and the effect of the inhibitor N^G-monomethyl-L-arginine (L-NMMA)

value). Nitrite concentrations were assayed using a colorimetric method based on the Griess reaction [1].

Results

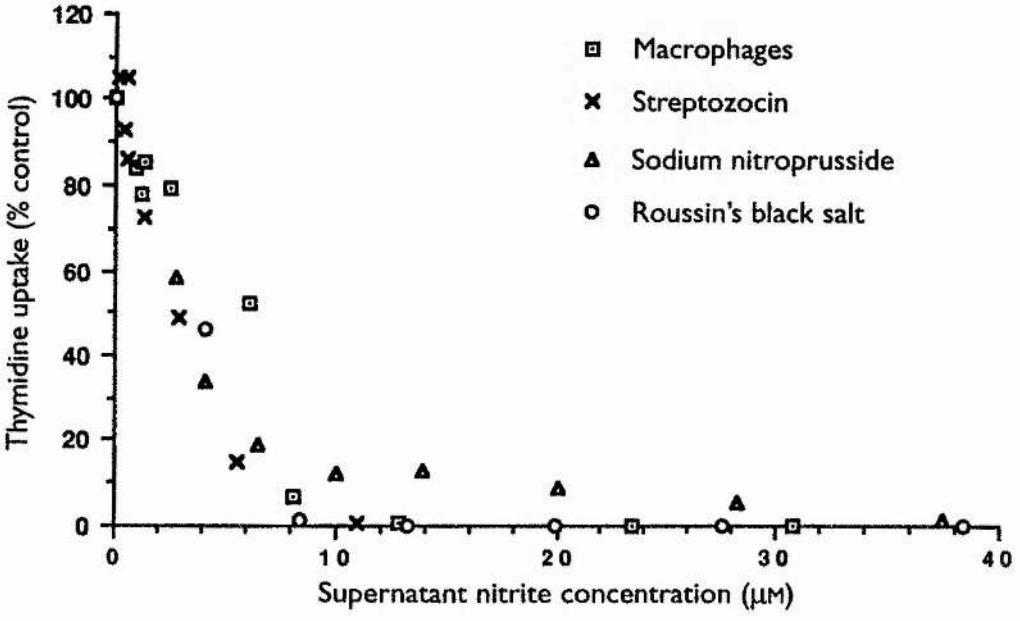
The effect of macrophages on lymphocyte proliferation is shown in Figure 1. Increasing numbers of macrophages in the cultures results in diminished thymidine uptake by lymphocytes and this effect is abrogated by the addition of 500 μM-L-NMMA to the medium. L-NMMA specifically inhibits the synthesis of L-arginine-derived NO by macrophages. The effects of co-incubation of lymphocytes with varying concentrations of NO-releasing compounds are shown in Figure 2.

Figure 2



Plots showing the effects of streptozocin (*a*), sodium nitroprusside (*b*) and Roussin's black salt (*c*) on lymphocyte proliferation

Figure 3



Plot showing the relationship between nitrite concentration in the cultures and lymphocyte thymidine uptake

Inhibition of lymphocyte proliferation by streptozocin, sodium nitroprusside and Roussin's black salt is independent of the amounts of initial, chemically bound, NO per molecule. The approximate concentrations at which the compounds inhibited 50% of the control thymidine uptake values were found to be 1.2 mM-streptozocin, 12 μM-sodium nitroprusside and 4 μM-Roussin's black salt. Figure 3 shows a correlation between nitrite levels in the culture supernatants and thymidine incorporation by the lymphocytes. Clearly, the effects on lymphocyte proliferation of equivalent amounts of NO released from streptozocin, sodium nitroprusside and Roussin's black salt are similar. Inhibition of lymphocyte proliferation by 50% is achieved by the release of less than 4 μM-NO during the 48 h of culture.

Discussion

The inhibition of lymphocyte proliferation by activated macrophages has been shown to be dependent on L-arginine-derived NO [2]. The results described here, however, show that NO-induced inhibition of lymphocyte proliferation is independent of the source of NO: inorganic and organic chemical sources as well as a biological source of NO were all effective. The correlation of inhibition of lymphocyte proliferation with concentration of nitrite ions in the culture supernatants is shown in Figure 3, with increasing nitrite concentrations reflecting decreased lymphocyte proliferation. Although these results do provide a substantial case for the direct effect of NO on the target lymphocytes, the possibility remains that a common intermediate derived from NO might be the effective agent. Our results suggest that the macrophage may be able to exert regulatory effects on local *in vivo* lymphocyte responses via the release of NO. In addition, as lymphocyte proliferation appears to be very sensitive to the release of NO from a variety of sources, this could constitute a useful model system for the evaluation of chemical and cellular agents, including anti-cancer agents which function via the release of NO.

We thank the Cancer Research Campaign for supporting this work.

