PROTECTION OF VASCULAR NITRIC OXIDE BY SUPEROXIDE DISMUTASE MIMETICS

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Andrew MacKenzie©

Clinical Research Initiative

West Medical Building

University of Glasgow

Glasgow G12 8QQ

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SUMMARY

1. The reduced species of molecular oxygen, superoxide anion, rapidly destroys nitric oxide and thereby impairs the vital vasodilator role played by the endothelium. Therapeutic treatment with the endogenous scavenger of superoxide, superoxide dismutase (SOD) is of limited value since it cannot penetrate cell membranes to protect nitric oxide intracellularly.

2. In this study, a number of structurally distinct membrane-permeant SOD mimetics were examined both biochemically for their ability to inhibit superoxide-catalysed reactions and functionally for their ability to protect nitric oxide from destruction by oxidant stress in rabbit aorta.

3. The SOD mimetics investigated were the nitroxide spin traps PTIYO (4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide) and tempol (4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl), the superoxide scavenger tiron (4,5-dihydroxy-1,3-benzene-disulfonic acid), the metal-based compounds CuDIPS (Cu [II]-[diisopropylsalicylate]$_2$) and MnTMPyP (Mn [III] tetrakis [1-methyl-4-pyridyl] porphyrin), and the metal salts $CuSO_4$ and $MnCl_2$.

4. These mimetics were investigated for their ability to inhibit the superoxide anion-catalysed reduction of cytochrome c and nitro blue tetrazolium. The rank order of potency in inhibiting the reduction of cytochrome c was $CuSO_4$ > $MnCl_2$ > CuDIPS > MnTMPyP > tiron ≥ tempol > PTIYO. The requirement of EDTA prevented the assessment of the activity of $CuSO_4$, $MnCl_2$ and CuDIPS in the assay involving inhibition of reduction of nitro blue tetrazolium. However, the rank order of potency for those agents which could be examined (MnTMPyP > tiron > tempol > PTIYO) was similar to that seen in the cytochrome c assay.

5. Novel models of oxidant stress were generated in rabbit isolated aortic rings by inactivating endogenous Cu/Zn SOD with diethyldithiocarbamate DETCA (60 min) either alone at 3 mM or at 0.3 mM in combination with superoxide generation using xanthine oxidase (XO; 4.8 mu ml^{-1}) and hypoxanthine (HX; 0.1 mM).

6. Addition of XO/HX to DETCA (0.3 mM)-treated tissues powerfully impaired both basal and acetylcholine-induced relaxation and exogenous SOD (250 u ml^{-1}) fully reversed the blockade, suggesting the oxidant stress was extracellular. CuDIPS (0.1 - 3 μM), $CuSO_4$ (0.3 - 3 μM), $MnCl_2$ (1 - 100 μM) and MnTMPyP (100 - 600 μM) also reversed the blockade powerfully. Tempol (30 μM – 1 mM) and tiron (0.3 – 10 mM) reversed the blockade weakly and PTIYO (10 – 300 μM) enhanced the blockade.

7. ACh-induced relaxation was inhibited by DETCA (3 mM, 60 min) and was not restored by exogenous SOD (250 u ml^{-1}), suggesting the oxidant stress was intracellular. MnTMPyP (600 μM and 1 mM) and $MnCl_2$ (100 μM) were the only agents to reverse the blockade of ACh-induced relaxation.

8. Thus, MnTMPyP was the only SOD mimetic to restore nitric oxide-dependent relaxation in conditions of both extracellular and intracellular oxidant stress. It was found, however, that MnTMPyP can paradoxically destroy basal nitric oxide through the generation of superoxide. This undoubtedly compromises its therapeutic potential in the treatment of vascular pathologies associated with oxidant stress.

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ABBREVIATIONS

1400W.....N-(3-(aminomethyl)benzyl)acetamidine

ACh.....acetylcholine

ALS.....amyotrophic lateral sclerosis

ATP.....adenosine triphosphate

bNOS.....brain nitric oxide synthase

CAT.....catalase

cGMP.....cyclic guanosine triphosphate

CuDIPS.....Cu [II]-[diisopropylsalicylate]$_2$

Cu/Zn SOD.....copper/ zinc superoxide dismutase

DETCA.....diethyldithiocarbamate

DNA.....deoxyribonucleic acid

EDRF.....endothelium-derived relaxing factor

EDTA.....ethylenediaminetetraacetic acid

eNOS.....endothelial nitric oxide synthase

FAD.....flavin adenine dinucleotide

FMN.....flavin mononucleotide

GSH.....glutathione

GSSG.....oxidised glutathione

GTP.....guanosine triphosphate

H_4B.....tetrahydrobiopterin

HX.....hypoxanthine

L-NAME.....N^G-nitro-L-arginine methyl ester

LY 83583.....6-anilino-5,8-quinolinedione

MLCK.....myosin light chain kinase

Mn SOD.....manganese superoxide dismutase

MnTMPyP.....Mn [III] tetrakis [1-methyl-4-pyridyl] porphyrin

mRNA.....messenger ribonucleic acid

NAD.....nicotinamide adenine dinucleotide (oxidised form)

NADH.....nicotinamide adenine dinucleotide (reduced form)

NADPH.....nicotinamide adenine dinucleotide phosphate

nNOS.....neuronal nitric oxide synthase

NOS.....nitric oxide synthase

NO.....nitric oxide

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

PE.....phenylephrine

PEG.....polyethylene glycol

PGH.....prostaglandin H

PTIYO.....4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide

SHR.....spontaneously hypertensive rat

SOD.....superoxide dismutase

tempol.....4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl

tiron.....4,5-dihydroxy-1,3-benzene-disulfonic acid

TRIM.....1-(2-trifluoromethylphenyl) imidazole

Tris buffer.....tris[hydroxymethyl]-aminomethane hydrochloride

XO.....xanthine oxidase

PUBLICATIONS

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DECLARATION

I declare that this thesis has been composed by myself and is a record of my work performed by myself. It has not been submitted previously for a higher degree.

This research was carried out in the Clinical Research Initiative, University of Glasgow, under the supervision of Professor W. Martin.

Andrew MacKenzie

November 1998

CHAPTER 1

INTRODUCTION

1.1 VASCULAR NITRIC OXIDE

1.1.1 The vascular endothelium

The luminal surface of the entire cardiovascular system, from the heart to capillaries, is lined by a smooth, single layer of endothelial cells, forming an interface between the blood and underlying tissue. These cells, collectively known as the endothelium, play an important role both in short- and long-term regulation of vascular homeostasis.

The endothelium provides anti-thrombogenic activity via the release of prostacyclin and nitric oxide, both of which inhibit platelet activation (Moncada *et al.*, 1976; Radomski *et al.*, 1987a). Additionally, nitric oxide inhibits platelet adhesion to the vessel wall (Radomski *et al.*, 1987b). Also released is tissue plasminogen activator which initiates the dissolution of blood clots by plasmin (Loskutoff & Edington, 1977). The endothelium also participates in haemostasis by releasing von Willebrand factor (Jaffe *et al.*, 1973), a factor VIII-related protein, which promotes adherence of platelets to a damaged vessel wall facilitating formation of a platelet plug. It also synthesises active hormones from inactive precursors such as in the conversion of angiotensin I to angiotensin II by the angiotensin converting enzyme (Caldwell *et al.*, 1976).

The endothelium can also modulate vascular tone by the production and release of the vasodilator mediators prostacyclin (Moncada *et al.*, 1976), endothelium-derived

hyperpolarising factor (Taylor & Weston, 1988), and endothelium-derived relaxing factor (Furchgott & Zawadzki, 1980) later found to be nitric oxide (Palmer *et al.*, 1987), as well as the vasoconstrictor peptide endothelin-1 (Yanagisawa *et al.*, 1988).

1.1.2 Endothelium-derived relaxing factor

Acetylcholine (ACh) is a potent vasodilator *in vivo* but does not always produce relaxation in isolated blood vessel preparations. Furchgott & Zawadzki (1980) reconciled this by their discovery that endothelial cells play an obligatory role in the relaxation. The simple explanation was that the previously over looked endothelial cells were almost always inadvertently rubbed off during isolation of blood vessels for tension recording. Using a 'sandwich' preparation of a rabbit endothelium-containing aortic (donor) strip co-mounted with an endothelium-denuded (detector) strip, they further demonstrated that ACh stimulated the endothelial cells to release a humoral substance which in turn acted on the smooth muscle of the detector strip to produce vasorelaxation. They termed this substance 'endothelium derived relaxing factor' (EDRF).

Endothelium-dependent relaxations involving EDRF were subsequently demonstrated in many vascular preparations from many species and in response to a variety of substances other than acetylcholine, such as bradykinin, substance P, calcium ionophore A23187, vasopressin, adenine nucleotides and histamine (see Moncada *et al.*, 1991).

Further characterisation of the nature of EDRF came from isolated tissue studies and variants of the 'sandwich' bioassay type of preparation described above. It was reported that EDRF had a half-life of only a few seconds in oxygenated physiological Krebs solution (Griffith *et al.*, 1984; Cocks *et al.*, 1985). The reduced species of oxygen, superoxide anion, appeared to contribute to the instability of EDRF since its relaxant effects in cascade bioassay systems were potentiated and prolonged by the addition of the scavenger of superoxide, superoxide dismutase (SOD; Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986b). SOD also prevented the action of other inhibitors of EDRF, such as pyrogallol and hydroquinone, suggesting that these act by generating superoxide (Moncada *et al.*, 1986). Another inhibitor of EDRF, haemoglobin, however, did not act via a superoxide related mechanism but was found to bind the EDRF molecule (Martin *et al.*, 1985b; Hutchinson *et al.*, 1987). An important discovery came from Rapport & Murad (1983) who reported that the endothelium-dependent relaxation of smooth muscle was mediated via the stimulation of soluble guanylate cyclase, with concurrent elevation of cyclic 3',5'-guanosine monophosphate (cGMP) levels. Indeed, the inactivator of soluble guanylate cyclase, methylene blue (Gruetter *et al.*, 1981), blocked endothelium-dependent relaxation (Martin *et al.*, 1985a). Methylene blue, however, is now known to have the additional action of superoxide anion generation (Wolin *et al.*, 1990) and has been superseded as the drug of choice by the more selective inhibitor of soluble guanylate cyclase, ODQ (Garthwaite *et al.*, 1995). Schmidt *et al.* (1998) report, however, that ODQ possibly has the additional action of interfering with the synthesis of nitric oxide. The ability of selective inhibitors of cGMP phosphodiesterase, such as M&B 22948 (zaprinast), to potentiate endothelium-

dependent relaxation further supported the role of cGMP in vascular relaxations (Martin *et al.*, 1986b). The most recently introduced inhibitor of cGMP phosphodiesterase 5, sildenafil (Viagra), has been shown to be some 100 – 240 fold more potent than zaprinast (Moreland *et al.*, 1998). This compound has great clinical utility in the treatment of male impotence (Goldstein *et al.*, 1998). This effect is derived from the involvement of nitric oxide in the relaxant responses that induce penile erection.

1.1.3 Chemical nature of endothelium-derived relaxing factor

In 1986, Furchgott and Ignarro both speculated, on the basis of almost identical pharmacological profiles and physical and chemical properties, that EDRF may be nitric oxide or a closely related species (see Furchgott 1988; Ignarro *et al.*, 1988). Conclusive evidence for this came from a series of compelling experiments conducted by Palmer *et al.* (1987). In these studies, nitric oxide was measured, by chemiluminesence, as the product of its reaction with ozone. It was reported that nitric oxide was released in a concentration-dependent manner from cultured endothelial cells in response to stimulation by bradykinin and that the levels of nitric oxide released were sufficient to account for the relaxation of vascular detector strips. In addition, both EDRF and nitric oxide caused a relaxation of the vascular strips that declined at the same rate during passage down a bioassay cascade. Furthermore, the relaxant actions of EDRF and nitric oxide were inhibited by haemoglobin and enhanced by SOD to a similar degree.

The chemical properties of nitric oxide could readily explain many of the characteristics of EDRF. The inactivation of EDRF by haemoglobin could be explained conveniently by the avid binding of nitric oxide to the haem iron of this molecule (Gibson & Roughton, 1957; Martin *et al.*, 1985b). In addition, the ability of superoxide anion to inactivate EDRF could be readily explained as a consequence of its extremely rapid reaction with nitric oxide, rate constant 6.9 x $10^9 M^{-1}s^{-1}$ (Huie & Padmaja, 1993). Furthermore, the vasorelaxant activity of nitrovasodilators, which is similar to that of EDRF, had long been suspected to be mediated by nitric oxide (Katsuki *et al.*, 1977; Arnold *et al.*, 1977).

1.1.4 Controversy over acceptance of nitric oxide as EDRF

For some, the finding that EDRF was nitric oxide seemed implausible. Many initial objections were founded on the basis that nitric oxide is a well known noxious agent. It is an environmental pollutant, contributes to acid rain and is found in cigarette smoke (Gibaldi, 1993). Such a labile, toxic gas seemed an unlikely endogenous biological mediator. Many researchers questioned, and provided evidence against, the conclusion that nitric oxide could account for the activity EDRF.

It was reported that EDRF from cultured endothelial cells only relaxed vascular smooth muscle whereas authentic nitric oxide also relaxed guinea pig vascular, tracheal and taenia coli smooth muscle (Shikano *et al.*, 1987; Dusting *et al.*, 1988). These findings were explained, however, by the much lower sensitivity to nitric

oxide of respiratory and gastrointestinal smooth muscle than of vascular smooth muscle (Gillespie & Sheng, 1988).

Another report suggested that EDRF, but not nitric oxide, was stabilised by acidification (Murray *et al.*, 1986). This was explained, however, by the reaction of nitric oxide with oxygen in physiological solution to form nitrite, which can generate nitric oxide when acidified. Nitrite has the same chromatographic mobility as that reported for the acidified 'stable' form of EDRF (Moncada *et al.*, 1991).

It was also suggested that EDRF may be a nitric oxide-releasing molecule, such as an unstable nitroso compound, rather than nitric oxide *per se* (Myres *et al.*, 1990). Based on comparisons of the potency of S-nitrosocysteine with that of nitric oxide on vascular strips and measurements of nitric oxide release by chemiluminesence, they suggested that EDRF was much more likely to be a nitrosylated compound than authentic nitric oxide. Another group (Kukreja *et al.*, 1993) compared the effects of nitric oxide and S-nitrosocysteine on tissues in relation to the associated increment in nitrite concentration and concluded that S-nitrosocysteine resembled EDRF more closely than nitric oxide. In contrast, however, more recent studies by Feelisch *et al.* (1994) propose that the actions of EDRF and nitric oxide, but the not S-nitrosothiols S-nitrosocysteine and S-nitrosocystcamine, were inactivated by L-cysteine, suggesting that EDRF and nitric oxide are identical.

It was recently suggested that nitric oxide, formed in neuronal tissue, is initially released as nitroxyl anion and that SOD plays an obligatory role in the conversion of

this to nitric oxide (Schmidt *et al.*, 1996). This finding, however, has been disputed by Xia & Zweier (1997b) who report that electron paramagnetic resonance spectroscopy measures directly authentic nitric oxide but not nitroxyl anion produced from neuronal tissue. These authors speculated that the above findings by Schmidt *et al,.* (1996) derive from the additional presence superoxide in the experimental procedure which would explain the necessity of SOD to detect nitric oxide.

It appears, therefore, that most of the evidence suggesting that EDRF is not authentic nitric oxide has been disputed. Nevertheless, even if EDRF is initially released as a precursor molecule, it seems virtually certain that its biological effects are ultimately mediated by nitric oxide.

1.1.5 Relaxant mechanisms of nitric oxide

Vascular smooth muscle cell tone is controlled primarily by the intracellular level of calcium. Free intracellular calcium binds to calmodulin and this complex attaches to and activates myosin light-chain kinase (MLCK). This protein kinase then utilises ATP to phosphorylate myosin which can then interact with actin. This initiates crossbridge cycling and the generation of force resulting in cell contraction (see Berne & Levy, 1998). It seems reasonable that the mechanism of vascular smooth muscle relaxation induced by nitric oxide occurs either via the lowering of free intracellular calcium levels or by reducing sensitivity to calcium.

Once produced, nitric oxide can readily pass through biological membranes where it can activate soluble guanylate cyclase in the smooth muscle cells. The endogenous receptor for nitric oxide on guanylate cyclase is the ferrous haem moiety (Ignarro *et al.*, 1982). The nitric oxide-guanylate cyclase bond dislocates the haem iron, thereby inducing a conformational change of the enzyme, which in turn activates the catalytic site (Tsai, 1994). The subsequent increase in formation of cGMP from guanosine triphosphate produces smooth muscle relaxation. The primary mechanism by which cGMP induces smooth muscle relaxation is by lowering intracellular calcium levels and phoshorylating MLCK (Collins *et al.*, 1986). It is likely that cGMP exerts its actions via a cGMP-dependent protein kinase. This activated protein kinase lowers calcium levels by: (i) stimulating calcium-ATPase activity to sequester calcium into the sarcoplasmic reticulum (Amrani *et al.*, 1995) and out of the cell (Ficus, 1988); (ii) stimulating calcium-activated potassium channels leading to a hyperpolarisation-mediated inhibition of calcium entry through L-type voltage-gated calcium channels (Robertson *et al.*, 1993); (iii) phosphorylation of phospholipase C resulting in inhibition of 1,4,5-trisphosphate formation and consequent release of calcium from sarcoplasmic reticulum (Hirata *et al.*, 1990); and (iv) inhibition of receptor-operated calcium channels (Godfraind, 1986). The protein kinase also lowers sensitivity to calcium by increasing the dephosphorylation of myosin light chain (Welch & Loscalzo, 1994). It has also been suggested that nitric oxide can directly open potassium channels in smooth muscle (Koh *et al.*, 1995), which would cause hyperpolarisation, leading to relaxation (see Figure 1.1).

1.1.6 Synthesis of nitric oxide

Synthesis of nitric oxide requires oxygen and the amino acid, L-arginine, as substrates. Cofactors required include calcium, calmodulin, flavin adenine dinucleotide, flavin mononucleotide, NADPH and tetrahydrobiopterin (Palmer *et al.*, 1988; Palmer & Moncada, 1989; Mayer *et al.*, 1989; Mulsch *et al.*, 1989; Bredt & Snyder, 1990; Hevel *et al.*, 1991; Stuehr *et al.*, 1991a; Leone *et al.*, 1991; Schmidt *et al.*, 1992b). See Figure 1.1.

The enzymatic generator of nitric oxide, nitric oxide synthase (NOS), appears to catalyse a five electron reduction of the terminal guanidino nitrogen atom of L-arginine to form nitric oxide. The formation of nitric oxide is likely to occur in at least a two step process. In the first step, L-arginine is converted to N^G-hydroxy-L-arginine by means of reduced NADPH- and tetrahydrobiopterin-dependent mono-oxygenation. In the second step, the oxidative cleavage of the C=N bond of N^G-hydroxy-L-arginine leads to the formation of nitric oxide and the byproduct, L-citrulline (Leone *et al.*, 1991; Stuehr *et al.*, 1991a; Marletta, 1993; Korth *et al.*, 1994) (see Figure 1.1).

Cells that synthesise nitric oxide possess an active uptake mechanism for L-arginine. Uptake is mediated by an active transporter, y+, which is also used by other cationic amino acids (Kerwin *et al.*, 1995; Bogle *et al.*, 1996). Some inhibitors of NOS, such as N^G-monomethyl-L-arginine, appear to exert an additional inhibitory action by competing with L-arginine for the cationic transporter (Kerwin *et al.*, 1995).

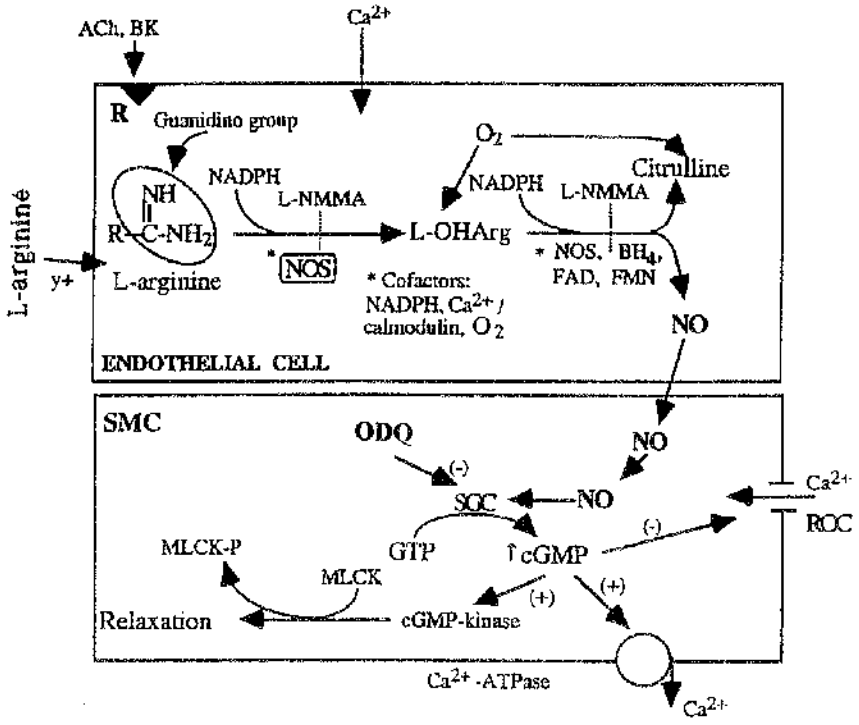


FIGURE 1.1 Biosynthesis of endothelial nitric oxide (NO). NO is formed from the terminal guanidino group of the amino acid L-arginine, which is taken into the cell by an active transport mechanism (y+). The activation of receptors (R) by agonists such as acetylcholine (ACh) or bradykinin (BK) leads to increased intracellular Ca^{2+} levels which activate the NOS to form NO and L-citrulline through the intermediate compound N^{G}-hydroxy-L-arginine (L-OHArg) and the use of cofactors. NOS is blocked by analogues of L-arginine, such as L-NMMA. In smooth muscle cells (SMC), NO activates soluble guanylate cyclase (SGC; which is blocked by ODQ), increasing cGMP levels. cGMP produces relaxation by several mechanisms including: inhibition of Ca^{2+} entry through receptor operated channels (ROC); stimulation of membrane Ca^{2+}-ATPases and inhibition of myosin light chain kinase (MLCK). See text for details. Cofactor abbreviations: BH_4 (tetrahydrobiopterin); FAD (flavin adenine dinucleotide); FMN (flavin mononucleotide). Taken from Marín & Rodríguez-Martínez, 1997.

1.1.7 Isoforms of nitric oxide synthase

Many non-endothelial cells are able to synthesise nitric oxide. To date, three isoforms of NOS have been identified as being responsible for this synthesis. The isoforms are termed NOS I, II, and III (see Förstermann *et al.*, 1994).

NOS I was first purified in brain tissue (Bredt & Snyder, 1990) and is therefore sometimes referred to as bNOS (for brain) or nNOS (for neuronal). These classifications, however, are somewhat imprecise since NOS I has been localised in the epithelium of lung, uterus and stomach (Schmidt *et al.*, 1992a) and in skeletal muscle (Nakane *et al.*, 1993). This enzyme is constitutively expressed and is regulated not only by calcium/calmodulin but also by calcium/calmodulin-dependent protein kinase II (which inhibits activity) and protein kinase C (which enhances activity) (Nakane *et al.*, 1991).

NOS II is not usually expressed but is induced in cells by bacterial lipopolysacchaarides and/or certain cytokines (Stuehr *et al.*, 1991a; Hevel *et al.*, 1991). This enzyme is sometimes referred to as iNOS (for inducible). NOS II expression is classically associated with macrophages but can be also be found in smooth muscle cells (Mitchell *et al.*, 1992), lymphocytes, neutrophils, Kupffer cells and endothelial cells (Bandaletova *et al.*, 1993). This enzyme is calcium-independent, and was originally believed also to be calmodulin-independent. It has been shown, however, to bind calmodulin so tightly that it is continuously activated by this cofactor even when calcium levels are low (Cho *et al.*, 1992). The regulation

of its activity is therefore by the induction of transcription (Xie *et al.*, 1992) and *de novo* synthesis of NOS II protein. This gives NOS II the characteristic of producing greater and longer lasting release of nitric oxide than the two constitutive isoforms.

NOS III is found in endothelial cells and is sometimes called eNOS (for endothelial). As with bNOS or nNOS, the term eNOS is largely an oversimplification since it has also been localised in platelets (Radomski *et al.*, 1990) and certain neuronal populations of the brain (Dinerman *et al.*, 1994). Unlike the other two soluble enzymes, it is more than 90 % particulate and anchored in the membrane (Förstermann *et al.*, 1991; Mitchell *et al.*, 1991a). Like NOS I, NOS III is constitutively expressed and is regulated by calcium/calmodulin. The pathway for the calcium increase in endothelial cells is unclear. It has been suggested, however, that entry may be through a receptor-operated ion channel (Johns *et al.*, 1987) and, to a lesser degree, calcium release from cyclopiazonic acid- and thapsigargin (calcium-ATPase inhibitors)-sensitive internal stores (Hutcheson & Griffith, 1997). It has been reported that the shear stress of flowing blood upregulates NOS III expression (Nishida *et al.*, 1992).

In humans, NOS I, II and III are encoded by separate genes located on chromosomes, 12 and 17, 7 respectively (Marsden *et al.*, 1993; Xu *et al.*, 1993). Despite this, the NOSs have many features in common and all show a high degree of amino acid sequence homology with NADH-cytochrome P_{450} reductase. Also, across species, the amino acid sequences for each isoform are well conserved (>90 % for NOS I and III and > 80 % for NOS II; see Förstermann *et al.*, 1994; Kerwin *et al.*, 1995). All

isoforms can be inactivated by high concentrations of nitric oxide. Therefore nitric oxide may function as a negative feedback modulator of its own synthesis (Rogers & Ignarro, 1992; Assreuy *et al.*, 1993). The mechanisms by which nitric oxide inactivates NOS is unknown but it may bind with the haem moiety of the enzyme or induce S-nitrosylation of a critical thiol.

1.1.8 Inhibitors of nitric oxide synthase

Considering the requirement for so many cofactors and the mechanisms by which it is regulated, inhibition of NOS can be realised in a variety of ways (see Fukuto & Chaudhuri, 1995). These can essentially be split into two groups, non-selective and isoform selective inhibitors.

Non-selective inhibitors operate by inactivating elements common to all isoforms. All NOS enzymes require flavin adenine dinucleotide and flavin mononucleotide. Therefore inhibitors of flavoproteins would be expected to inhibit NOS activity. Indeed, one such inhibitor, diphenyleneiodonium, has been shown to inhibit NOS I in nitrergic nerves (Rand & Li, 1993), NOS II in macrophages and NOS III in endothelial cells (Stuehr *et al.*, 1991b). In addition, inhibition of tetrahydrobiopterin biosynthesis, by 2,4-diamino-6-hydroxypyrimidine, also leads to a loss of NOS II activity in macrophages (Sakai *et al.*, 1993) and NOS III in endothelial cells (Schmidt *et al.*, 1992b). Furthermore, analogues of L-arginine represent another route to inhibit NOS activity. These include N^G-methyl-L-arginine, N^G-nitro-L-arginine and N^G-nitro-L-arginine methyl ester which have been shown to be

competitive inhibitors of NOS I, NOS II and NOS III (Gross *et al.*, 1990; Rees *et al.*, 1990).

Isoform-specific inhibitors of NOS operate by inactivating elements unique to the isoenzyme. For example, inhibition of calmodulin would be expected to impair NOS I and III function but not NOS II since this enzyme is not regulated by this agent. Indeed, the calmodulin inhibitors, calmidazolium and fendiline, inhibit ACh-induced relaxation in rat aorta but have no effect on cytokine-induced NOS II expression and nitric oxide production (Schini & Vanhoutte, 1992). In addition, some L-arginine analogues inhibit certain NOS isoforms more than others. N^G-nitro-L-arginine, inhibits NOS I more powerfully than NOS II (Furfine *et al.*, 1993). Moreover, N-amino-L-arginine and N-nitro-L-arginine analogues were about 100 fold more potent in inhibiting NOS III than the other isoenzymes (Gross *et al.*, 1991). Recently, however, much more selectively potent inhibitors have become available, such as the NADPH-dependent agent, N-(3-(aminomethyl)benzyl)acetamidine (1400W), which has been described as 1000-fold more potent against NOS II than NOS III (Garvey *et al.*, 1997). Aminoguanidine (Misko *et al.*, 1993) and L-N6-(1-iminoethyl)lysine (Moore *et al.*, 1994) have also has been described as a highly selective inhibitors of NOS II while 7-nitroindazole (Moore *et al.*, 1993; Nanri *et al.*,1998) and 1-(2-trifluoromethylphenyl) imidazole (TRIM) (Handy *et al.*, 1995; Handy *et al.*, 1996) selectively inhibit NOS I.

1.1.9 Physiological functions of nitric oxide synthase isoforms

Selective inhibition of the NOS isoforms has uncovered many of the physiological functions of these enzymes.

Nitric oxide produced from NOS I mediates neurotransmission both in the central and peripheral nervous systems. In the central system, nitric oxide has been associated with long-term potentiation, a process connected with the formation of memory (Shibuki & Okada, 1991; Izumi *et al.*, 1992). Indeed, inhibitors of NOS impair learning and can produce amnesia in chicks and rats (Holscher & Rose, 1992; Bölune *et al.*, 1993). In the periphery, nitric oxide appears to mediate neurotransmission in a particular set of non-adrenergic non-cholinergic nerves. These 'nitrergic' nerves (Rand, 1992) innervate some smooth muscle of the gastrointestinal tract, pelvic viscera and airways and their stimulation results in muscle relaxation (see Gillespie *et al.*, 1990; Martin & Gillespie, 1991).

Induction of NOS II produces large amounts of nitric oxide in macrophages or other cells types. These high concentrations of nitric oxide play a primary role in the defence against micro-organisms, parasites or tumour cells. Nitric oxide can bind to iron in proteins of target cells which can inhibit key enzymes including ribonucleotide reductase (involved in DNA replication) and iron-sulphur cluster dependent enzymes involved in mitochondrial electron transport e.g. aconitase (Nathan & Hibbs, 1991). In addition, nitric oxide can have a direct effect on the DNA of target cells causing stand breaks and fragmentation (Wink *et al.*, 1991;

Fehsel *et al.*, 1993). There is also clear evidence that superoxide anion generated from macrophages can react with co-released nitric oxide to from the potent oxidant, peroxynitrite (Xia & Zweier, 1997a). Peroxynitrite can cause nitration of proteins leading to altered protein function, increased protein turnover and alteration of tyrosine phosphorylation (Crow & Beckman, 1995). A combination of these effects probably explains the cytotoxic effects of nitric oxide. Why macrophages are more resistant to nitric oxide than their target cells is not yet known.

NOS III is constitutively active and nitric oxide is, therefore, constantly produced from the endothelium. This results in a tonic dilatory influence on the vascular smooth muscle (Eglème *et al.*, 1984; Griffith *et al.*, 1984; Rubanyi *et al.*, 1985; Martin *et al.*, 1986a). Nitric oxide production can be further stimulated by a number of agonists (see section 1.1.2) and physical stimuli such as the shearing of flowing blood (Pohl *et al.*, 1986; Rubanyi *et al.*, 1986; Busse & Fleming, 1998) or hypoxia (Pohl & Busse, 1989; Igari *et al.*, 1998). The importance of endothelial NOS III in the control of vascular tone is clearly demonstrated when the L-arginine/nitric oxide pathway is disrupted by infusion of inhibitors of NOS. This leads to peripheral vasoconstriction and increased blood pressure (Rees *et al.*, 1989; Gardiner *et al.*, 1990; Tresham *et al.*, 1991). Nitric oxide produced from NOS III in the endothelium can also inhibit platelet adhesion to the vascular wall (Radomski *et al.*, 1987b).

1.1.10 Vascular disorders associated with nitric oxide

Inappropriate or over production of nitric oxide can disrupt normal vascular tone and, in severe cases, can result in major pathology. For example, nitric oxide is thought to play a key role in migraine. It is proposed that 5-hydroxytryptamine induces endothelial cells of the cerebral vasculature to release nitric oxide. The nitric oxide can then activate directly sensory fibres to release their transmitters e.g. substance P and calcitonin gene-related peptide, causing oedema formation and inducing pain. Overall, nitric oxide seems to induce a 'sterile' inflammatory response (see Fozard, 1995). This proposal is supported by the finding that some nitric oxide donors, such as amyl nitrite, cause a migraine-like headache (Olesen *et al.*, 1994).

Another condition associated with overproduction of nitric oxide is septic shock. This is a clinical syndrome of excessive hypotension and associated cardiovascular failure. Bacterial endotoxins are likely to induce an over expression of NOS II which causes over production of nitric oxide. This excess of nitric oxide may cause cytotoxicity and extensive vasodilation resulting in myocardial depression and circulatory failure (see Moncada *et al.*, 1991; Förstermann *et al.*, 1994). In animal models of septic shock, the nitric oxide overproduction caused by NOS II induction can be blocked by inhibitors of NOS, thus restoring blood pressure and vascular responses to normal levels (Kilbourne *et al.*, 1994). Such treatment, however, must be used with caution since a general inhibitor of NOS will inactivate NOS I and III as well as isoform II. Such treatment could result in additional complications for the patient. Therefore, development of a highly selective inhibitor of NOS II, such as

1400W (Garvey *et al.*, 1997; Hamilton & Warner, 1998) or aminoguanidine (Misko *et al.*, 1993) offer great therapeutic potential.

Under production and/or activity of nitric oxide may also contribute to vascular pathologies. Indeed, many pathologies are associated with reduced endothelium-dependent relaxation. For example, endothelium-dependent relaxation to acetylcholine is reduced in spontaneously hypertensive rats (Dohi *et al.*, 1990; Grunfield *et al.*, 1995). Human hypertensive subjects also have impaired vasorelaxation to acetylcholine (Linder *et al.*, 1990) and reduced vasoconstriction to inhibitors of NOS, suggesting reduced influence of basal nitric oxide activity as compared to normotensives (Panza *et al.*, 1993). Hypercholesterolaemia and atherosclerosis are also associated with reduced endothelium-dependent vasorelaxation (Shimokawa & Vanhoutte, 1989; Wennmaln, 1994; Ohara *et al.*, 1995). Moreover, reduced endothelium-dependent relaxation has been reported for human and animal diabetic (Calver *et al.*, 1992; Heygate *et al.*, 1995; Kamata & Kobayashi, 1996) and heart failure subjects (Kubo *et al.*, 1991; Lindsay *et al.*, 1992; Hirooka *et al.*, 1994). Recent findings suggest that a defect in the nitric oxide pathway can promote abnormal vascular remodelling and may facilitate pathological changes in vessel wall morphology associated with diseases such as hypertension and atherosclerosis (Taddei *et al.*, 1998).

Consideration of the source(s) of the nitric oxide dysfunction in the above diseases could perhaps open up novel therapeutic possibilities. As already mentioned, nitric oxide is rapidly destroyed by the reduced species of molecular oxygen, superoxide anion (Gryglewski *et al.*, 1986). The loss of vasodilator function following the

interaction of nitric oxide with superoxide anion (Kaustic & Vanhoutte, 1989; Ohlstein & Nichols, 1989, Mian & Martin, 1995a) had led to the suggestion that this process may be involved in many pathological process. Consideration of this possibility requires an understanding of reactive oxygen species and endogenous antioxidant mechanisms.

1.2 REACTIVE OXGEN SPECIES

1.2.1 Oxygen

All aerobic organisms are vulnerable to damaging assault by oxygen. This toxicity is largely due to reactive intermediates of oxygen reduction. These reactive oxygen species include the free radicals, superoxide anion and hydroxyl radical, as well as hydrogen peroxide. By definition this last species is not a free radical but is nonetheless an important damaging oxidant. Free radicals can be defined as 'any species capable of independent existence that contain one or more unpaired electrons' (Halliwell & Gutteridge, 1989). The presence of unpaired electrons usually causes radicals to be unstable and highly reactive species. Molecular oxygen, containing two unpaired electrons, certainly qualifies as a free radical. Due to its outer electron configuration, however, it tends to accept electrons one at a time making it react rather sluggishly with many non-radicals (see Fridovich, 1983; Halliwell & Gutteridge, 1989; Fridovich, 1989; Warner, 1994; Yu *et al.*, 1994; Marin & Rodriguez-Martinez, 1995; Crow & Beckman, 1995; Kerr *et al.*, 1996).

1.2.2 Superoxide anion

Superoxide anion (O_2^-) is formed by a one step reduction (addition of an electron; e^-) of molecular oxygen (O_2).

$$O_2 + e^- \rightarrow O_2^-$$

The term 'superoxide' does not relate to its activity but was coined to describe its electron configuration (Pauling, 1979). In fact, with only one unpaired electron, superoxide is actually less of a radical than oxygen itself. Indeed, many of its deleterious effects are thought to be due to its conversion to more reactive oxygen species such as hydrogen peroxide or hydroxyl radical (see below). Superoxide has been shown, however, to directly cause the oxidation of catecholamines as well as initiating the peroxidation of unsaturated lipids. Importantly, superoxide destroys the vasorelaxant actions of nitric oxide (Gryglewski *et al.*, 1986; Kaustic & Vanhoutte, 1989; Ohlstein & Nichols, 1989, Mian & Martin, 1995a).

Spontaneous dismutation of superoxide to hydrogen peroxide is a very rapid process, with a rate constant 5 x 10^5 M^{-1} s^{-1} at pH 7.8 in aqueous solution, and this is dramatically increased (10,000-fold) by the endogenous enzyme, superoxide dismutase (SOD). This gives superoxide a very short half life, 1 x 10^{-6} s at 37 °C, and a steady state cellular concentration of approximately 1 x 10^{-11} M (see Fridovich, 1983; Halliwell & Gutteridge, 1989; Yu *et al.*, 1994; Marin & Rodriguez-Martinez, 1995).

Superoxide anion is produced biologically from a number of sources: membrane bound NADH/NADPH oxidase (Pagano *et al.*, 1995; Rajagopalan *et al.*, 1996), xanthine oxidase (M^{c}Cord & Fridovich, 1968), cyclo-oxygenase (Kontos *et al.*, 1985; Cosentino *et al.*, 1994), lipoxygenase (Kukreja *et al.*, 1986), nitric oxide synthase (Pou *et al.*, 1992; Xia *et al.*, 1996; Xia & Zweier, 1997a), and electron spillage during electron transport chain from mitochondria (Boveris, 1977). In addition, autoxidation of compounds such as haemoglobin (Misra & Fridovich, 1972a), catecholamine (Misra & Fridovich, 1972b), thiols (Misra, 1974), sulfhydryl compounds (Jia & Furchgott, 1993) and guanidino containing compounds (Aoyagi *et al.*, 1989) result in formation of superoxide anion.

1.2.3 Hydrogen peroxide

Addition of an electron to superoxide anion gives the peroxide ion (O_2^{2-}). The oxygen-oxygen bond in this ion is relatively weak, and in biological systems, is usually stabilised by a couple of hydrogen ions (H^+) forming hydrogen peroxide (H_2O_2).

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide is formed mainly from the dismutation of superoxide but can also be generated directly from such enzymes as glycollate oxidase, D-aminoacid oxidase, and urate oxidase. Hydrogen peroxide is not a free radical (it does not

contain unpaired electrons) but is a weak oxidant. It is more stable than superoxide and can easily diffuse through cell membranes. It is, however, potentially more cytotoxic than superoxide due to its ability to inactivate many enzymes directly via the oxidation of thiols. Moreover, it directly causes increased permeability of endothelial monolayers (Berman & Martin, 1993) and impairs contraction and endothelium-dependent relaxation in rabbit aorta (Dowell *et al.*, 1993). Its major route of damage, however, is thought to be via the generation of hydroxyl anion, either by spontaneous decomposition or by reaction with transition metals (see below and for review see Halliwell & Gutteridge, 1989; Yu *et al.*, 1994; Marin & Rodriguez-Martinez, 1995; Kerr *et al.*, 1996).

In addition to its actions as a oxidant, hydrogen peroxide can also induce vascular relaxation by endothelium-dependent and -independent mechanisms (Furchgott, 1991; Zembowicz *et al.*, 1993; Mian & Martin, 1995a; Mian & Martin, 1995b).

1.2.4 Hydroxyl radical

Addition of an electron to hydrogen peroxide can generate hydroxyl radical. This reaction is, however, slow but can be catalysed *in vivo* by the combined presence of superoxide anion and trace amounts of transition metals, specifically iron or copper. This is known as the superoxide-assisted Fenton reaction or the transition metal-catalysed Haber-Weiss reaction. Here, free ferric iron (Fe^{3+}) or cupric copper (Cu^{2+}) is reduced by superoxide anion to ferrous iron (Fe^{2+}) or cuprous copper (Cu^{+}) (Step 1). The reduced iron or copper is then oxidised by hydrogen peroxide (H_2O_2) to form

hydroxyl radical ($OH^{\bullet}$) and the inert hydroxide anion (OH^-) (Step 2). (Halliwell & Gutteridge, 1989).

Step 1: $Fe^{3+} / Cu^{2+} + O_2^{\bullet-} \rightarrow Fe^{2+} / Cu^{+} + O_2$

Step 2: $Fe^{2+} / Cu^{+} + H_2O_2 \rightarrow Fe^{3+} / Cu^{2-} + OH^{\bullet} + OH^-$

Net: $O_2^{\bullet-} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + O_2$

Hydroxyl radical is considered to be potentially the most potent oxidant encountered in biological systems and can react with virtually all classes of biological molecule. Some authors argue that its effective diffusion radius of only a few angstroms, along with its half life of approximately $1x10^{-9}$s at 37 °C, make it too reactive to elicit any specific damage. During its brief life, however, it may initiate a propagating chain of reactions that can lead to lipid peroxidation, DNA alteration and damage to other vital materials such as proteins.

The hydroxyl radical-mediated cytotoxicity of superoxide and hydrogen peroxide is influenced, therefore, by the distribution of free metal ions. Consequently, tightly controlled sequestration of metal ions in 'safe' forms could prevent formation of this damaging radical. Generation of hydroxyl radical by hydrogen peroxide can be catalysed, however, by iron-containing proteins such as haemoglobin. In addition, superoxide anion can cause ferritin, an iron storage molecule, to release iron which can then participate in the Haber-Weiss reaction. Most copper complexes present *in vivo*, however, are not able to contribute to the generation of hydroxyl radical and free copper levels are kept low by binding proteins such as caeruloplasmin and albumin. Nevertheless, the Cu/Zn isoform of SOD is inactivated by prolonged

exposure to hydrogen peroxide perhaps due to the generation of hydroxyl radical which could destroy the histidine residues essential for the catalytic mechanism (for review see Fridovich, 1983; Halliwell & Gutteridge, 1989; Fridovich, 1989; Warner, 1994; Yu *et al.*, 1994; Marin & Rodriguez-Martinez, 1995; Crow & Beckman, 1995; Kerr *et al.*, 1996). Hydroxyl radical can also be formed by a metal-independent means, via the decomposition of peroxynitrite (see below).

Hydroxyl radical has been reported to play a role in endothelium-dependent acetylcholine (ACh)-induced relaxation (Rubanyi & Vanhoutte, 1986b). This conclusion is derived from the ability of the hydroxyl radical scavenger, mannitol, to impair relaxation. Prasad & Bharadwaj (1996) also report that hydroxyl radical is involved in ACh-induced relaxation and propose that it is formed via breakdown of peroxynitrite, generated from the interaction of superoxide anion with ACh-stimulated release of nitric oxide (see below). The inability, however, of exogenously applied SOD to affect ACh-induced relaxation (Ohlstein *et al.*, 1989; Abrahamsson *et al.*, 1991; Mian & Martin, 1995a) would suggest that superoxide (and hence peroxynitrite and hydroxyl radical) play no role in the endothelium-dependent relaxation. Indeed, the evidence that ACh-induced relaxation is mediated by nitric oxide and not some other agent is overwhelming.

1.2.5 Peroxynitrite

The reaction of nitric oxide with superoxide anion forms peroxynitrite ($ONOO^-$)

$$NO + O_2^{\cdot} \rightarrow ONOO^{-}$$

Peroxynitrite is stable (for weeks) in alkaline solutions but at physiological pH has a half-life of 1 – 2 s. It also has properties identical to hydroxyl radical (Beckman *et al.*, 1990). These authors proposed that the protonated form of peroxynitrite can decompose to form hydroxyl radical and nitrogen dioxide (NO_2).

$$ONOO^{-} + H^{+} \rightarrow ONOOH$$

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

Decomposition of peroxynitrite, therefore, forms an alternative pathway to the Fenton or Haber-Weiss reaction (section 1.2.4) for formation of hydroxyl radical.