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Evidence for the involvement of arginine-dependent nitric oxide formation in autodestructive processes

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Introduction

In type 1 diabetes the loss of insulin-producing β-cells is associated with the infiltration of pancreatic islets by macrophages and lymphocytes. In animal models

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Photochemical release of nitric oxide from iron-sulphur cluster nitrosyls: laser potentiation of vasodilator actions on rat isolated tail artery

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The rat isolated tail artery (RTA) preparation was used as a bioassay system to show that the release of nitric oxide (NO·) from an iron-sulphur cluster nitrosyl (Roussin's 1858 'black' salt, or RBS; see Fig. 1 in Flitney *et al.* 1990) is accelerated during exposure to laser light ($\lambda = 514.5$ and 457.9 nm). The experiments were performed in a darkened laboratory. RTA segments were perfused with Krebs solution containing phenylephrine (5–20 μM; flow rate: 2 ml.min^{-1}). The addition of RBS (0.3–1 μM) to the internal perfusate caused the vessel to dilate. Exposure of RBS solutions to laser light en route to the artery produced an additional vasodilator response during the period of irradiation (5 min). The amplitude of the light-induced vasodilator response (or LIVR) increased with increasing intensities of illumination and was greater at the shorter wavelength. The photochemical efficiency of NO· production was measured by monitoring the decrease in absorbance (360 nm: $\varepsilon = 14,940$ M^{-1} cm^{-1}) of RBS solutions following exposure to light under conditions which exactly simulated those used for recording LIVRs. The steady-state [NO·]s formed during photolysis (means ± S.E.M.) were found to be 22.96 ± 1.43 nM.mW^{-1} (514.5 nm) and 88.62 ± 2.73 nM.mW^{-1} (457.9 nm) for 1 μM RBS; and 8.16 ± 2.15 nM.mW^{-1} (514.5 nm) and 43.2 ± 2.15 nM.mW^{-1} (457.9 nm) for 0.3 μM RBS. These values were used to construct log dose–response curves, relationg the amplitude of the LIVR to [NO·] produced during irradiation. A correction was made for loss of NO· in transit from the site of photolysis to the detector artery (time delay = 45 s): the half-life for NO· in our apparatus was measured and found to be 33 s. The [NO·] producing a half-maximal LIVR (ED_{50} value) was 15.6 nM.

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"REPRIMING" OF VASCULAR SMOOTH MUSCLE PHOTORELAXATION IS DEPENDENT ON ENDOGENOUS OR EXOGENOUS NITRIC OXIDE.

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Precontracted vascular smooth muscle relaxes on exposure to light (Furchgott et al., 1961 Journal of General Physiology 44, 499). The phenomenon is thought to involve activation of soluble guanylate cyclase. Irradiation of precontracted, internally perfused segments of rat tail artery with laser light (514.5 nm; 6min; photon flux = 2.7×10^{14} photons. $sec^{-1}.mm^{-2}$) causes a transient vasodilation which recovers fully during illumination (within ca. 4 mins). The amplitude of subsequent photorelaxations (PRs) is dependent on the time interval between consecutive exposures: the "repriming" process requires 2-3 hrs. The nitric oxide (NO) synthase inhibitor N^G-monomethyl-L-arginine (LNMMA; 100μM) prevents repriming. Bolus injections (10μl) of S-nitroso-N-acetyl penicillamine (SNAP; 1mM) or stimulation of endogenous NO production with a 5 min. perfusion of carbachol (0.1mM) both accelerate repriming, producing enhanced PRs within ca. 10 min. Ferrohaemoglobin, a scavenger of NO, suppresses PRs. These results suggest that PR is due to the photochemical release of NO from a molecular store within the tissue. The store can be depleted and then replenished, either by endothelium-derived NO (slow repriming) or from an NO donor drug, such as SNAP (fast repriming).

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THE VASODILATOR ACTION OF *S*-NITROSOGLUTATHIONE: EVIDENCE FOR NO TRANSFER

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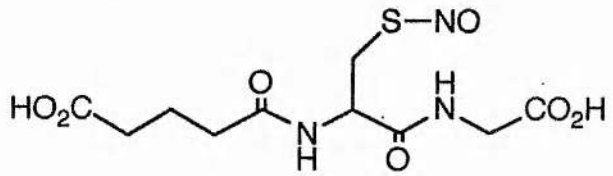
Nitrosothiols are effective NO-donor drugs but, once in solution, are thermally unstable and therefore difficult to use. A survey has shown that amongst the most stable is *S*-nitrosoglutathione (SNOG). Its vaso-



SNOG

dilator action on precontracted isolated rat tail artery has been examined and compared with that of related compounds. *In vitro* studies have shown that, in the presence of an ionised thiol (RS^-), there is transfer of the NO group from SNOG to form RSNO, which may decompose to NO and a disulphide far more readily than SNOG itself. Thus, RS^- is a 'catalyst' in the production of NO from SNOG. We have examined the *in vitro* chemistry of the production of NO from SNOG in the presence of *inter alia* cystein, cystein methyl ester, *N*-acetylcystein, penicillamine, and *N*-acetylpenicillamine and the results are consistent with the slow step being the decomposition of the nitrosothiol formed by NO transfer from SNOG. Further, an examination of the vasodilator action of SNOG on precontracted isolated rat tail artery in the presence of a thiol in the perfusate is consistent with the vasodilator action of SNOG alone being due to NO transfer to endogenous cystein residues in the cells of the artery.