In addition, peroxynitrite is a powerful cytotoxic oxidant. It can cause oxidation and nitration of tyrosine residues in proteins (it is thought to inactivate Mn-based superoxide dismutase (SOD) in this manner; MacMillan-Crow *et al.*, 1998) and alcohols in sugars. Moreover, it directly attacks sulfhydryl groups of molecules and proteins and initiates lipid peroxidation (see Marin & Rodriguez-Martinez, 1995; Crow & Beckman, 1995).

Even under physiological conditions, peroxynitrite is stable enough to diffuse several cell diameters and cross lipid membranes to damage critical cellular machinery either directly or by decomposing to form hydroxyl radical. Clearly, this agent is highly

toxic but its activity can be controlled by impairing the ability of superoxide anion to react with nitric oxide.

In vivo, macrophages may utilise peroxynitrite, formed from co-released nitric oxide and superoxide, to attack invading organisms or other pathogens (Xia & Zweier, 1997a). In addition, peroxynitrite is also a powerful vasorelaxant. Dowell *et al.* (1997; 1998) report that this is due to peroxynitrite reacting with an alcohol functional group or thiol to form novel relaxant species. The physiological relevance of this action, if any, remains to be resolved.

1.2.6 Other reactions involving nitric oxide

Nitric oxide is itself a free radical and in addition to its reaction with superoxide to form peroxynitrite, it also reacts with other oxygen-derived species.

Nitric oxide can react with molecular oxygen to from nitrogen dioxide (NO_2)

$$2NO + O_2 \rightarrow 2NO_2$$

In the gas phase, this reaction occurs rapidly resulting in the characteristic brown fumes of nitrogen dioxide. Indeed, one of the objections to nitric oxide accounting for the actions of EDRF was that it would be immediately converted to nitrogen dioxide. In the aqueous phase, however, this reaction is slow with a half-life of

hours. The short physiological half-life of nitric oxide is not, therefore, likely to be due to destruction by molecular oxygen (Butler *et al.*, 1995).

Nitric oxide can also react with ozone (O_3) leading to the production of activated (high energy state) nitrogen dioxide.

$$NO + O_3 \rightarrow NO_2 \text{ (excited)} + O_2$$

$$NO_2 \text{ (excited)} \rightarrow NO_2 + \text{light}$$

This generation of light can be easily detected by chemiluminescence. Indeed, this reaction formed the basis by which the original experiments were conducted to identify endothelium-derived relaxing factor as nitric oxide in biological systems (Palmer *et al.*, 1987; see section 1.1.3).

1.3 DEFENCES AGAINST REACTIVE OXYGEN SPECIES

Agents which act to counteract the risks induced by reactive oxygen species are known generally as antioxidants. These can be defined as 'any substance that, when present in low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate' (Halliwell & Gutteridge, 1989). These include a diverse range of agents that work by quite varied chemical process, but essentially can be divided into two groups: first, those which prevent the generation of reactive species, such as inhibitors of enzymes that generate radicals or agents which tightly bind metals which could act as catalysts in the formation of

reactive species. The second group act to scavenge the radicals once they are formed. These include specific enzymes such as superoxide dismutase which scavenges superoxide, and catalase and glutathione peroxidase both of which act to scavenge hydrogen peroxide. Removal of superoxide anion and hydrogen peroxide prevents the formation of hydroxyl radical.

1.3.1 Superoxide dismutase

The major endogenous antioxidant mechanism against superoxide anion is the enzyme superoxide dismutase (SOD). This enzyme catalyses the oxidation of one molecule of superoxide to form molecular oxygen, and reduction of another molecule of superoxide (with the additional involvement of hydrogen ions) to form hydrogen peroxide. The rate constant for the reaction is about 2×10^9 M^{-1} s^{-1} and is insensitive to pH over a wide range (Fridovich, 1983). It is also proposed that SOD has a cluster of positive charges surrounded by a broad distribution of negative charges elsewhere on the protein which attracts the negatively charged superoxide anion to its active site (Getzoff *et al.*, 1983).

Three isoforms of SOD have been identified and located in mammalian organisms and each is encoded by a separate gene: intracellular Cu/Zn SOD (Heckl *et al.*, 1988); extracellular Cu/Zn SOD (Hjalmarsson *et al.*, 1987); and Mn SOD located mainly in the mitochondria (Ho & Crapo, 1988). A fourth Fe-containing isoform is found only in bacteria. All isoforms of SOD catalyse the same reaction with comparable efficiency (Fridovich, 1989). For the mammalian types of SOD, the

copper and manganese metals are the active sites of these enzymes. Both metals undergo redox cycling to catalyse the reaction described above, i.e.

$$SOD\text{-}Cu^{2+}/Mn^{3+} + O_2^- \rightarrow SOD\text{-}Cu^{+}/Mn^{2+} + O_2$$
$$SOD\text{-}Cu^{+}/Mn^{2+} + O_2^- + 2H^+ \rightarrow SOD\text{-}Cu^{2+}/Mn^{3+} + H_2O_2$$

Net: $$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Intracellular Cu/Zn SOD is found in, and synthesised by, virtually every cell type in the body. Each enzyme is composed of two identical subunits with an overall molecular weight of 32,000 daltons. Each subunit contains one copper and one zinc molecule.

The extracellular Cu/Zn SOD enzyme is a tetrametric glycoprotein with a molecular weight of 135,000 daltons and is found in plasma, lymph and synovial fluid. This enzyme is secreted by only a few cell types, such as fibroblasts and glia cells, to diffuse around and reversibly bind to heparin on the surface of most tissue types and in the interstitial matrix (Marklund, 1990).

Both intracellular and extracellular isoforms of Cu/Zn SOD are inactivated by the copper chelator diethyldithiocarbamate. This irreversibly binds copper at the active site and removes the metal from the enzyme (Heikkila *et al.*, 1976; Cocco *et al.*, 1981; Kelner *et al.*, 1989). The zinc appears to stabilise the enzyme.

Mn SOD is a tetramer of molecular weight 88,000 daltons and is located strictly in the inner membrane of mitochondria. This enzyme acts to scavenge superoxide overflowing from the electron transport chain. In contrast to Cu/Zn SOD, Mn SOD is not inhibited by diethyldithiocarbamate but is destroyed by chloroform and ethanol (Halliwell & Gutteridge, 1989).

The importance of the antioxidant action of SOD to cells is clearly demonstrated in mutant organisms in which this enzyme is omitted. Such organisms are highly susceptible to damage by superoxide anion and this injury is prevented following the reintroduction of the SOD gene (Warner, 1994). Altered genetic expression of SOD is linked to two known diseases in humans. The first is familial amyotrophic lateral sclerosis (ALS) which is an age-dependent degenerative disorder of motor neurones in the cortex and spinal cord. This disorder is linked to mutated gene expression on chromosome 21. It results in SOD levels being less than 50 % of normal (Rosen *et al.*, 1993). The pathology associated with ALS may therefore be due to accumulation of superoxide anion in motor neurones. The second disorder is Down's syndrome which is associated with 3 copies of chromosome 21 in cells. In contrast to ALS, this leads to a 50 % elevation in levels of Cu/Zn SOD (Garber *et al.*, 1979). With no similar concurrent increase in catalase activity, this could lead to overproduction of hydrogen peroxide (Warner, 1994). Indeed this condition, perhaps as an adaptive response, is associated with elevated levels of glutathione peroxidase. Cells from individuals with Down's syndrome have increased lipid peroxidation, perhaps due to elevated hydrogen peroxide, which can lead to premature cell death especially in the brain (Ceballos *et al.*,1988).

Findings following the use of the use of, diethyldithiocarbamate, which inactivates both extracellular and intracellular isoforms of Cu/Zn SOD suggests that this enzyme exerts a vital role in protecting nitric oxide produced by the vascular endothelium (Omar *et al.*, 1991; Mügge *et al.*, 1991a; Abrahamsson *et al.*, 1992; Mian & Martin, 1995a) and nitrergic nerves (Martin *et al.*, 1994; Lilley & Gibson, 1995) from destruction by superoxide anion. Deficiency in Cu/Zn SOD activity thus provides a mechanism by which inactivation of nitric oxide by superoxide anion could occur. Indeed, decreased expression of Cu/Zn SOD mRNA has been correlated with impaired endothelium-dependent relaxation on diabetic rats (Kamata & Kobayashi, 1996).

Levels of Cu/Zn SOD associated with ALS and Down's syndrome suggest that the expression of this enzyme is constitutive and directly related to copy number. However, cells and organisms, exposed to reactive oxygen species can display increased expression of either Cu/Zn SOD or Mn SOD. For example, exposure to elevated levels of superoxide induces greater expression of Mn SOD in neonatal rat lungs (Stevens & Autor, 1977). In addition, the superoxide-generator, paraquat, induces enhanced Cu/Zn SOD expression in leukocytes (Niwa *et al.*, 1990). The factors controlling expression of antioxidant enzymes are poorly understood but may be related to levels of cytokines such as interleukin-1 and tumour necrosis factor (Fridovich, 1983; Warner, 1994).

1.3.2 Catalase

The endogenous enzyme catalase acts to remove hydrogen peroxide. This enzyme is present in all major organs of the body and is especially concentrated in liver and erythrocytes. It is largely located in peroxisomes (subcellular organisms bounded by a single membrane) which contain hydrogen peroxide-generating enzymes, such as glycollate oxidase.

Catalase is a tetrameric protein having a molecular weight of 60,000 daltons. Each protein subunits contains a haem (Fe^{3+}-protoporphyrin) group at its active site and a molecule of NADH which stabilises the enzyme. It catalyses the reduction of hydrogen peroxide, via an intermediate compound (compound I), by converting it to water (H_2O) and molecular oxygen at a rate constant of 2×10^7 M^{-1} s^{-1} (see Fridovich, 1983; Halliwell & Gutteridge, 1989; Fridovich, 1989; Warner, 1994; Yu *et al.*, 1994)

$$\text{catalase-Fe}^{3+} + H_2O_2 \rightarrow \text{compound I}$$

$$\text{compound I} + H_2O_2 \rightarrow \text{catalase-Fe}^{3+} + H_2O + O_2$$

net: $$2H_2O_2 \rightarrow 2H_2O + O_2$$

The exact structure of compound I is uncertain but it is perhaps a two electron oxidation product of catalase-Fe^{3+}, i.e. catalase-Fe^{5+}.

Catalase can be inhibited by sodium azide or cyanide but a more specific inhibitor is aminotriazole (Mian & Martin, 1995b; Mian & Martin, 1997). This agent exerts its actions by binding to and irreversibly inhibiting compound I. Therefore, it will only inhibit catalase if hydrogen peroxide is available to allow generation of this intermediate.

As with SOD, catalase activity can be upregulated in cells exposed to reactive oxygen species. For example, it was reported that there was a 20-fold increase in catalase activity in Chinese hamster fibroblasts exposed to high concentrations of hydrogen peroxide (Spitz *et al.*, 1992).

1.3.3 Glutathione peroxidase

Glutathione peroxidase is an endogenous selenium-containing enzyme which offers an alternative pathway of hydrogen peroxide removal. It is a tetrameric protein with a molecular weight of about 76,000 daltons. It is fairly ubiquitous but found in high concentrations in heart, lung and brain. The enzyme catalyses the oxidation of the thiol, glutathione, at the expense of hydrogen peroxide at a rate constant of 5×10^7 $M^{-1} s^{-1}$.

In its reduced state, glutathione (GSH), is a low molecular weight tripeptide (glutamic acid-cysteine-glycine). Glutathione peroxidase catalyses the oxidation of two molecules of GSH. This liberates two hydrogen ions from the –SH groups of cysteine which then reduce hydrogen peroxide to water. In the process, the two GSH

molecules join together by forming the disulphide bridge of oxidised glutathione (GSSG).

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

Most free glutathione *in vivo* is present in the reduced GSH form rather than oxidised GSSG. This is due to the presence of the enzyme glutathione reductase which catalyses the reduction of GSSG to GSH at the expense of NADPH. Glutathione reductase is a dimeric protein with each subunit containing a flavin adenine dinucleotide (FAD) at its active site. The NADPH reduces the FAD which then passes the electrons onto the disulphide bridge between the two cysteine residues in GSSG. This breaks the bridge, reforming the -SH groups and the reduced GSH.

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

Both catalase and glutathione peroxidase function to remove hydrogen peroxide. In addition to differences in their catalytic action, these two enzymes have different substrate affinities. Catalase has a high capacity for the reduction of hydrogen peroxide but a low affinity for its substrate. Whereas, glutathione peroxidase has a low capacity but a high affinity for hydrogen peroxide. It is thought, therefore, that low levels of hydrogen peroxide are removed preferentially by glutathione peroxidase while high levels are reduced by catalase (see Halliwell & Gutteridge, 1989; Fridovich, 1989; Warner, 1994; Yu *et al.*, 1994; Marin & Rodriguez-Martinez, 1995)

1.3.4 Other antioxidants

In addition to the enzymes described above that deal with specific substrates, other antioxidants exist that are known to protect against reactive oxygen species.

In addition to its actions as a vitamin, ascorbate (vitamin C) is a powerful water soluble reducing agent. This provides ascorbate with the ability to neutralise many reactive species. For example, ascorbate is known to reduce superoxide anion and hydroxyl radical forming the semidehydroascorbate radical (oxidised ascorbate) in the process. Ascorbate may play an important physiological antioxidant role in the lens of the human eye where its levels are high and expression of SOD is low (Halliwell & Gutteridge, 1989).

Glutathione, in addition to its role as the substrate for the hydrogen peroxide-removing enzyme glutathione peroxidase (section 1.3.3), also directly scavenges hydroxyl radical itself. Furthermore, the end product of purine metabolism, uric acid, is also known to be a powerful scavenger of hydroxyl radical (Halliwell & Gutteridge, 1989). Vitamin E is also reported to have antioxidant actions. Indeed, vascular relaxations to ACh were impaired in Vitamin E deprived rats and normal relaxation was restored following vitamin E supplementation (Rubino & Burnstock, 1994). Furthermore, the lipid soluble vitamins A and E are thought to play a vital role in preventing reactive oxygen species-induced lipid peroxidation and low density lipoprotein oxidation (Halliwell & Gutteridge, 1989).

Interestingly, it has been hypothesised that basal release of nitric oxide may act to scavenge superoxide anion thereby providing antioxidant protection to stimulated release of nitric oxide (Laight *et al.*, 1998). Any antioxidant actions of nitric oxide by this method would, however, produce the damaging species peroxynitrite.

1.4 REACTIVE OXYGEN SPECIES IN PATHOLOGY

1.4.1 Oxidant stress and impaired endothelium-dependent relaxation

The fragile equilibrium that exists between production of reactive oxygen species and endogenous antioxidant activity may be altered toward oxidative stress in pathology. Oxidant stress can be defined as 'an imbalance between oxidants and antioxidants in favour of the oxidants, leading to damage' (Sies, 1996). As determined by plasma markers of radical damage such as malondialedchyde, many diseases (see below) are associated with oxidant stress. It is likely that the enhanced generation of superoxide anion in these diseases will destroy greater amounts of nitric oxide. The attraction between these two radicals is enormous. Indeed, the rate constant for this reaction (6.9×10^9 M^{-1} s^{-1}; Huie & Padmaja, 1993) exceeds even that for the reaction of superoxide with its endogenous scavenger, superoxide dismutase (2×10^9 M^{-1} s^{-1}; Fridovich, 1983).

It is not surprising therefore that enhanced destruction of endothelium-derived nitric oxide by superoxide anion forms a common defect in a number of conditions. Indeed, hypertension (Nakazono *et al.*, 1991; Grunfeld *et al.*, 1995; Bouloumie *et al.*,

1997), atherosclerosis (Sharma *et al.*, 1992; Ohara *et al.*, 1993), ischaemia-reperfusion injury (Downey, 1990), diabetes (Hattori *et al.*, 1991; Kamata & Kobayashi, 1996) and heart failure (McMurray *et al.*, 1990; Katz *et al.*, 1993) are all associated with increased free radical production and reduced nitric oxide-dependent relaxation. The source of the superoxide in each case may be different, endothelial and smooth muscle NADPH/NADH oxidase in angiotensin II-induced hypertension (Rajagopalan *et al.*, 1996), endothelial xanthine/xanthine oxidase and neutrophil NADPH oxidase in ischaemia-reperfusion injury (Downey, 1990), advanced glycosylation productions in diabetes (Bucala *et al.*, 1991), and mitochondrial respiration and NADH oxidoreductase in heart failure (Mohazzab *et al.*, 1995).

1.4.2 Therapeutic intervention

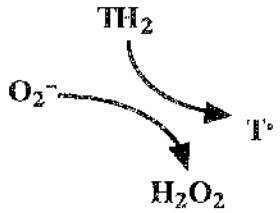
As detailed in section 1.3.1, the integrity of endogenous Cu/Zn SOD is of vital importance in protecting endothelium-derived nitric oxide from destruction by superoxide anion. Endogenous levels of this enzyme may therefore be critical in the aetiology of vascular pathologies associated with oxidant stress. Consequently, stable elevation of the activity of SOD might form the basis of a rational strategy for therapeutic intervention in these cardiovascular pathologies. It is likely, however, that therapeutic treatment with Cu/Zn SOD itself will be of limited effectiveness. This is because SOD is not orally active, is rapidly cleared from the circulation and, in view of its large size, is unable to penetrate cell membranes to protect nitric oxide from an intracellular oxidant stress (Gardner *et al.*, 1996). Greater therapeutic

potential may lie, however, with low molecular weight, membrane-permeant, compounds that exhibit SOD-like activity.

1.4.3 Membrane-permeant superoxide dismutase mimetics

A diverse range of membrane-permeant superoxide dismutase mimetics has been described. These include superoxide scavengers, nitroxide spin traps and metal-based compounds.

The superoxide anion scavenging agent tiron (4,5-dihydroxy-1,3-benzene-disulfonic acid; Ledenev *et al.*, 1986) has been shown to inhibit superoxide-induced damage in neutrophils (Gyllenhammer, 1987) and lymphocytes (Devlin *et al.*, 1981). Tiron (TH_2) is thought to scavenge superoxide by reducing it to hydrogen peroxide leaving the tiron radical (T•) (Ledenev *et al.*, 1986).



Nitroxide spin traps are agents used in electron spin resonance (ESR) studies. They react with highly reactive radicals to produce another long-lived radical that can be detected by ESR. Nitroxides, such as PTIYO (4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide) and tempol (4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl), have been reported to have superoxide anion-scavenging ability in

biochemical and cultured cell studies (Mitchell *et al.*, 1990; Ewing & Janero, 1995). Nitroxides (RN-O; where R denotes the rest of the molecule) are thought to oxidise superoxide to molecular oxygen. The reduced nitroxide can then be reoxidised back to the parent nitroxide (Samuni, 1991).

$$RN\text{-}O + O_2^{\cdot-} + H^{+} \rightarrow RN\text{-}OH + O_2$$

The metal-based agents CuDIPS (Cu [II]-[diisopropylsalicylate]$_2$; Huber *et al.*, 1987; Sorenson, 1995) and MnTMPyP (Mn [III] tetrakis [1-methyl-4-pyridyl] porphyrin; Faulkner *et al.*, 1994; Gardner *et al.*, 1996) as well as the simple metal salts of Cu and Mn (Huber *et al.*, 1987; Beyer & Fridovich, 1990) also possess SOD-like activity in biochemical and isolated cell studies. Their modes of action are thought to mimic the catalytic sites of Cu/Zn SOD and Mn SOD, respectively (see section 1.3.1).

Before commencement of this study, none of the above putative SOD mimetics had been investigated for their potential ability to protect endothelium-derived nitric oxide from destruction by superoxide anion.

1.5 AIM

The aim of this study was to determine whether SOD mimetics were potentially useful in protecting vascular nitric oxide from destruction in conditions of oxidant stress.

CHAPTER 2

GENERATION OF OXIDANT STRESS IN RABBIT AORTA

2.1 INTRODUCTION

Endothelium-derived nitric oxide is of vital importance in the regulation of vascular tone and blood pressure (see Moncada *et al.*, 1991). Nitric oxide is destroyed, however, by the reduced species of molecular oxygen, superoxide anion (Gryglewski *et al.*, 1986). In eukaryotic cells, three isoforms of superoxide dismutase (SOD) have evolved to cope with the stress induced by the oxygen-rich environment: an intracellular Cu/Zn-containing form, an extracellular Cu/Zn-containing form, and a Mn-containing form found in the mitochondria, and all dismutate superoxide at equivalent rates (Fridovich, 1989). Endogenous levels of Cu/Zn SOD have been shown to be critically important in protecting nitric oxide from destruction by superoxide in a number of isolated blood vessels including the aorta of the rabbit (Mügge *et al.*, 1991a) and rat (Mian & Martin, 1995a) and the bovine pulmonary (Cherry *et al.*, 1990) and coronary arteries (Omar *et al.*, 1991).

The loss of vasodilator function following the interaction of nitric oxide with superoxide has led to the suggestion that this process is involved in a number of cardiovascular pathologies. For example, hypertension (Nakazono *et al.*, 1991; Grunfeld *et al.*, 1995; Bouloumie *et al.*, 1997), atherosclerosis (Sharma *et al.*, 1992; Ohara *et al.*, 1993), ischaemia-reperfusion injury (Downey, 1990), diabetes (Hattori *et al.*, 1991; Kamata & Kobayashi, 1996) and heart failure (McMurray *et al.*, 1990; Katz *et al.*, 1993) are all associated with increased free radical production and reduced nitric oxide-dependent relaxation. Endogenous levels of SOD may therefore be of critical importance in the aetiology of vascular pathologies associated with

oxidant stress. Stable elevation of the activity of SOD might, therefore, form the basis of a rational strategy for therapeutic intervention in these cardiovascular pathologies. Therapeutic treatment with Cu/Zn SOD itself, however, is of limited effectiveness. This is because SOD is not orally active, is rapidly cleared from the circulation and, in view of its large size, is unable to penetrate cell membranes to protect nitric oxide from an intracellular oxidant stress (Gardner *et al.*, 1996). Greater therapeutic potential may lie, however, with low molecular weight, membrane-permeant, compounds that exhibit SOD-like activity.

A diverse range of these compounds has been described but before the putative SOD mimetics can be assessed for their ability to protect endothelium-derived nitric oxide, conditions must be created where nitric oxide-dependent relaxation is impaired by oxidant stress. These models of oxidant stress should hopefully mimic the pathological process associated with vascular dysfunction. Oxidant stress can be defined as 'an imbalance between oxidants and antioxidants in favour of the oxidants, leading to damage' (Sies, 1996). In this particular study, the roles of the oxidant, superoxide anion, and the antioxidant, SOD, were the subject of investigation. There are two potential strategies, therefore, which can be adopted to develop oxidant stress: the first, to decrease the activity of the antioxidant enzyme SOD. This could be achieved by use of a copper chelator such as diethyldithiocarbamate (DETCA). DETCA irreversibly inactivates Cu/Zn SOD both in the intracellular and extracellular space by chelating the copper from the enzyme, but has no effect on Mn SOD (Heikkila *et al.*, 1976; Cocco *et al.*, 1981; Halliwell & Gutteridge, 1989). Inactivation of endogenous SOD leads to increased levels of

superoxide in the vasculature, as determined by chemiluminesesce (Cherry *et al.*, 1990; Omar *et al.*, 1991; Pagano *et al.*, 1993). The second strategy is to elevate levels of superoxide anion by its exogenous generation. Many superoxide anion-generators are available but some of the better characterised include: the xanthine oxidase/hypoxanthine (XO/HX) system in which XO oxidises HX to form xanthine and superoxide anion and xanthine is then further oxidized by XO to produce uric acid and another superoxide anion (Halliwell & Gutteridge, 1989). Also available are LY 83583 and pyrogallol, both are quinones and can create oxidative stress by undergoing redox cycling, i.e. the quinone autoxidises to its semiquinone which then reacts reversibly with oxygen to form quinone and superoxide anion (Halliwell & Gutteridge, 1989). Together, these strategies can be employed in an attempt to recreate the pathological processes associated with impaired nitric oxide-dependent relaxation and oxidant stress.

This chapter is concerned, therefore, with characterisation of the effects of oxidant stress on the activity of basal and agonist-stimulated endothelium-derived nitric oxide in rabbit aorta. Once established, these models will serve as a basis with which to assay the efficacy of the putative SOD mimetics in protecting endothelium-derived nitric oxide in oxidant stress.

2.2 METHODS

2.2.1 Preparation of Tissues

Male New Zealand White rabbits (2.5 - 3.5 kg) were killed with an injection of sodium pentobarbitone (200 mg kg^{-1}) into the marginal ear vein. The thoracic aorta was then carefully removed and immersed in Krebs bicarbonate buffer solution consisting of (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11, and pre-gassed with 95% O_2 and 5% CO_2. The aorta was then cleaned of fat and connective tissue and care was taken not to damage inadvertently the intimal surface of the aorta where the endothelium is located. Some tissues were used that day while others were stored in oxygenated Krebs solution overnight at 4 °C for use the following day. The aorta was then cut into transverse rings (2.5 mm wide) using a razor blade slicing device. In some experiments, the endothelium was removed to abolish the influence of endothelium-derived nitric oxide. This was achieved by locating the aortic ring between two stainless steel hooks, with the base hook weighted with a 2 g weight, and gently rubbing the initial surface with a matchstick moistened with Krebs solution. Removal of the endothelium was deemed successful if, following development of phenylephrine-induced tone, no relaxation took place in response to the endothelium-dependent relaxant, acetylcholine (10 nM - 3 μM).

2.2.2 Tension recording

The aortic rings were then mounted under 2 g resting tension on stainless steel hooks within 10 ml tissue baths containing Krebs solution gassed with 95% O_2 and 5% CO_2. The Krebs solution was maintained at 37 °C by a water jacket heated by a thermostatically controlled pump (Techne water circulator model C-85D). Tension was recorded isometrically with Grass FTO3C transducers and responses displayed and recorded on a MacLab (E Series, AD Instruments) or on a Grass polygraph model 7D. Tissues were allowed to equilibrate for 60 min before experiments were carried out, during which time the resting tension was re-adjusted to 2 g, as required.

2.2.3 Induction of tone

Following the 60 min equilibration period, aortic rings were contracted with the stable α_1–adrenoceptor agonist, phenylephrine (PE). Once induction of submaximal (40 - 50% of maximal) PE (30 - 300 nM)-induced tone had stabilised, relaxant responses could be obtained. Relaxant responses are expressed as a percentage (%) relaxation of the PE-induced tone existing immediately prior to addition of the relaxant. A number of the experimental procedures employed affected the level of tone induced by PE. For example, endothelial removal or treatment with the inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methyl ester (L-NAME; Rees *et al.*, 1990), both lead to an increase in sensitivity to PE due to the loss of the vasodepressor influence of basal nitric oxide. In order to take account for these changes in sensitivity, the concentration of PE employed in each individual

experiment was adjusted to ensure that the level of tone achieved was 40 - 50 % of the maximum observed on control, endothelium-containing rings. This procedure ensured that physiological antagonism, resulting from differences in levels of tone, did not contribute to the results obtained. Following completion of each concentration-response curve, the baths were repeatedly washed out and the tissues allowed to re-equilibrate for at least 30 min before further experimentation.

2.2.4 Assessment of basal activity of nitric oxide

Basal activity of nitric oxide was assessed indirectly by measuring the endothelium-dependent depression of PE-induced vasoconstriction on rabbit aortic rings. The rationale for these experiments is that basal nitric oxide produced by the endothelium results in a tonic vasodepressor effect opposing the actions of vasoconstrictor agents (Elglème *et al.*, 1984; Martin *et al.*, 1986a). Consequently, this endothelium-dependent depression of vasoconstriction would be expected to be blocked or enhanced by agents which block or enhance the activity of nitric oxide, respectively. Specifically, submaximal tone was generated using PE (0.1 μM) in endothelium-containing rings and basal nitric oxide activity was assessed by the augmentation of tone seen following addition of the inhibitor of nitric oxide synthase, L-NAME (100 μM).

2.2.5 Assessment of agonist-stimulated release of nitric oxide

Agonist-stimulated activity of endothelial-derived nitric oxide was determined by assessing acetylcholine (ACh)-induced relaxation. Specifically, cumulative concentration-response curves to ACh (10 nM - 3 μM; in increments of 0.5 log units) were constructed on endothelium-containing rings following induction of sub-maximal PE (30 – 300 nM)-induced tone.

2.2.6 Generation of oxidant stress by inhibition of endogenous Cu/Zn superoxide dismutase

In certain experiments the effects were examined of inactivation of the endogenous SOD on basal and agonist-induced activity of nitric oxide. There are two major forms of this enzyme, a Cu/Zn containing form which is located both extracellularly and intracellularly and a Mn-containing form which mainly resides in the mitochondria (Fridovich, 1989). Cu/Zn SOD can be selectively inactivated by use of a copper chelator such as diethyldithiocarbamate (DETCA; Cocco *et al.*, 1981; Halliwell & Gutteridge, 1989; Mian & Martin, 1995a). Since DETCA is a specific copper chelator it will have no effect on Mn SOD. DETCA (0.3 - 10 mM) was added to the Krebs solution bathing the aortic rings for a period of 60 min to allow time for this agent to permeate the tissue and penetrate cells.

Since DETCA inhibits Cu/Zn SOD irreversibly, there is no need for this agent to be present in the tissue bath once it has produced its effects. Consequently, following

the 60 min incubation period, the tissues were extensively washed with fresh Krebs solution to remove DETCA. This procedure ensured that in experiments in which exogenous Cu/Zn SOD was added in an attempt to reverse the actions of DETCA, there was no risk of this added enzyme being inactivated by DETCA.

Following the 60 min incubation period and washout of DETCA, the tissues were submaximally contracted with PE (0.1 μM). The effects of DETCA on basal activity of nitric oxide were assessed by measuring the augmentation of tone seen following addition of the inhibitor of nitric oxide synthase, L-NAME (100 μM).

The effects of DETCA on agonist-stimulated activity of nitric oxide were assessed by cumulative concentration-response curves to ACh (10 nM - 3 μM) constructed on endothelium-containing rings following induction of submaximal PE (30 – 300 nM)-induced tone.

2.2.7 Generation of oxidant stress by superoxide anion production

Tissues were exposed to three well characterised superoxide anion generating systems: xanthine oxidase (XO)/hypoxanthine (HX), LY 83583 and pyrogallol.

XO/HX is a commonly used enzymatic system (McCord & Fridovich, 1968; Halliwell & Gutteridge, 1989; Mian & Martin, 1995a) in which XO oxidises HX to form xanthine and superoxide anion. Xanthine is then further oxidized by XO to produce uric acid and another superoxide anion (Halliwell & Gutteridge, 1989). In

experiments involving XO/HX, XO was added either to precontracted tissues or to the tissues prior to contraction with PE and tone was then induced. In either case XO was in contact with the tissue for at least 20 min to allow time for this enzyme to permeate the interstitial space of the tissues before addition of its substrate, HX.

Both LY 83583 and pyrogallol are quinones and can generate superoxide anion by undergoing redox cycling (Halliwell & Gutteridge, 1989)

The effects of superoxide anion generation on basal nitric oxide activity were assessed differently to the protocols detailed in section 2.2.4. In these experiments, aortic rings were submaximally contracted with PE (0.1 μM) and when the contraction had stabilised the effects of applied XO (4.8 mu ml^{-1})/HX (0.1 mM), LY 83583 (0.3 μM) or pyrogallol (0.1 mM) were examined on tone. Since superoxide destroys nitric oxide and therefore its vasorelaxant activity, any augmentation of tone observed upon the addition of these agents gives an indirect measurement of the activity of basal nitric oxide.

The effects of superoxide anion generation on agonist-stimulated activity of nitric oxide were also assessed. Immediately after stabilisation of submaximal PE (30 - 300 nM)-induced tone and following the addition of either XO (4.8 mu ml^{-1})/HX (0.1 mM), LY 83583 (0.3 μM) or pyrogallol (0.1 mM), cumulative concentration-response curves to ACh (10 nM - 3 μM) were constructed and relaxation compared with that seen on control rings.

2.2.8 Generation of oxidant stress by inactivation of endogenous Cu/Zn superoxide dismutase and superoxide anion production

In certain experiments we wished to examine the effects of superoxide anion generation in tissues in which endogenous Cu/Zn SOD was inhibited. Here Cu/Zn SOD was inactivated by treatment with a low concentration of DETCA (0.3 mM). This concentration produced only slight inhibition (<20 % inhibition of maximal relaxation, see Results section) of ACh-induced relaxation by itself.

Following induction of submaximal PE (30 - 300 nM)-induced tone in tissues pretreated with DETCA (0.3 mM, 60 min, washout), XO (4.8 mu ml^{-1})/HX (0.1 mM), LY 83583 (0.3 μM) or pyrogallol (0.1 mM) were added. The augmentation of tone observed was used as an indirect measure of basal nitric oxide activity. Following addition of superoxide anion generators and immediately after stabilisation of tone, cumulative concentration-response curves to ACh (10 nM – 3 μ M) were constructed and relaxation compared with that seen in control rings.

2.2.9 Treatment with exogenous superoxide dismutase

The effects of exogenously applied Cu/Zn SOD were assessed on basal and ACh-stimulated activity of nitric oxide. In these experiments, SOD (250 u ml^{-1}) was added to submaximally PE-contracted rings at least 20 min before the assessment of basal or ACh-stimulated activity of nitric oxide. Basal nitric oxide activity was assessed by the augmentation of tone observed following the addition of L-NAME (100 μM).

Agonist-induced activity of nitric oxide was assessed by the construction of cumulative concentration-response curves to ACh (10 nM - 3 μM).

In some experiments, an examination was made of the ability of exogenously applied Cu/Zn SOD to reverse the inhibition of basal or ACh-stimulated activity of nitric oxide induced by treatment with DETCA (3 mM, 60 min, washout) alone; with the superoxide anion generators XO (4.8 mu ml^{-1})/HX (0.1 mM), LY 83583 (0.3 μM) or pyrogallol (0.1 mM) alone; or with the superoxide generators in combination with treatment with DETCA (0.3 mM; 60 min; washout). In these experiments, SOD (250 u ml^{-1}) was added to the tissue after incubation and washout of DETCA but 20 min before the addition of the superoxide generators. Basal nitric oxide activity was assessed by the augmentation of tone seen following addition of superoxide anion generators. ACh-induced activity of nitric oxide was assessed by construction of cumulative concentration-response curves to ACh (10 nM - 3 μM).

All experiments involving DETCA, XO/HX, LY 83583 or pyrogallol were conducted in the presence of catalase (1000 u ml^{-1}), given as a pretreatment to prevent accumulation of hydrogen peroxide.

2.2.10 Drugs and solvents

Acetylcholine chloride (ACh), catalase (bovine liver), diethyldithiocarbamate (DETCA), N^{G}-nitro-L-arginine methyl ester (L-NAME), phenylephrine hydrochloride (PE), hypoxanthine (HX), superoxide dismutase (SOD, Cu/Zn-

containing enzyme from bovine erythrocytes) and xanthine oxidase (XO, buttermilk) were obtained from Sigma (Poole, UK). 6-anilino-5,8-quinolinedione (LY 83583) was obtained from Calbiochem (Nottingham, U.K.). Pyrogallol was obtained from BDH Ltd. (Poole, U.K.).

All drugs were dissolved in normal saline (0.9 %), except for hypoxanthine (50 mM stock) and LY 83583 (20 mM stock) which were dissolved in 0.1% sodium hydroxide and ethanol, respectively. Control experiments demonstrated that the solvents, sodium hydroxide and ethanol, did not account for the effects observed with hypoxanthine and LY 83583, respectively. All dilutions were made in saline (0.9 %). Concentrations of drugs are expressed as the final molar concentration in the tissue bath.

2.2.11 Statistical analysis of data

Results are expressed as the mean ± s.e. mean of n separate experiments. Statistical comparisons were made by one-way analysis of variance followed by the Bonferroni post-test. A value of $P<0.05$ was considered significant. Concentration-response curve fitting and statistical analyses were performed using a computer-based programme (Graph Pad, Prism).

2.3 RESULTS

2.3.1 Basal activity of nitric oxide in the rabbit aorta

Basal release of nitric oxide in rabbit aortic rings can be assessed indirectly by measuring the endothelium-dependent depression of phenylephrine (PE)-induced tone (Martin *et al.*, 1986a). This is clearly observed in Figure 2.1 where endothelium-denuded rings are considerably more sensitive to PE than endothelium-containing rings. Consequently, addition of PE (0.1 μM) generated 2.02 ± 0.23 g of tension (n = 30) on endothelium-containing rings but induced substantially greater levels of tone on denuded rings: 3.54 ± 0.15 g; n = 29 (Figure 2.3). Furthermore, treatment of submaximally contracted aortic rings with the inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) significantly enhanced tone in endothelium-containing but not endothelium-denuded rings (Figure 2.2 and 2.3).

2.3.2 Effect of diethyldithiocarbamate and superoxide dismutase on basal activity of nitric oxide

Treatment for 60 min (followed by washout) with diethyldithiocarbamate (DETCA; 0.3, 1 and 3 mM) to inhibit endogenous Cu/Zn superoxide dismutase (SOD) did not affect the enhancement of tone induced by treatment with L-NAME (100 μM) on PE (0.1 μM)-contracted endothelium-containing aortic rings (Figure 2.4).

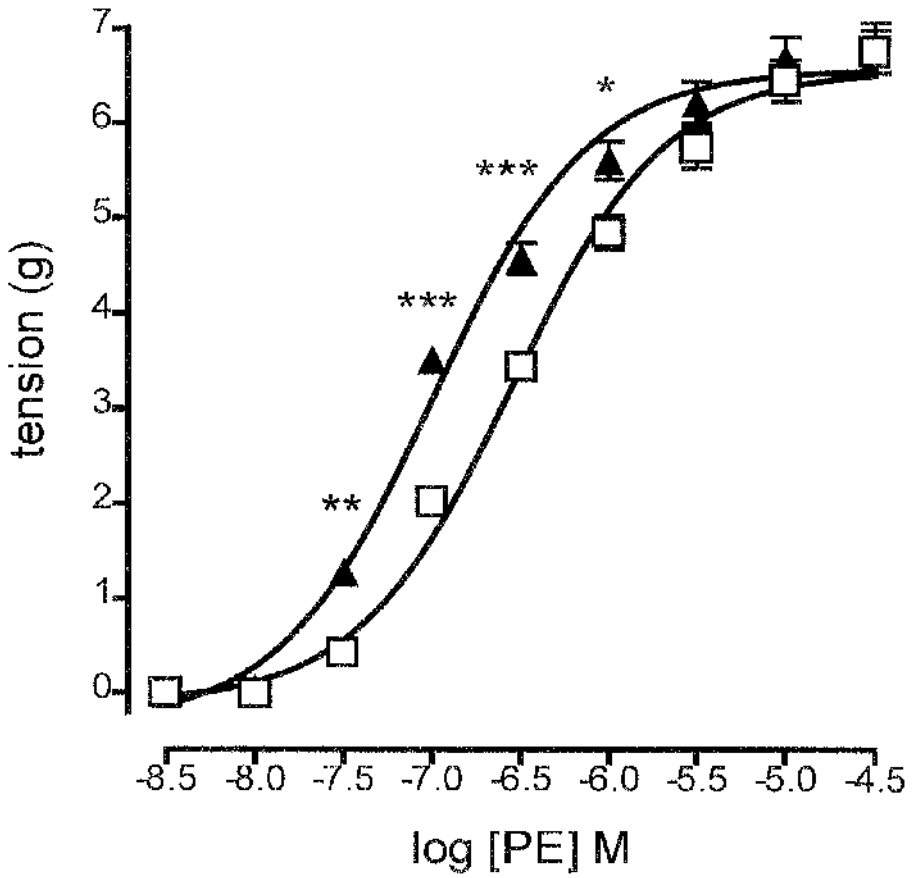


FIGURE 2.1 Concentration-response curves showing the contractile effect of phenylephrine (PE) on endothelium-containing (□) and endothelium-denuded (▲) rings of rabbit aorta. Each point is the mean ± s.e. mean of 29-30 observations. *P<0.05, **P<0.01 and ***P<0.001 indicate significant difference from the level of tone induced by PE in endothelium-containing rings.

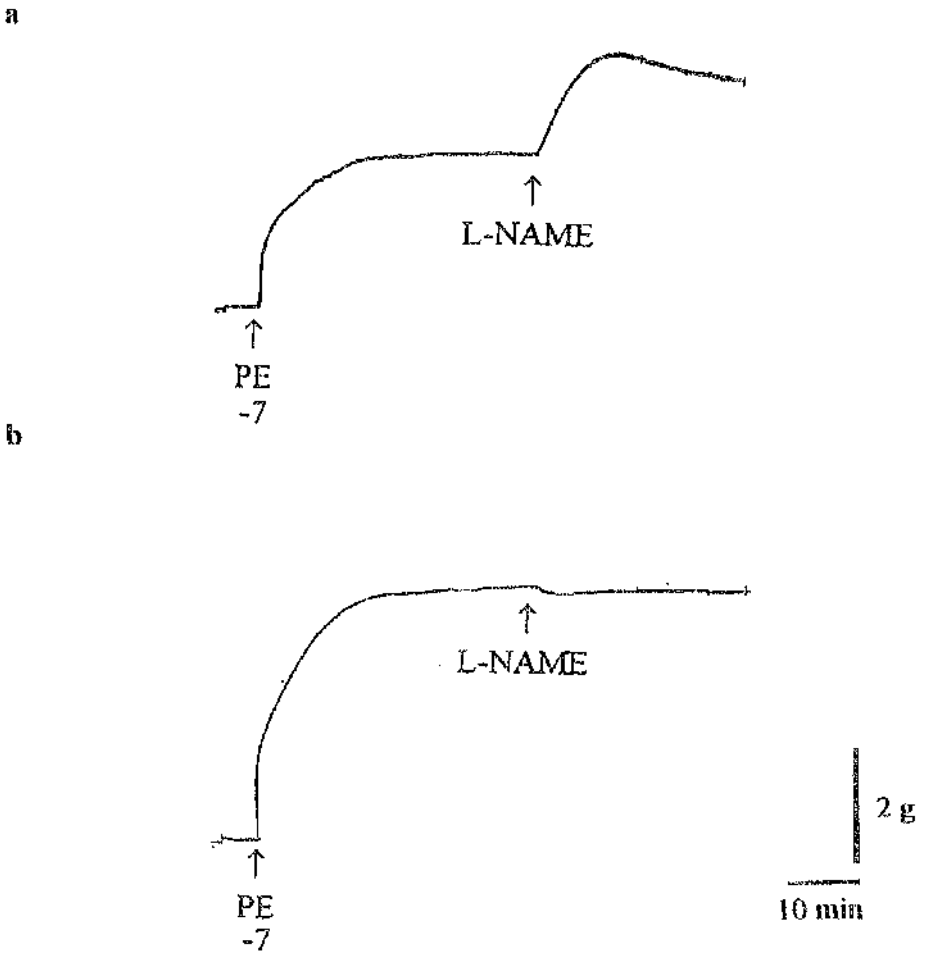


FIGURE 2.2 Individual experimental traces showing the ability of the inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM), to enhance phenylephrine (PE)-induced tone on an endothelium-containing ring of rabbit aorta (**a**) but not an endothelium-denuded ring (**b**). Drug concentrations are expressed in log molar units.

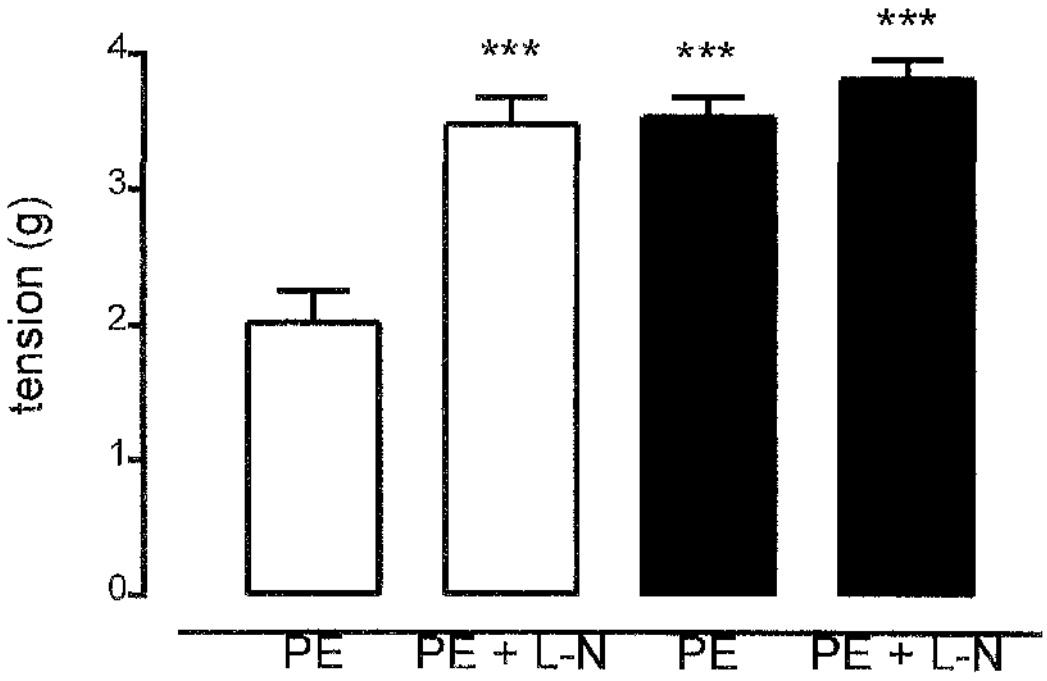


FIGURE 2.3 The inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methyl ester (L-N; 100 μM), enhanced phenylephrine (PE; 0.1 μM)-induced tone on endothelium-intact (open columns) but not endothelium-denuded (filled columns) rings of rabbit aorta. Data are expressed as mean ± s.e. mean of 28 observations. ***P<0.001 indicates a significant difference from PE-induced tone in endothelium-intact rings.

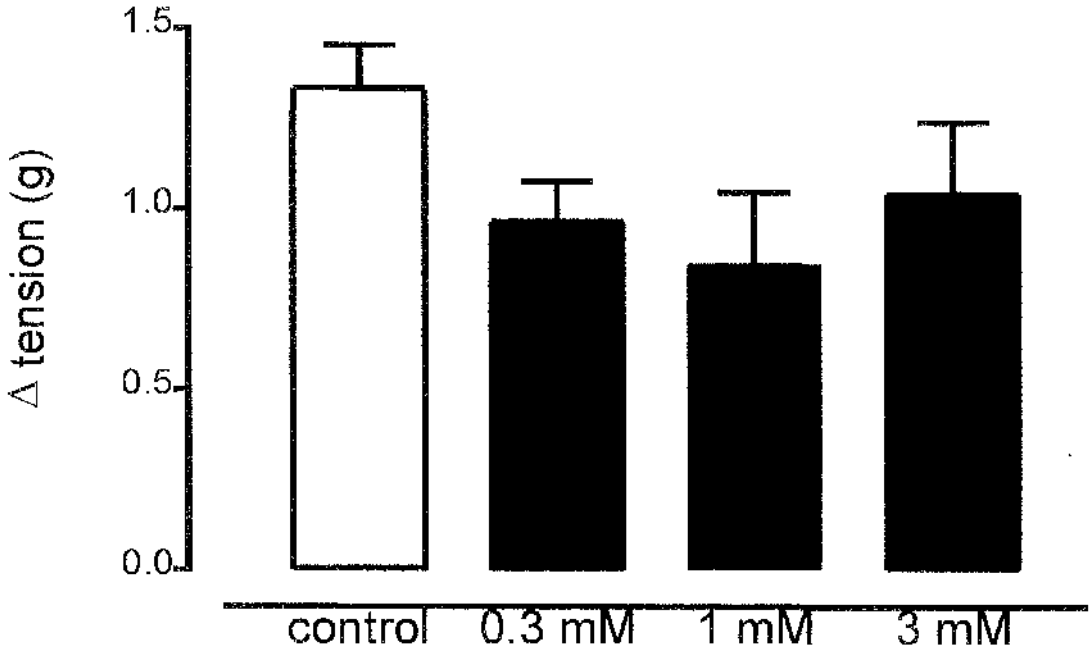


FIGURE 2.4 The change in phenylephrine (0.1 μM)-induced tone (Δ tension) resulting from addition of N^G-nitro-L-arginine methyl ester (100 μM) to endothelium-intact rings of rabbit aorta was unaffected by treatment with diethyldithiocarbamate at 0.3, 1 and 3 mM (60 min; washout; filled columns). Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Data are expressed as mean ± s.e. mean 8-12 observations.

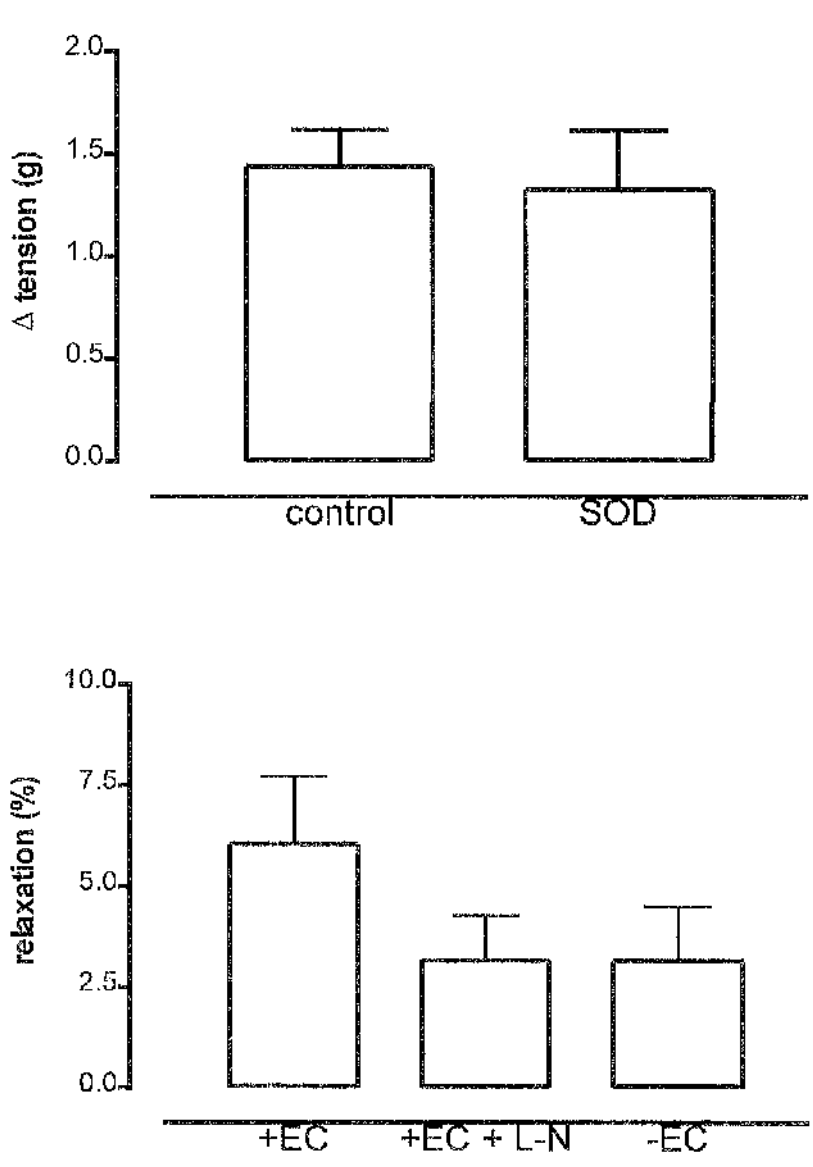


FIGURE 2.5 (**a**) Pretreatment for 20 min with exogenously applied superoxide dismutase (SOD; 250 u ml^{-1}) did not change the ability of N^G-nitro-L-arginine methyl ester (L-N; 100 μM) to enhance phenylephrine (PE; 0.1 μM)-induced tone (Δ tension) in endothelium-intact rings of rabbit aorta. (**b**) The relaxation induced by treatment with SOD in PE-contracted endothelium containing (+EC) rings was unaffected by treatment with L-N or endothelial removal (-EC). Data are expressed as mean ± s.e. mean 8 observations.

These findings suggest that basal action of nitric oxide was not impaired by treatment with DETCA.

Pretreatment for 20 min with exogenously applied SOD (250 u ml^{-1}) did not affect the L-NAME-induced enhancement of tone (Figure 2.5a). The addition of SOD (250 u ml^{-1}) to submaximally PE-contracted aortic rings resulted in a small relaxation (less than 10 %; Figure 2.5b). This relaxation was unaffected by treatment with L-NAME (100 μM) or by endothelial removal, however, there was a tendency for the SOD-induced relaxation to be reduced in both conditions. These findings suggests that in rabbit aorta, SOD-induced relaxation does not involve nitric oxide.

2.3.3 Effects of superoxide anion generation by xanthine oxidase/hypoxanthine on basal activity of nitric oxide

Following submaximal PE-induced tone, addition of xanthine oxidase (XO; 4.8 mu ml^{-1}) resulted in a substantial, but transient, relaxation of tone in endothelium-containing aortic rings (Figure 2.6). This fall in tone was reduced in tissues treated with L-NAME (100 μM) and absent following endothelial removal. Furthermore, this relaxation was present only upon the first addition of the enzyme to the tissue. This relaxation is known to be due to contamination of XO with a trace amount of trypsin, which itself produces endothelium-dependent relaxation (Thomas & Ramwell, 1987).

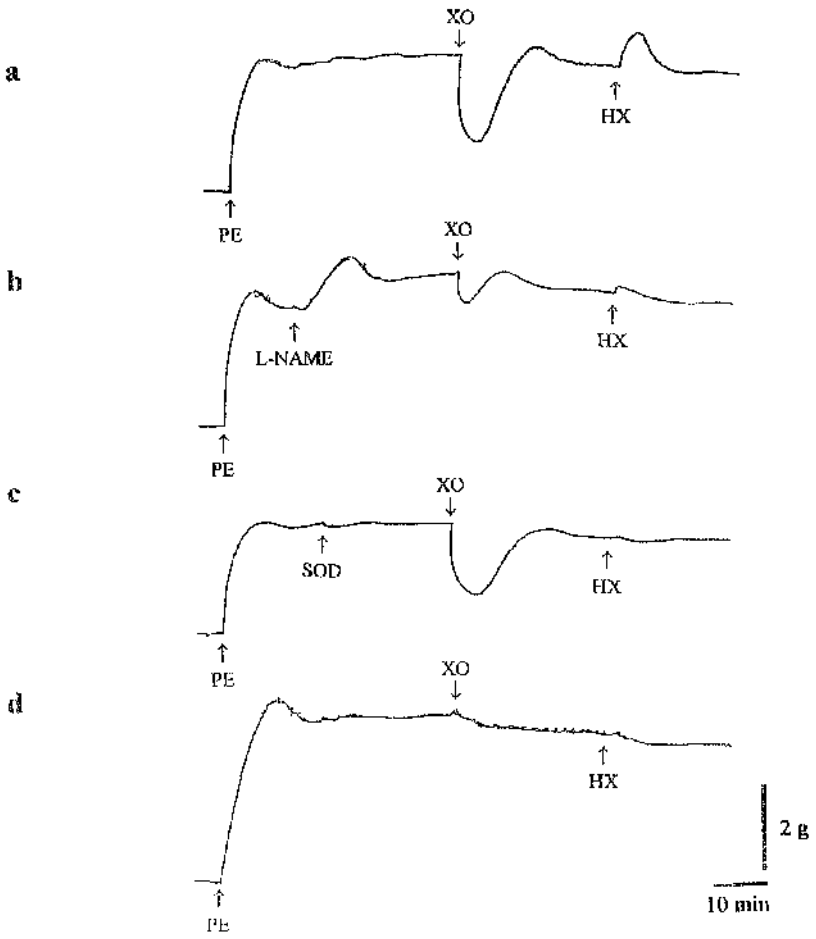


FIGURE 2.6 Individual experimental traces showing that a transient relaxation was seen following addition of xanthine oxidase (4.8 mu ml^{-1}) to PE-contracted endothelium-containing rings of rabbit aorta. Subsequent addition of hypoxanthine (0.1 mM) led to transient enhancement of a phenylephrine (PE)-induced tone. The transient relaxation to XO and the enhancement of tone by HX were inhibited by treatment for 20 min with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) (**b**) or by endothelial removal (**d**). Treatment with SOD (250 u ml^{-1}) for 20 min had no effect on the transient relaxation to XO but abolished the enhancement of tone to HX (**c**). All experiments were conducted in the presence of catalase (CAT; 1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Drug concentrations are expressed in log molar units.

Addition of hypoxanthine (HX; 0.1 mM) to precontracted endothelium-containing aortic rings already treated with XO (4.8 mu ml^{-1}) resulted in a transient enhancement of tone (Figures 2.6). This enhancement was reduced in tissues treated for 20 min with L-NAME (100 μM) and absent in tissues treated with SOD (250 u ml^{-1}) or following endothelial removal. The enhancement of tone was, therefore, likely to be due to destruction of basal nitric oxide by superoxide anion.

2.3.4 Effects of the combination of diethyldithiocarbamate and superoxide anion generation on basal activity of nitric oxide

Treatment with DETCA did not impair the enhancement of PE contraction induced by L-NAME (100 μM; see section 2.3.2 and Figure 2.4). This suggests that inactivation of endogenous Cu/Zn SOD did not lead to destruction of basal nitric oxide activity in rings of rabbit aorta. From the results of section 2.3.3 it would appear, however, that superoxide anion generation leads to inactivation of basal nitric oxide activity. If endogenous Cu/Zn SOD is important in protecting basal nitric oxide activity then its inactivation by DETCA would be expected to potentiate the inhibitory effects of the superoxide generating systems. Consequently, this possibility was investigated by examining the actions of the superoxide anion generators XO/HX, LY 83583 and pyrogallol on PE-induced tone in aortic rings previously treated with DETCA.

Following treatment of rabbit aortic rings with DETCA (0.3 mM; 60 min; washout) and XO (4.8 mu ml^{-1}), the enhancement of PE (0.1 μM)-induced tone seen upon

addition of HX (0.1 mM) was greatly augmented (Figure 2.7). Treatment with SOD (250 u ml^{-1}) for 20 min abolished the enhancement of PE-induced tone seen with XO/HX in control tissues and tissues treated with DETCA. Treatment with LY 83583 (0.3 μM) or pyrogallol (0.1 mM) also led to enhancement of PE (0.1 μM)-induced tone on endothelium-containing rings of rabbit aorta (Figure 2.8). As with XO/HX, the enhancement of tone induced by LY 83583 or pyrogallol was augmented in tissues treated with DETCA (0.3 mM; 60 min; washout). The enhancement of tone induced by these agents in control tissues and in tissues treated with DETCA was abolished following pretreatment with SOD (250 u ml^{-1}).

These results suggest that basal nitric oxide is inactivated by superoxide and that endogenous Cu/Zn SOD plays a role in protecting it.

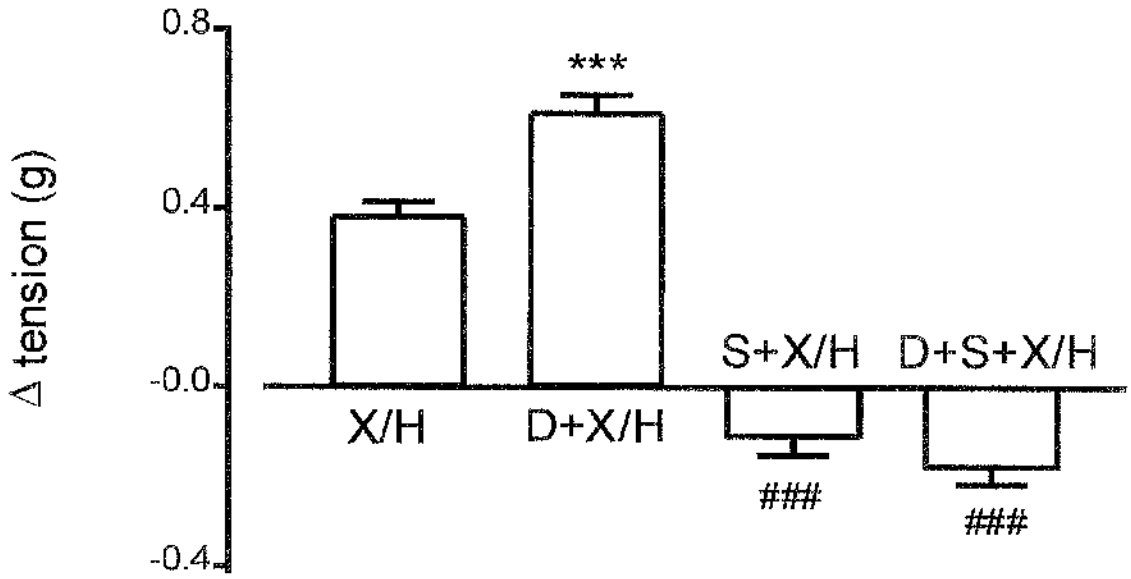


FIGURE 2.7 The enhancement of phenylephrine (0.1 μM)-induced tone (Δ tension) seen following treatment with xanthine oxidase (X; 4.8 mu ml^{-1})/hypoxanthine (H; 0.1 mM) was greater in tissues treated with diethyldithiocarbamate (D; 0.3 mM; 60 min; washout) than in control tissues. Pretreatment for 20 min with superoxide dismutase (S, 250 u ml^{-1}) abolishes the enhancement of tone seen upon addition of xanthine oxidase/ hypoxanthine to control tissues and tissues treated with DETCA. Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Data are expressed as mean ± s.e. mean of 8 observations. ***P<0.001 indicates significant differences from the change in tone induced by xanthine oxidase/ hypoxanthine alone. ###P<0.001 indicates a significant blockade by superoxide dismutase.

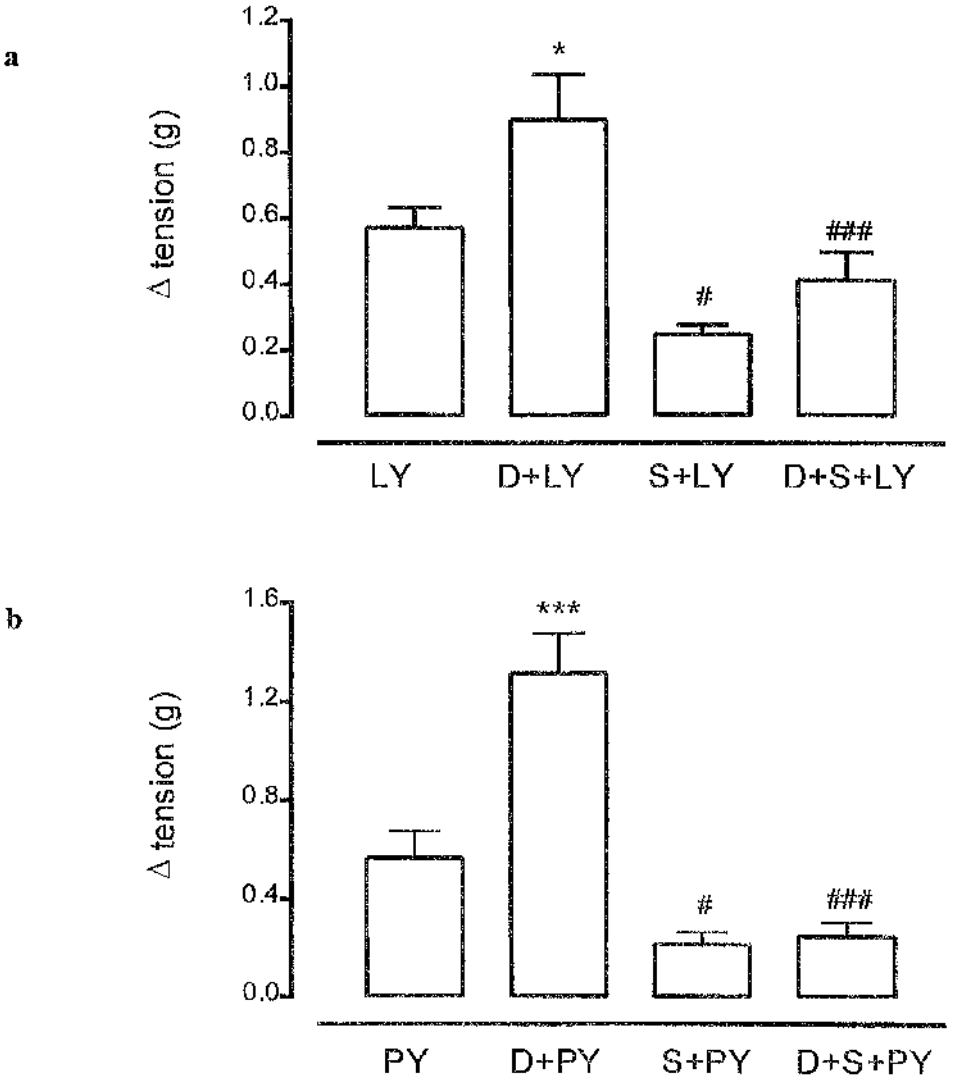


FIGURE 2.8 The enhancement of phenylephrine (0.1 μM)-induced tone (Δ tension) seen following treatment with LY 83583 (LY; 0.3 μM; **a**) or pyrogallol (PY; 0.1 mM; **b**) was greater in tissues treated with diethyldithiocarbamate (D; 0.3 mM; 60 min; washout) than in control tissues. Pretreatment for 20 min with superoxide dismutase (S, 250 u ml^{-1}) abolishes the enhancement of tone seen upon addition of LY 83583 or pyrogallol to control tissues and tissues treated with DETCA. Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Data are expressed as mean ± s.e. mean of 6-8 observations. *P<0.05 and ***P<0.001 indicate significant differences from the change in tone induced by treatment with LY 83583 or pyrogallol alone. #P<0.05 and ###P<0.001 indicate a significant blockade by superoxide dismutase.

2.3.5 Agonist-induced activity of nitric oxide in rabbit aorta

Submaximal tone (40 - 50 % of maximum; 2.5 - 3.0 g tension) was induced by PE (30 - 300 nM) in endothelium-containing rings of rabbit aorta. When this tone had stabilised, acetylcholine (ACh; 10 nM - 3 μM) was added, and produced powerful concentration-dependent relaxations (Figures 2.9a and 2.10): the maximum relaxation was 96.2 ± 2.1 %; n = 10. Following a 20 min treatment with the inhibitor of nitric oxide synthase, L-NAME (100 μM), the ability of ACh to induced relaxation was powerfully impaired, and after endothelial removal it was abolished (Figures 2.9 and 2.10).

These results show that in conditions where nitric oxide synthase activity is removed, the ability of ACh to induce relaxation is almost abolished.

2.3.6 Effects of diethyldithiocarbamate and superoxide dismutase on acetylcholine-induced activity of nitric oxide

The ability of ACh to induce relaxation was investigated in endothelium-containing aortic rings in which Cu/Zn SOD was inactivated following treatment with DETCA. Treatment with DETCA for 60 min at 0.3, 3 and 10 mM (followed by washout) resulted in a concentration-dependent impairment of the ability of ACh (10 nM - 3 μ M) to induce relaxation (Figure 2.11). The blockade induced by treatment with a submaximal concentration of DETCA (3 mM) was sustained over time as determined by a subsequent concentration response curve obtained 60 min later (Figure 2.11).

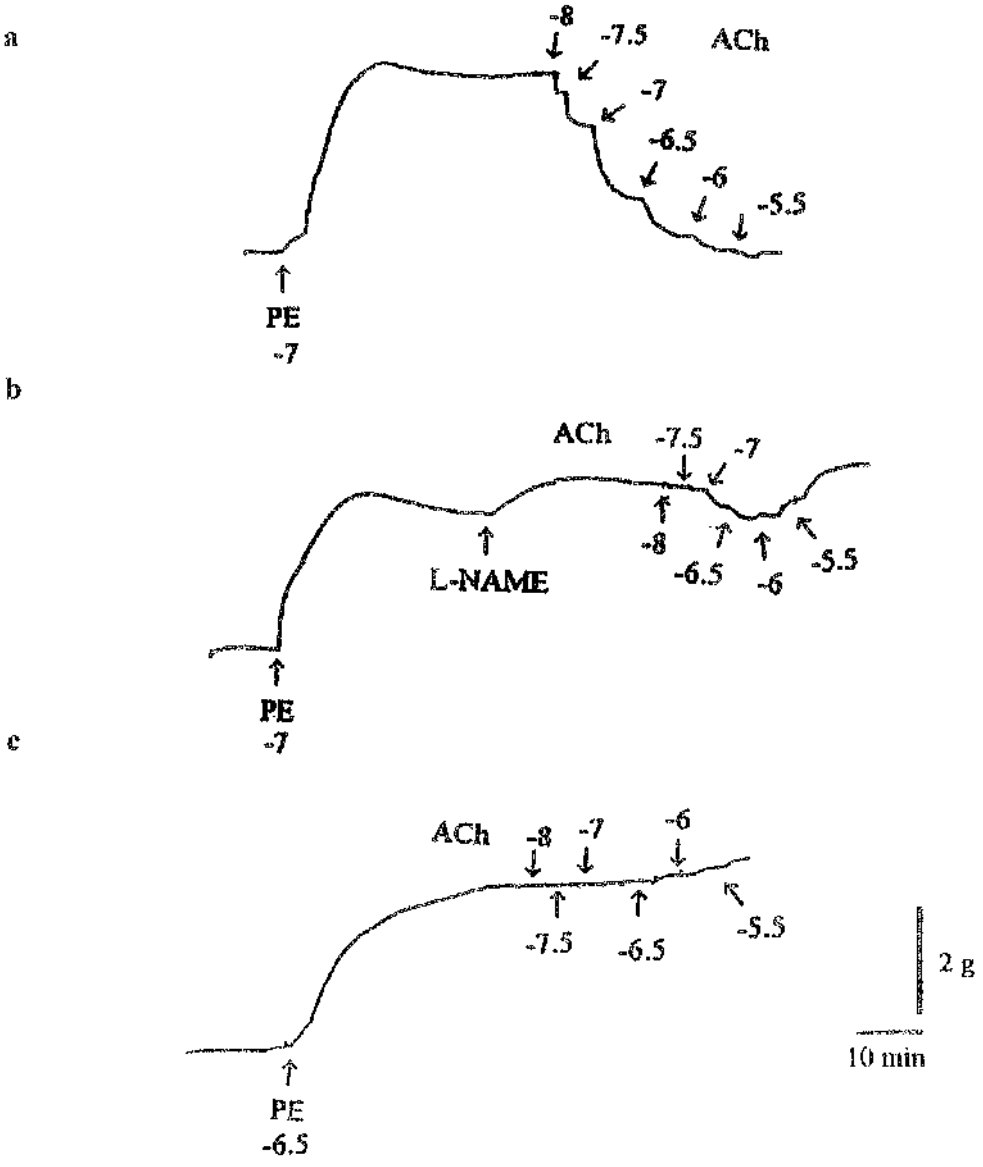


FIGURE 2.9 Individual experimental traces showing concentration-dependent relaxation to acetylcholine (ACh) on phenylephrine (PE)-contracted endothelium-containing rings of rabbit aorta (**a**) and the inhibition of this relaxation by treatment with the inhibitor of nitric oxide synthase, N^{G}-nitro-L-arginine methyl ester (L-NAME; 100 μM; **b**) or endothelial removal (**c**). Drug concentrations are expressed in log molar units.

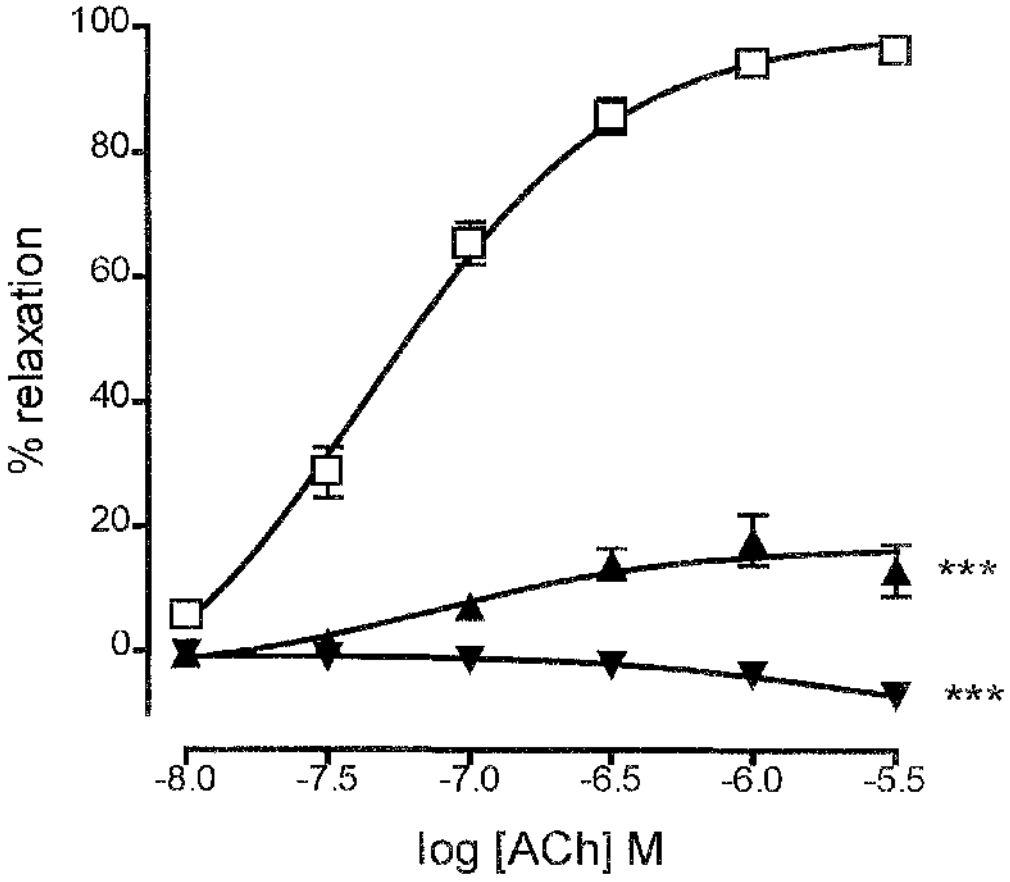


FIGURE 2.10 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta. Treatment with the inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methyl ester (100 μM; ▲), powerfully impaired the relaxation while endothelial removal (▼) abolished it. Each point is the mean ± s.e. mean of 10-18 observations. ***P<0.001 indicates a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

In an attempt to replenish lost stores of endogenous Cu/Zn SOD, exogenously applied SOD was given as a 20 min treatment to tissues already exposed to a submaximal concentration of DETCA (3 mM; 60 min; washout). Exogenous SOD (250 or 750 u ml^{-1}) did not restore ACh-induced relaxation to any degree (Figure 2.12). Consequently, since exogenously applied SOD cannot penetrate cells, these findings suggest that treatment with DETCA results in destruction of nitric oxide mainly in the intracellular space. Control experiments showed that exogenously applied SOD (250 u ml^{-1}) did not affect ACh-induced relaxation in untreated endothelium-containing rings (Figure 2.13).

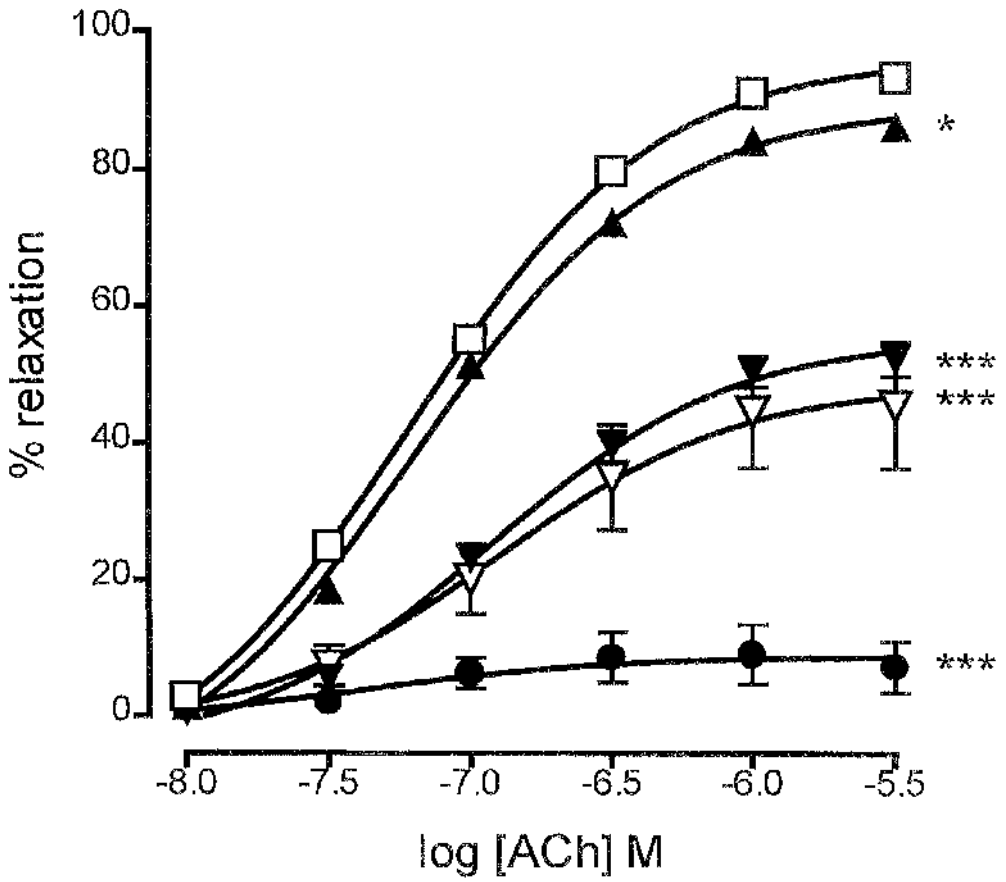


FIGURE 2.11 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta. Inhibition of endogenous Cu/Zn superoxide dismutase with diethyldithiocarbamate (60 min, wash) at 0.3 mM (▲), 3 mM (▼) and 10 mM (●) blocked ACh-induced relaxation in a concentration-dependent manner. The blockade induced by 3 mM diethyldithiocarbamate was sustained as was seen from a subsequent concentration-response curve obtained 60 min later (▽).Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 5-10 observations. *P<0.05 and ***P<0.001 indicate a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

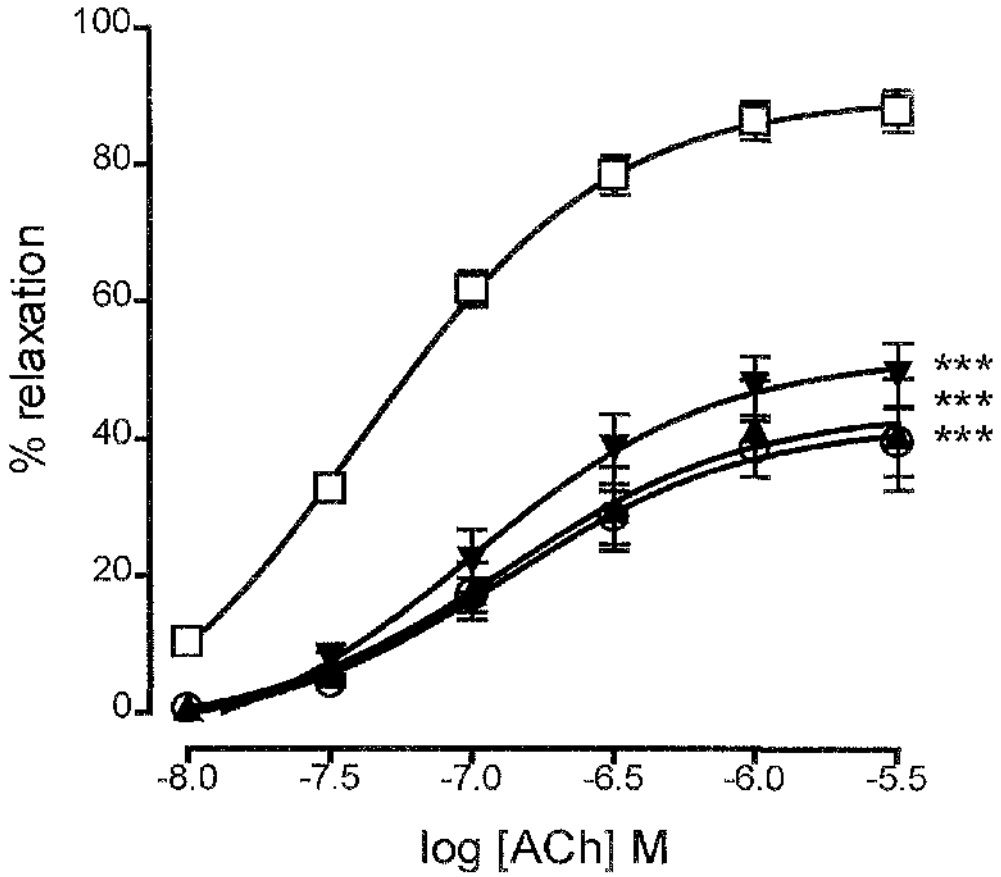


FIGURE 2.12 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and the blockade of this relaxation following treatment with diethyldithiocarbamate (3mM; 60 min; washout; ○). Treatment with exogenously applied superoxide dismutase for 20 min at 250 (▲) or 750 u ml^{-1} (▼) did not restore impaired ACh-induced relaxation. Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 6-13 observations. ***P<0.001 indicates a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

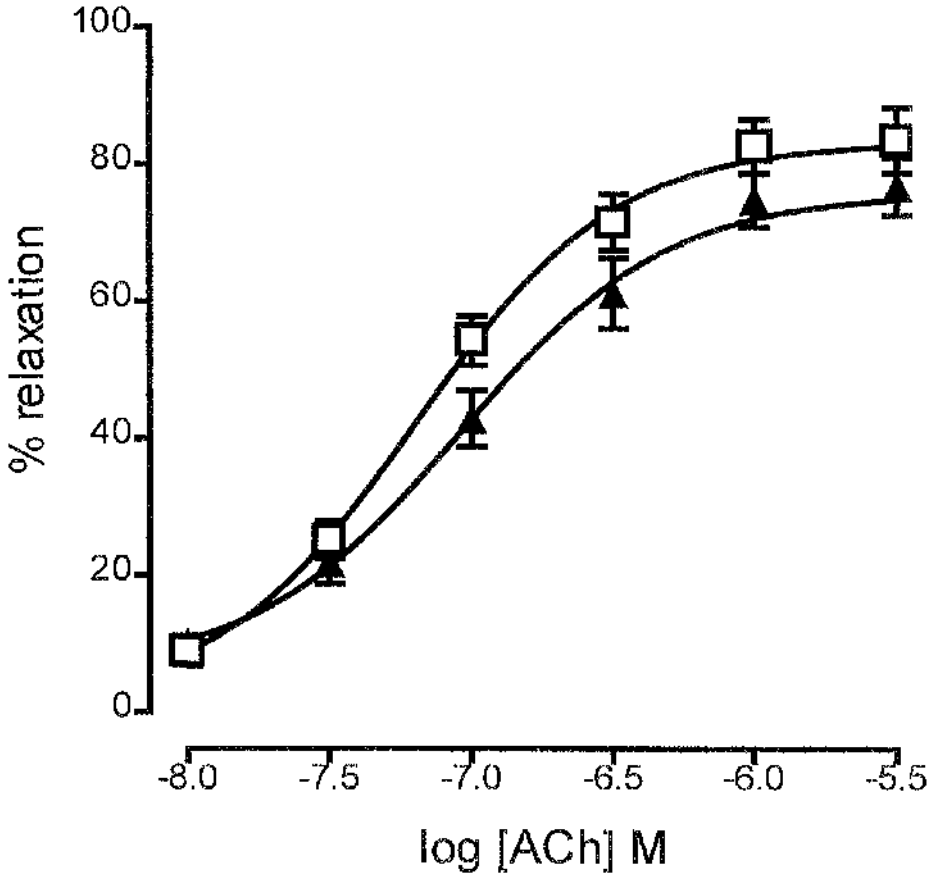


FIGURE 2.13 Concentration-response curves showing relaxation to acetylcholine (ΛCh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta. Treatment with exogenously applied superoxide dismutase (250 u ml^{-1}; ▲) had no effect on relaxation. Each point is the mean ± s.e. mean of 8 observations.

2.3.7 Effects of superoxide anion generation on acetylcholine-induced activity of nitric oxide

Following induction of submaximal PE (30 - 300 nM)-induced tone, the addition of the superoxide anion generating systems, xanthine oxidase (XO; 4.8 mu ml^{-1})/hypoxanthine (HX; 0.1 mM) and LY 83583 (0.3 μM), resulted in substantial impairment of the ability of ACh (10 nM - 3 μM) to induced relaxation (Figures 2.14 and 2.15, and 2.16a, respectively). It was important to establish if the blockade induced by XO/HX and LY 83583 was due to superoxide anion-mediated destruction of nitric oxide. To test this the tissues were pretreated with exogenous SOD (250 u ml^{-1}) for 20 min before addition of XO/HX or LY 83583. Treatment with SOD offered complete protection against the blockade induced by both superoxide generators (Figures 2.14 and 2.15, and 2.16a, respectively). Pyrogallol, another superoxide anion generator, did not impair ACh-induced relaxation to any degree when tested at the concentration of 0.1 mM (Figure 2.16b). Perhaps this concentration of pyrogallol generated insufficient superoxide to impair ACh-induced relaxation. Concentrations of pyrogallol of 0.3 mM or greater, however, caused such powerful falls in tone that relaxation to ACh could not be examined.

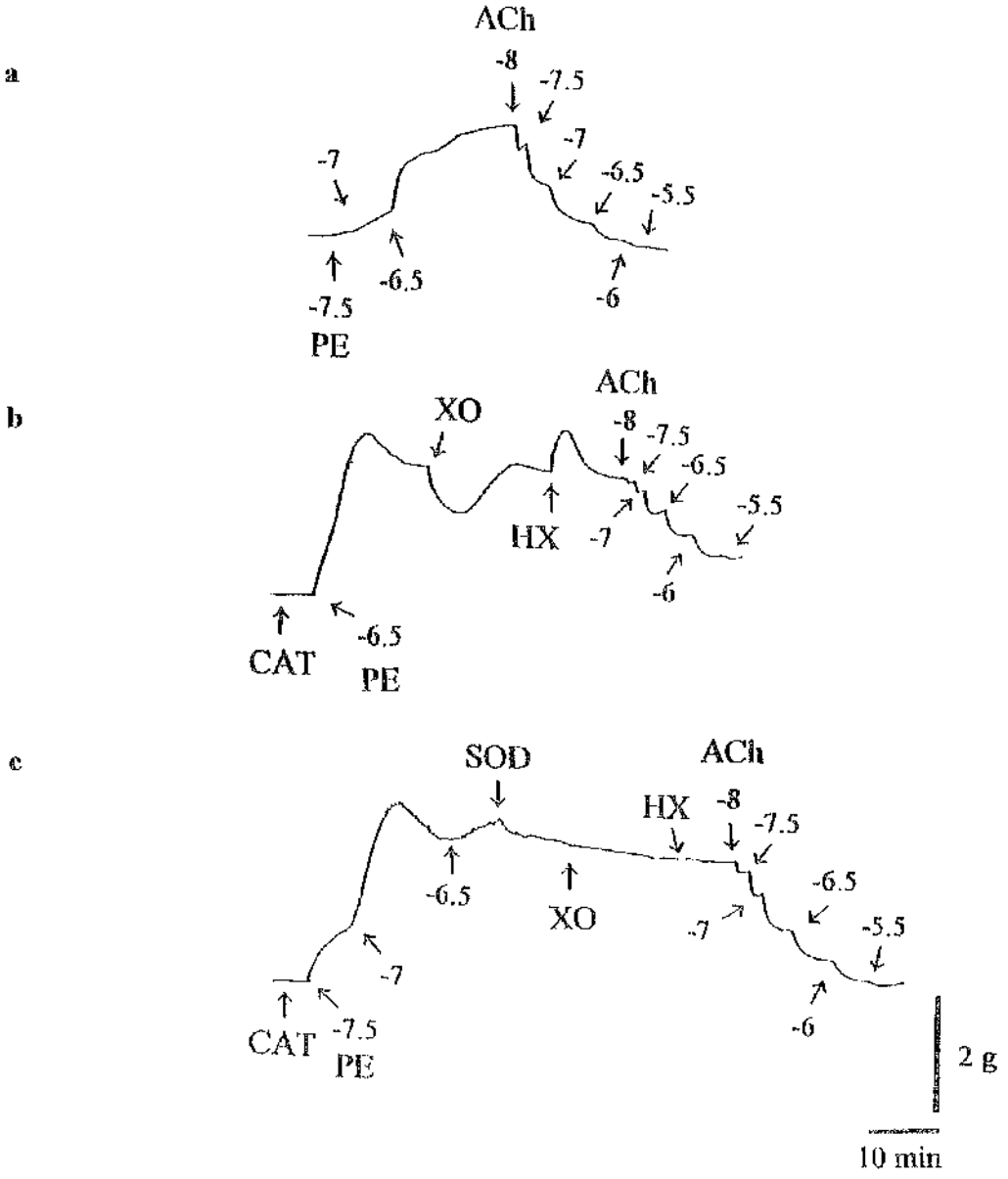


FIGURE 2.14 Sequential experimental traces from a single phenylephrine (PE)-contracted endothelium-containing ring of rabbit aorta showing that control (**a**) concentration-dependent relaxations to acetylcholine (ACh) were impaired by treatment with xanthine oxidase (XO; 4.8 mu ml^{-1})/hypoxanthine (HX; 0.1 mM) (**b**). Treatment with superoxide dismutase (SOD; 250 u ml^{-1}) prevented this inhibition (**c**). Experiments were conducted in the presence of catalase (CAT; 1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Drug concentrations are expressed in log molar units.

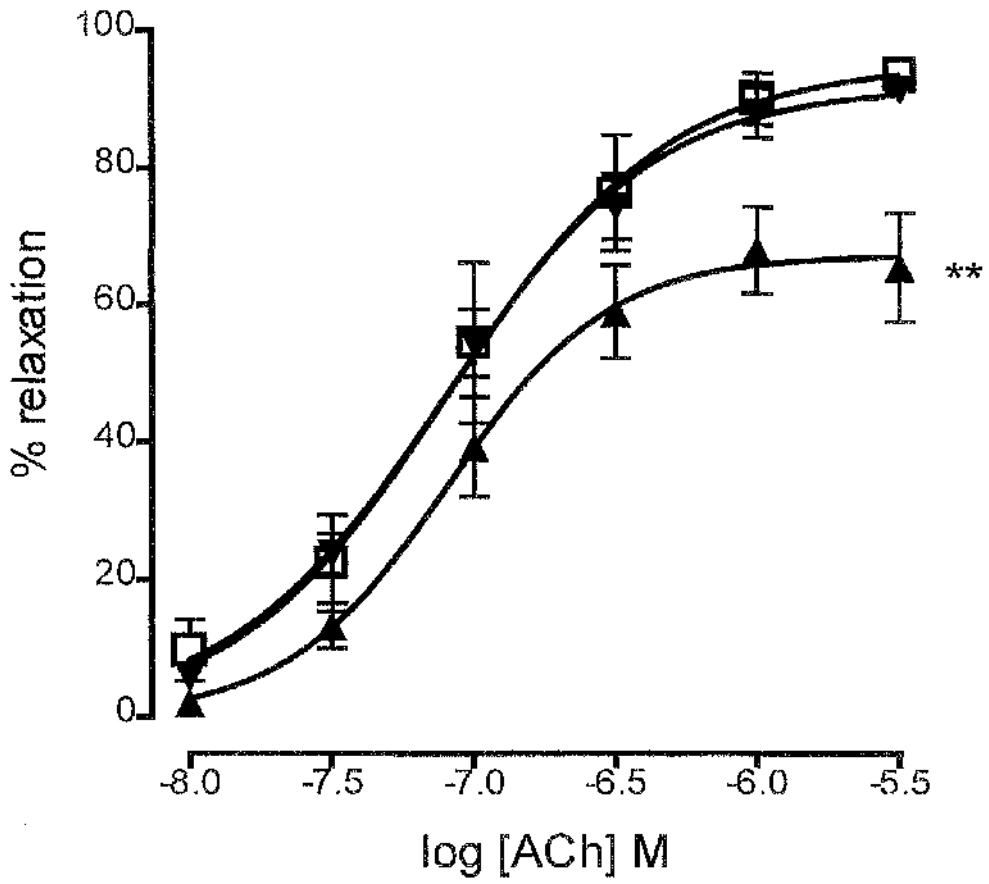


FIGURE 2.15 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta, and the ability of the superoxide anion generating system, xanthine oxidase (4.8 mu ml^{-1})/hypoxanthine (0.1 mM) (▲), to inhibit this relaxation. This inhibition was prevented by pretreatment for 20 min with exogenously applied superoxide dismutase (250 u ml^{-1}; ▼). Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 6 observations. **P<0.01 indicates a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

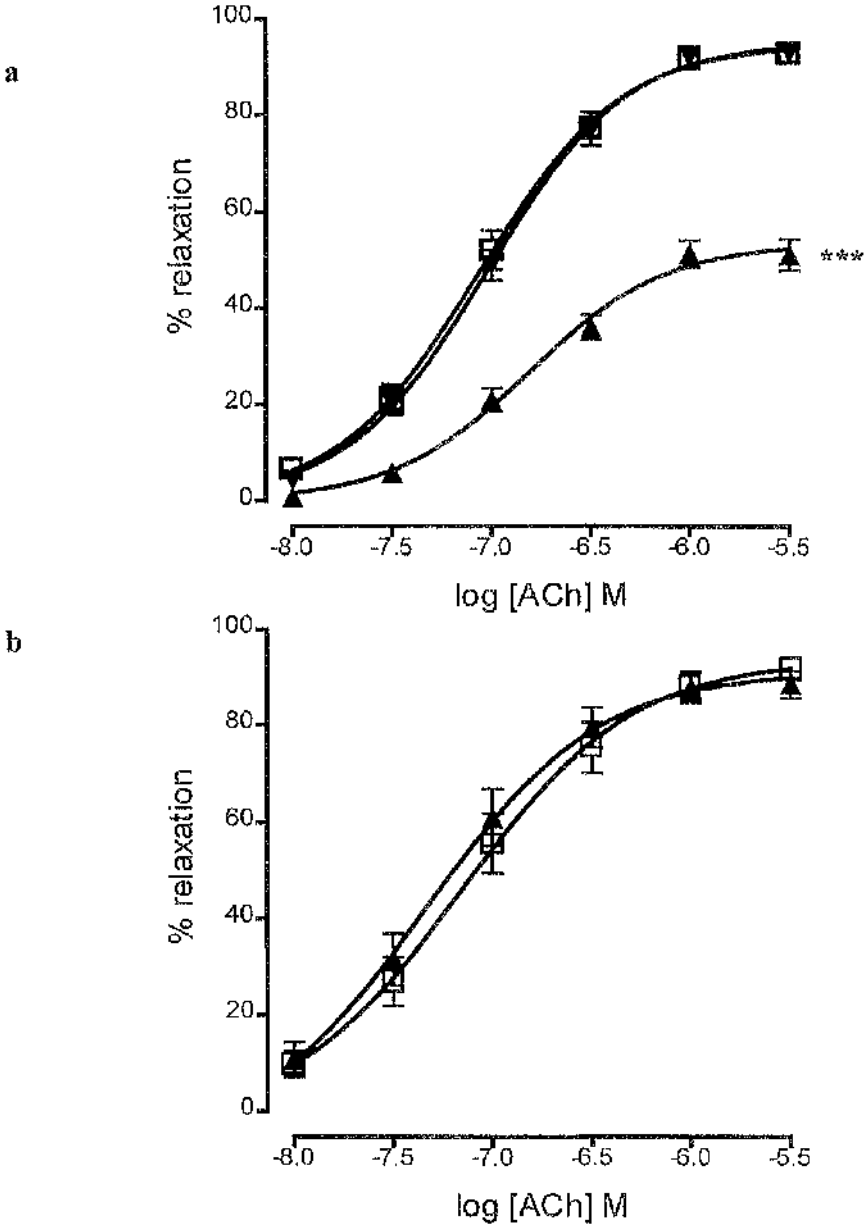


FIGURE 2.16 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and (**a**) the ability of LY 83583 (0.3 μM; ▲) to block this relaxation. Prevention of this blockade by pretreatment with superoxide dismutase (250 u ml^{-1}; ▼) is also shown. Treatment with pyrogallol (0.1 mM; ▲) did not affect ACh-induced relaxation (**b**). Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 6-12 observations. ***P<0.001 indicates a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

2.3.8 Effects of the combination of diethyldithiocarbamate and superoxide anion generation on acetylcholine-induced activity of nitric oxide

If endogenous Cu/Zn SOD is important in protecting nitric oxide against destruction by superoxide anion then its inactivation by DETCA would be expected to potentiate the inhibitory effects of the superoxide generating systems. This possibility was investigated.

The ability of XO (4.8 mu ml^{-1})/HX (0.1 mM) or LY 83583 (0.3 μM) to impair ACh-induced relaxation was powerfully potentiated in tissues that were pretreated with a concentration of DETCA (0.3 mM; 60 min; washout) which itself caused little impairment of relaxation (Figures 2.17 and 2.18a, respectively). The enhanced blockade induced following the combined treatment of DETCA with XO/HX or LY 83583 was abolished following treatment for 20 min with exogenous SOD (250 u ml^{-1}) (Figures 2.17 and 2.18a, respectively). Although pyrogallol (0.1 mM) had no effect by itself, it produced powerful blockade of ACh-induced relaxation in tissues pretreated with DETCA (0.3 mM; Figure 2.18b). This enhanced blockade was also completely prevented by pretreatment with exogenous SOD (250 u ml^{-1}; Figure 2.18b).

These findings suggest that endogenous Cu/Zn SOD plays a vital role in protecting nitric oxide from destruction by superoxide anion following its release by ACh.

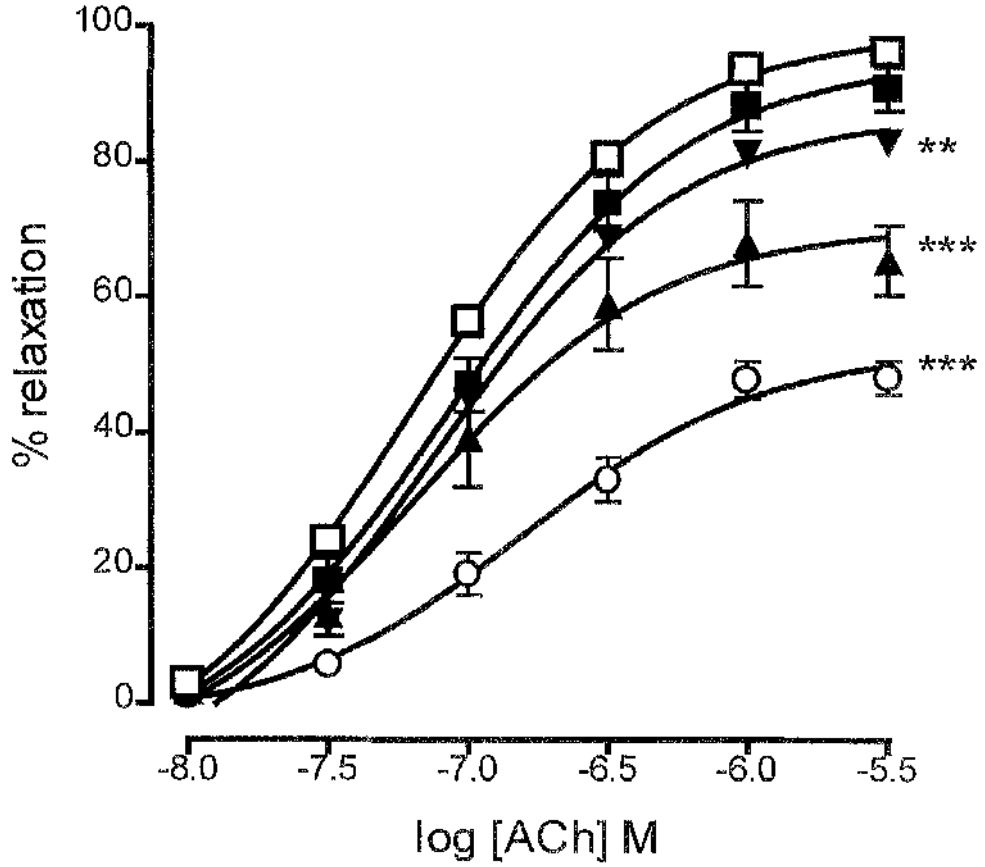


FIGURE 2.17 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta, and the ability of xanthine oxidase (XO; 4.8 mu ml^{-1})/hypoxanthine (HX; 0.1 mM) (▲), to block this relaxation. Following treatment with diethyldithiocarbamate (0.3 mM; 60 min; washout; ▼) ACh-induced relaxation was partially inhibited and subsequent treatment with XO/HX now produced profound blockade (○). This enhanced blockade was prevented by pretreatment for 20 min with exogenously applied superoxide dismutase (250 u ml^{-1}; ■). Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 5-10 observations. **P<0.01 and ***P<0.001 indicate a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

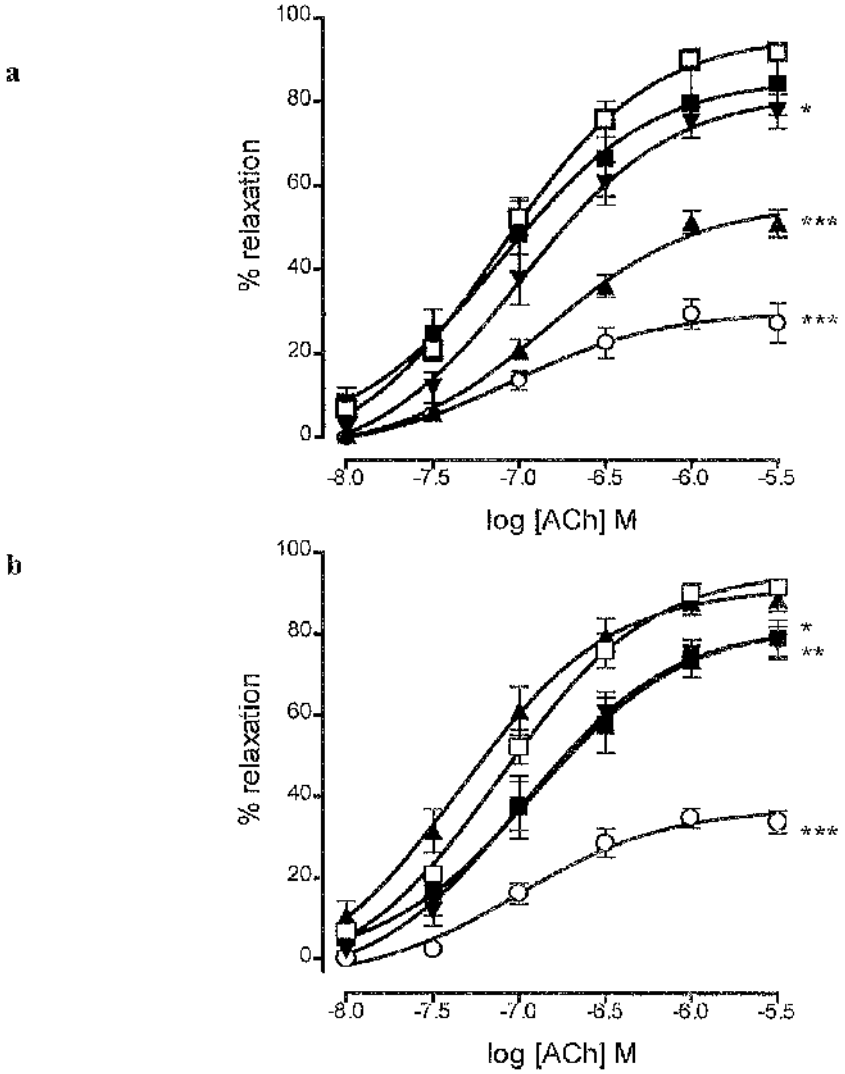


FIGURE 2.18 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta, and the ability of (**a**) LY 83583 (0.3 μM; ▲) and inability of (**b**) pyrogallol (0.1 mM; ▲) to block this relaxation. Following treatment with diethyldithiocarbamate (0.3 mM; ▼) ACh-induced relaxation was partially inhibited and subsequent treatment with LY 83583 or pyrogallol (○; a and b, respectively) now produced profound blockade. This enhanced blockade was prevented by pretreatment with exogenously applied superoxide dismutase (250 u ml^{-1} ; ■). Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 6-14 observations. *P<0.05, **P<0.01 and ***P<0.001 indicates a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

2.4 DISCUSSION

Acetylcholine (ACh) is a potent vasodilator *in vivo* but does not always produce relaxation in isolated blood vessel preparations. Furchgott & Zawadzki (1980) reconciled this anomaly by their discovery that endothelial cells play an obligatory role in the relaxation to ACh. The simple explanation was that the previously ignored layer of endothelial cells was almost always inadvertently rubbed off during isolation of blood vessel preparations. Since then the importance of the endothelium and release of the 'endothelium-derived relaxing factor' (EDRF) has revolutionised our understanding of how vascular tone is regulated locally. Some seven years later, Palmer *et al.* (1987) demonstrated that the lipophilic free radical gas, nitric oxide, accounts for the biological actions of EDRF.

The discovery that superoxide anion causes a reduction of the vasodilator effects of endothelium-derived nitric oxide in cascade bioassay systems, while superoxide dismutase (SOD) enhances it, has stimulated interest in the interaction between superoxide anion and nitric oxide and the potential protective role of SOD (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986b). Three isoforms of SOD are present in eukaryotic cells: an intracellular Cu/Zn-containing form, an extracellular Cu/Zn-containing form, and a Mn-containing form found in the mitochondria (see Fridovich, 1989). Endogenous levels of Cu/Zn SOD have been shown to be critically important in protecting nitric oxide from destruction by superoxide in a number of isolated blood vessels (Cherry *et al.*, 1990; Mügge *et al.*, 1991a; Omar *et al.*, 1991; Mian & Martin, 1995a). An enhanced superoxide-mediated destruction of nitric

oxide is thought to underlie the vascular pathology associated with many cardiovascular diseases (see Marín & Rodríguez-Martínez, 1997).

The aim of this section of the study was to develop models of oxidant stress in isolated rabbit aorta in which nitric oxide is destroyed by superoxide anion. It was hoped that these might provide an insight into the loss of nitric oxide associated with cardiovascular pathology. Essentially two strategies were employed to create oxidant stress: 1) inactivation of endogenous Cu/Zn SOD; and 2) generation of superoxide anion.

2.4.1 Basal release of endothelium-derived nitric oxide relaxes rabbit aortic rings

Basal (non-stimulated) production of nitric oxide exerts a tonic vasodilator influence in precontracted endothelium-containing aortic rings (Eglème *et al.*, 1984; Martin *et al.*, 1986a). This influence can be assessed indirectly when considering that the endothelium-dependent depression of vasoconstriction would be expected to be blocked or enhanced by agents which block or enhance the activity of nitric oxide, respectively. This vasodepressor influence of nitric oxide can be removed by endothelial denudation or the use of pharmacological inhibitors such N^G-nitro-L-arginine methyl ester (L-NAME; Rees *et al.*, 1990). The influence of basal nitric oxide was observed in this study where a set concentration of PE (0.1 μM) was shown to generate almost double the tension in endothelium-denuded compared with endothelium-containing rings. Also, L-NAME (100 μM) induced a rise of tone in

precontracted endothelium-containing but not endothelium-denuded rings of aorta. This rise in tone almost doubled the tension in aortic rings. Both of these procedures uncover the extent of the vasodepressor influence of basal nitric oxide. The influence of basal nitric oxide, however, is reportedly greater in rat aorta than in rabbit (Martin *et al.*, 1986a); where endothelium-denuded rings are 4- to 6- fold more sensitive to PE at the EC_{30} level. Although, not as substantial as in rat, this study confirmed that rabbit aorta does exhibit a measurable basal nitric oxide activity.

Having characterised basal nitric oxide activity in rabbit aortic rings, the effects of exogenous application of Cu/Zn SOD, inhibition of endogenous Cu/Zn SOD and superoxide anion generation were then investigated.

2.4.2 Exogenous application of superoxide dismutase has no effect on basal nitric oxide activity

SOD scavenges superoxide anion by converting it to hydrogen peroxide (Halliwell & Gutteridge, 1989), and it is well established that exogenous SOD potentiates the activity of nitric oxide in cascade bioassay systems (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986b).

Exogenously applied SOD (250 u ml^{-1}) produced a small relaxation of PE (30 – 300 nM)-induced tone in endothelium-containing aortic rings. There was a tendency for this relaxation to be reduced by treatment with L-NAME (100 μM) or endothelial removal but this remained statistically insignificant. This SOD-induced relaxation

was, therefore, independent of the endothelium and nitric oxide. In contrast to its action in rabbit aorta, exogenously applied SOD (at the same concentration; 250 u ml^{-1}) results in substantial relaxation (approximately 70 %) of precontracted rings of rat aorta (Ohlstein & Nichols, 1989; Mian and Martin, 1995a and see Chapter 5). This relaxation is likely to be due to removal of superoxide generated in the tissue or oxygenated Krebs solution, which was destroying basally produced nitric oxide. The different effects of exogenous SOD in these two animal preparations is likely to be due to the greater basal production of superoxide (and therefore depression of vasoconstriction) in rat with respect to rabbit.

Exogenous application of SOD had no effect on the L-NAME (100 μM)-induced enhancement of tone in rabbit aortic rings and, by inference, no effect on basal activity of nitric oxide. This provides independent confirmation that background levels of superoxide anion must be too low to inactivate nitric oxide, otherwise, addition of SOD would have been expected to potentiate the effects of nitric oxide, leading to greater L-NAME-induced enhancement of tone.

2.4.3 Diethyldithiocarbamate has no effect on basal nitric oxide activity

One strategy employed to create oxidant stress was to inactivate the endogenous scavenger of superoxide, SOD. This was achieved by use of diethyldithiocarbamate (DETCA). This compound irreversibly inactivates Cu/Zn SOD, but not Mn SOD, both in the intracellular and extracellular space by chelating the copper from the enzyme (Heikkila *et al.*, 1976; Cocco *et al.*, 1981; Halliwell & Gutteridge, 1989).

Inactivation of endogenous SOD leads to increased levels of superoxide in the vasculature as determined by chemiluminesesce (Cherry *et al.*, 1990; Omar *et al.*, 1991; Pagano *et al.*, 1993).

A 60 min treatment with DETCA (0.3 - 3 mM; washout) had no effect on the L-NAME (100 μM)-induced enhancement of tone. This suggests that basal nitric oxide activity remained intact when endogenous Cu/Zn SOD was inactivated. This finding suggests that either endogenous Cu/Zn SOD provides no protection to basal nitric oxide or the background levels of superoxide anion must be too low to inactivate nitric oxide. An alternative explanation, however, is that basally released endothelium-derived relaxing factor (EDRF) may not be nitric oxide *per se* but a different, superoxide anion-resistant, compound. The later possibility was investigated directly by assessing whether basal nitric oxide activity was impaired in conditions of exogenous superoxide anion generation.

2.4.4 Superoxide anion generators impairs basal nitric oxide activity

The effects of superoxide anion generation by the well characterised systems of xanthine oxidase (XO)/hypoxanthine (HX), LY 83583 and pyrogallol were investigated on basal activity of nitric oxide in rabbit aorta.

Oxidation of HX by XO forms superoxide and xanthine. Xanthine is then further oxidised by XO to produce uric acid and another superoxide. The quinones, LY 83583 and pyrogallol, generate superoxide by undergoing redox cycling, i.e. the

quinone autoxidises to it semiquinone which then reacts reversibly with oxygen to form quinone and superoxide anion (Halliwell & Gutteridge, 1989).

Following induction of submaximal PE-induced tone, XO (4.8 mu ml^{-1}) induced a transient relaxation which was mediated by endothelium-derived nitric oxide since it was impaired by treatment with L-NAME or endothelium removal. This relaxation was largely limited to the first addition of XO to the aortic ring, since subsequent additions to the same ring after washout gave much smaller or no relaxations. Similar finding have been reported in rabbit aorta by Furchgott (1994) and in dog coronary artery by Rubanyi & Vanhoutte (1986a). A possible explanation for this relaxation is that commercially available XO has been reported to be contaminated by trypsin, phoshoplipases and chelating agents such as EDTA (Halliwell & Gutteridge, 1989). Furthermore, Thomas & Ramwell (1987) report that endothelium-dependent relaxation elicited by XO is blocked by trypsin inhibitors and mimicked by authentic trypsin, suggesting that this action of XO is probably due to contaminating trypsin.

Following induction of PE-tone in endothelium-containing XO-treated rings of rabbit aorta, addition of HX (0.1 mM) produced a substantial but transient augmentation of tone. This effect was absent following treatment with L-NAME or in endothelium-denuded tissues. The absolute dependence of the endothelium for this XO/HX-mediated enhancement of PE-tone suggests that it is due to destruction of basal nitric oxide. Furthermore, exogenously applied SOD completely prevents this enhancement in tone. This suggests that the XO/HX-induced rise in tone indeed resulted from a superoxide-mediated destruction of basal nitric oxide. Furthermore,

since exogenously applied SOD is active only in the extracellular space, it follows that generation of superoxide anion by XO/HX, and subsequent destruction of nitric oxide occurs solely in the extracellular space. Logically, this would seem to be the case since the exogenously applied enzyme, XO, would also be too large to diffuse into cells. As with XO/HX, the enhancement of tone induced by LY 83583 (0.3 μM) and pyrogallol (0.1 mM) was also impaired by treatment with exogenously applied SOD, suggesting that the destruction of basal nitric oxide activity in each case was due to the generation of superoxide in the extracellular space. These experiments indicate clearly, that basal activity is destroyed by superoxide, thus precluding the need to suggest that the mediator is anything other than nitric oxide *per se*.

As reported in section 2.4.3, endogenous Cu/Zn SOD appears to be unimportant for the normal integrity of basal nitric oxide in rabbit aorta. This was therefore likely to indicate that levels of endogenous superoxide are too low to inactivate nitric oxide. If exogenous Cu/Zn SOD is, however, important in protecting basal activity of nitric oxide against destruction by superoxide anion then its inactivation by DETCA would be expected to potentiate the inhibitory effects of the superoxide generating systems. Consequently, experiments were conducted to investigate the effects of DETCA on the ability of XO/HX, LY 83583 and pyrogallol to inhibit basal nitric oxide activity. It was found that following pretreatment with a low concentration of DETCA (0.3 mM; 60 min; washout), which by itself had no effect on basal nitric oxide activity, the ability of XO (4.8 mu ml^{-1})/HX (0.1 mM), LY 83583 (0.3 μM) or pyrogallol (0.1 mM) to enhance PE-tone was now powerfully potentiated. It was likely that this enhanced augmentation of tone occurred as a consequence of elevated levels of

superoxide anion since it was blocked following pretreatment with exogenous SOD (250 u ml^{-1}). These augmentations of tone were also equivalent in magnitude to the rise observed upon the addition of L-NAME (100 μM; approximately 1 g tension), suggesting that basal nitric oxide activity was virtually abolished by the superoxide anion generators in DETCA-treated tissues. These experiments not only demonstrate that endogenous Cu/Zn SOD does indeed play a vital role in protecting basal nitric oxide from destruction by superoxide anion, but also provide a convenient model in which basal nitric oxide is destroyed by an extracellular oxidant stress.

2.4.5 Acetylcholine induces endothelium-dependent relaxation in rabbit aortic rings

Production and release of nitric oxide from the vascular endothelium is stimulated by a large number of mediators such as ACh (Furchgott & Zawadzki, 1980) and by the physical shearing force the flowing blood (Rubanyi *et al.*, 1986).

Although in normal physiology ACh is unlikely to stimulate production of endothelium-derived nitric oxide, *in vitro* it provides a convenient tool for assessing stimulated (non-basal) activity of nitric oxide. It was found that ACh (10 nM - 3 μM) produced a concentration-dependent and virtually complete relaxation of submaximally contracted endothelium-containing rings. This relaxation was impaired by inhibition of nitric oxide synthase following treatment with L-NAME (100 μM), and was abolished by endothelial removal. This ability of ACh to induce relaxation in rabbit aorta is consistent with the first report of EDRF described by

Furchgott & Zawadzki (1980) and indicate that the endothelium-dependent mediator released is nitric oxide.

Having characterised agonist-stimulated activity of nitric oxide in rabbit aortic rings, the effects of inhibition of endogenous Cu/Zn SOD and superoxide anion generation were then investigated.

2.4.6 Exogenous application of superoxide dismutase has no effect on acetylcholine-induced nitric oxide activity

Exogenous application of SOD (250 u ml^{-1}) had no effect on ACh-induced relaxation of rings of rabbit aorta. A similar lack of effect was reported in rabbit aorta by Ohlstein *et al.* (1989) and Abrahamsson *et al.* (1991). This suggests that background levels of superoxide anion must be too low as to not significantly inactivate nitric oxide, otherwise, addition of SOD would have been expected to potentiate the effects of nitric oxide and so enhance ACh-induced relaxation. As with ACh-induced relaxation, basal nitric oxide activity was also unaffected by exogenous SOD (Section 2.4.1). Exogenously applied SOD has also been shown to be without effect on ACh-induced relaxation of rat aorta (Mian & Martin, 1995a).

2.4.7 Diethyldithiocarbamate impairs acetylcholine-induced nitric oxide activity

In a number of blood vessels the inhibitor of Cu/Zn SOD, DETCA, leads to severe impairment of agonist-stimulated nitric oxide-dependent relaxation (Cherry *et al.*, 1990; Mügge *et al.*, 1991a; Omar *et al.*, 1991; Mian & Martin, 1995a). This loss of nitric oxide activity was unlikely to have resulted from reduced synthesis of nitric oxide since the release of total nitrogen oxides, as measured by chemiluminesence, was unaffected (Mügge *et al.*, 1991a). These authors concluded that the impairment of relaxation was due to the destruction of nitric oxide by superoxide anion as a consequence of inactivation of the protective Cu/Zn SOD enzyme. As with these previous reports, we found that treatment with DETCA (0.3 – 10 mM) alone produced a concentration-dependent blockade of ACh-induced relaxation. None of the above investigators, however, attempted to restore nitric-oxide dependent relaxation by replenishing SOD in the tissues. This was attempted in our study but we found that the blockade of ACh-induced relaxation produced by treatment with a submaximal concentration of DETCA (3 mM) was not reversed at all by treatment with exogenously applied Cu/Zn SOD (250 or 750 u ml^{-1}). This suggests that the impaired relaxation does not result from inhibition of endogenous extracellular SOD since reapplication would be expected to restore relaxation. The blockade may, therefore, result from impairment of the intracellular isoform of Cu/Zn SOD which might allow background levels of superoxide inside cells to rise and destroy nitric oxide intracellularly, i.e. an intracellular oxidant stress may have been generated. Consistent with this proposed intracellular action of high concentrations of DETCA is its ability to impair the actions of nitrovasodilators, such as glyceryl trinitrate,

which promote relaxation by the generation of nitric oxide inside smooth muscle cells (Omar *et al.*, 1991). Alternatively, the effects of DETCA may have resulted from non-selective, i.e. SOD-independent, effects in the tissue. Kelner *et al.* (1989) have already shown that DETCA can deplete intracellular glutathione and inactivate key enzymes by forming alkylates with important thiol group. In addition, elevated levels of superoxide anion, and consequently peroxynitrite, associated with treatment with DETCA have been reported to inactivate nitric oxide synthase (Mitchell *et al.*, 1991b, Rengasamy & Johns, 1993, Pasquet *et al.*, 1996).

Proof that the blocking action of DETCA indeed resulted from the inhibition of intracellular SOD would be obtained, however, if ACh-induced relaxation could be restored by a strategy which led to elevation of intracellular SOD or SOD-like activity. This will be addressed in Chapter 4 by investigating if a number of putative membrane-permeant SOD mimetics can restore nitric oxide-dependent relaxation following inactivation by DETCA.

2.4.8 Superoxide anion generators impair acetylcholine-induced nitric oxide activity

Treatment with the superoxide anion generators, XO (4.8 mu ml^{-1})/HX (0.1 mM) and LY 83583 (0.3 μM), each led to a substantial blockade of ACh-induced relaxation in PE-contracted endothelium-containing aortic rings. As in previous studies (Rubanyi & Vanhoutte, 1986a, Abrahamsson *et al.*, 1991; Mian & Martin, 1995a), the inhibition of relaxation is likely to have been due to superoxide anion-mediated destruction of nitric oxide. This was confirmed by the ability of exogenously applied

SOD to completely prevent the inhibitory effects of XO/HX or LY 83583. It should be noted also that the inhibitory effect of superoxide anion is not due to breakdown of ACh (Rubanyi & Vanhoutte, 1986a). The superoxide generator, pyrogallol at 0.1 mM, did not impair ACh-induced relaxation to any degree. Concentrations of pyrogallol of 0.3 mM or greater, however, caused such a dramatic fall in tone that it prevented examination of the relaxant effects of ACh.

If exogenous Cu/Zn SOD is important in protecting agonist-induced activity of nitric oxide against destruction by superoxide anion then its inactivation by DETCA would be expected to potentiate the inhibitory effects of the superoxide generating systems. Consequently, experiments were conducted to investigate the effects of DETCA on the ability of XO/HX, LY 83583 and pyrogallol to inhibit ACh-induced relaxation. It was found that following pretreatment with a low concentration of DETCA (0.3 mM; 60 min; washout), which by itself caused little inhibition of ACh-induced relaxation, then the ability of XO (4.8 mu ml^{-1})/HX (0.1 mM) and LY 83583 (0.3 μM) to impair relaxation was now powerfully potentiated. Also, treatment with pyrogallol (0.1 mM), which by itself had no effect on relaxation, now resulted in substantial impairment of ACh-induced relaxation. It was likely that these inhibitions had occurred as a consequence of elevated levels of superoxide anion since they were blocked following pretreatment with exogenous SOD. Since exogenous SOD, which cannot penetrate cells, completely restores ACh-induced relaxation, it is likely that this enhanced blocking ability of XO/HX, LY 83583 and pyrogallol in DETCA (0.3 mM)-treated tissues resulted from inhibition of extracellular SOD. This confirms previous findings that endogenous Cu/Zn SOD is vital in protecting agonist-induced nitric oxide from destruction by superoxide anion (Cherry *et al.*, 1990; Mügge *et al.*,

1991a; Omar *et al.*, 1991; Mian & Martin, 1995a). Furthermore, these experimental conditions provide convenient models in which ACh-induced activity of nitric oxide is destroyed by an extracellular oxidant stress.

Another important point to consider is the concentration dependence of the effects of DETCA. Specifically, high concentrations of DETCA (≥ 3 mM) produced blockade of ACh-induced relaxation which was not reversed by exogenous membrane-impermeant SOD, probably reflecting inhibition of intracellular Cu/Zn SOD (see section 2.4.3). In contrast, a low concentration (0.3 mM) facilitated blockade by the extracellular superoxide anion generators, and the augmented blockade was entirely reversed by exogenous membrane-impermeant SOD. It is likely therefore that a low concentration of DETCA can preferentially inhibit Cu/Zn SOD extracellularly whereas higher concentrations will inhibit both extracellularly and intracellularly.

2.4.9 Differential effect of diethyldithiocarbamate on basal and acetylcholine-induced activity of nitric oxide

The results from this chapter have clearly demonstrated that both basal and ACh-induced activity of nitric oxide are impaired by superoxide anion and both are protected against destruction by endogenous Cu/Zn SOD. Surprisingly, however, inactivation of endogenous Cu/Zn SOD, by treatment with DETCA (3 mM) alone, powerfully inactivates ACh-induced activity but not basal activity of nitric oxide. This apparent discrepancy could be explained if endogenous levels of superoxide anion were different in the two experimental conditions i.e. if higher levels of superoxide anion were present during stimulation with ACh than during unstimulated

conditions where basal activity of nitric oxide was measured. This could potentially occur if ACh-induced stimulation of endothelial cells lead to activation of cellular processes which stimulated superoxide production. One potential source of superoxide is cyclooxygenase, the enzyme that leads to production of prostaglandins, prostacyclin, and thromboxane (Berne & Levy, 1998). Generation of prostaglandin H (PGH) via PGH synthase forms free radical intermediates that react with $NADH^{\cdot}$ to form the radical $NAD^{\bullet}$. This radical is known to react in turn with oxygen to produce superoxide anion (Cosentino *et al.*, 1994). This finding is consistent with the demonstrated ability of the endothelial cyclooxygenase pathway in cerebral arteries to produce superoxide anion (Kukreja *et al.*, 1986). Furthermore, activated protein kinase C can lead to generation of superoxide anion in endothelial cells (Matsubara & Ziff, 1986). Thus, if ACh does lead to the generation of superoxide anion as well as of nitric oxide then this could explain why ACh-induced relaxation but not basal relaxation is impaired when endogenous Cu/Zn SOD has been compromised by treatment with DETCA.

To conclude, in addition to the characterisation of the effects of endothelium-derived nitric oxide in rabbit aorta, this chapter also describes the development of two distinct models of oxidant stress: an extracellular model (involving a low concentration of DETCA 0.3 mM in combination with superoxide generation) in which agonist-induced and basal activity of nitric oxide is inactivated; and an intracellular model (involving a high concentration of DETCA 3 mM alone) in which agonist-induced activity but not basal activity of nitric oxide is impaired. These models can be used to assay the efficacy of the ability of the putative SOD mimetics to protect nitric oxide from destruction by oxidant stress *in vitro*.

CHAPTER 3

BIOCHEMICAL ASSESSMENT OF SUPEROXIDE DISMUTASE ACTIVITY

3.1 INTRODUCTION

Chapter 2 confirms previous findings that superoxide anion impairs nitric oxide-dependent relaxation (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986b; Katusic & Vanhoutte, 1989; Ohlstein & Nichols, 1989; Mian & Martin, 1995a) and that endogenous Cu/Zn superoxide dismutase (SOD) plays a vital role in protecting endothelium-derived nitric oxide from destruction (Cherry *et al.*, 1990; Mügge *et al.*, 1991a; Omar *et al.*, 1991; Mian & Martin, 1995a). This process of oxidant stress-mediated destruction of nitric oxide is thought to be involved in the vascular dysfunction associated with many diseases. For example, hypertension (Nakazono *et al.*, 1991; Grunfeld *et al.*, 1995; Bouloumie *et al.*, 1997), atherosclerosis (Sharma *et al.*, 1992; Ohara *et al.*, 1993), ischaemia-reperfusion injury (Downey, 1990), diabetes (Hattori *et al.*, 1991; Kamata & Kobayashi, 1996) and heart failure (McMurray *et al.*, 1990; Katz *et al.*, 1993) are all associated with increased free radical production and reduced nitric oxide-dependent relaxation. A case therefore exists for augmenting SOD activity in the hope of restoring nitric oxide-dependent relaxation in pathologies associated with oxidant stress. SOD itself is unlikely to be of therapeutic benefit under such circumstances because its large size prevents it from entering cells to protect nitric oxide from intracellular oxidant stress. However, interest is growing in the development of membrane permeant SOD mimetics as therapeutic agents. A number of different classes of SOD mimetic have been described, including the nitroxide spin traps PTIYO (4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide) and tempol (4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl) (Mitchell *et al.*, 1990; Ewing & Janero, 1995), the superoxide scavenger tiron (4,5-dihydroxy-1,3-

benzene-disulfonic acid; Ledenev *et al.*, 1986), the metal-based compounds CuDIPS (Cu [II]-[diisopropylsalicylate]2; Huber *et al.*, 1987; Sorenson, 1995) and MnTMPyP (Mn [III] tetrakis [1-methyl-4-pyridyl] porphyrin; Faulkner *et al.*, 1994; Gardner *et al.*, 1996), and the metal salts $CuSO_4$ and $MnCl_2$ (Huber *et al.*, 1987; Beyer & Fridovich, 1990). The copper and manganese based compounds mimic the catalytic site of Cu/Zn SOD and Mn SOD, respectively.

The previous chapter was concerned with the development of models of oxidant stress in which nitric oxide-dependent relaxation was impaired by superoxide anion. These models were to be used to investigate the ability of putative SOD mimetics to restore impaired nitric oxide activity under conditions of oxidant stress. Before this was attempted, it was regarded as advantageous to rank the activity of each of the putative SOD mimetics in a biochemical assay.

Direct determination of SOD activity can be assessed only by highly specialised equipment such as is used in pulse radiolysis or nuclear magnetic resonance (Halliwell & Gutteridge, 1989). It is far simpler, however, to use indirect assays to detect SOD activity. In these, superoxide anion is generated by a system which then reacts with a detector molecule. The presence of SOD, or SOD-like compounds, removes the superoxide and therefore inhibits the reaction with the detector. Two well established distinct assays based on this system were employed. The first was based on the ability of the superoxide-generating system NADH and phenazine methosulphate to reduce nitro blue tetrazolium (Halliwell & Gutteridge, 1989; Ewing & Janero, 1995). The second was based on the xanthine oxidase/hypoxanthine

superoxide generating system to reduce cytochrome c (McCord & Fridovich, 1968; Halliwell & Gutteridge, 1989; Laight *et al.*, 1997).

The aim of this section was to assess the rank order of potency of the SOD mimetics in these biochemical assays. The information gained would subsequently be compared with their ability to restore nitric oxide-dependent relaxation in conditions of oxidant stress.

3.2 METHODS

Superoxide dismutase (SOD)-like activity was assessed by the use of two separate assays which measure the ability of agents to inhibit superoxide anion-mediated reactions. The agents assessed were authentic SOD and the putative SOD mimetics, PTIYO, tempol, tiron, $CuSO_4$, CuDIPS, $MnCl_2$ and MnTMPyP. Both assays are based on the principle that generation of superoxide anion, a reducing agent, promotes reduction of a detector molecule. This causes the detector molecule to change colour which can be detected spectrophotometrically. If authentic SOD or a SOD-like agent is present in the assay it will scavenge the superoxide anion produced and thereby inhibit the change in colour. SOD activity, therefore, can be expressed as a function of its ability to inhibit the reduction of the detector agent, i.e. 0 % inhibition corresponds to zero SOD activity while 100 % inhibition corresponds to maximum SOD activity (i.e. all of the generated superoxide anion was scavenged).

The assays employed were based on the ability of superoxide anion to reduce either nitro blue tetrazolium or cytochrome c. Both assays were conducted in final volumes of 300 µl in 96 well plates, incubated at room temperature on an orbital shaker. Following incubation, changes in absorbance were measured with a microplate reader (Dynex Ltd.).

In preliminary experiments, a time course was constructed for the reduction of nitro blue tetrazolium and cytochrome c (Figure 3.1). This was done to establish suitable incubation times for assays involving SOD and the SOD mimetics.

3.2.1 Reduction of nitro blue tetrazolium

This assay was based on the ability of superoxide anion, generated by NADH and phenazine methosulphate, to reduce yellow nitro blue tetrazolium to blue formazan dye (Halliwell & Gutteridge, 1989; Ewing and Janero, 1995).

$$NBT^{2+} \text{ (yellow)} + O_2^{\cdot-} \rightarrow \text{formazan (blue)}$$

The complete assay system consisted of (final concentrations in parenthesis): 10 µl of 1.5 mM nitro blue tetrazolium (50 µM), 10 µl of 2.34 mM NADH (78 µM), 100 µl of 9.9 µM phenazine methosulphate (3.3 µM), taken to a total volume of 300 µl with phosphate buffer (50 mM, pH 7.4), containing ethylenediaminetetraacetic acid (EDTA, 0.1 mM) in order to chelate contaminating heavy metals which interfere with the reduction of nitro blue tetrazolium. For the assay of SOD activity, 10 µl of a range of concentrations of SOD or a SOD mimetic was added to the assay system at

the expense of 10 µl of phosphate buffer. Each plate included two separate blanks to correct for the intrinsic absorbance of the reagents used: the first, consisted of all the reagents with the exception of the phenazine methosulphate and the test agent (i.e. SOD or the SOD mimetic). The second, consisted of all the reagents together with the highest concentration used of the test chemical (i.e. SOD or the SOD mimetic) but with the exception of the phenazine methosulphate. Zero standards (containing no SOD or SOD mimetic) were also included in each plate to assess the maximum reduction of the detector agent during the incubation period, i.e. no mimetic present. This control was then used to calculate SOD activity, which was expressed as a percentage inhibition of the reduction of nitro blue tetrazolium. Each protocol had n = 8 replicates on each plate. The reaction was initiated with the addition of freshly prepared phenazine methosulphate. Following incubation for 5 min the absorbance of the solution was measured at 550 nm.

3.2.2 Reduction of cytochrome c

This assay was based on the ability of superoxide anion, generated by xanthine oxidase/hypoxanthine (XO/HX), to reduce cytochrome c. Commercially available cytochrome c is supplied largely in the oxidised form, ferricytochrome c, and superoxide anion causes its reduction to form ferrocytochrome c. The reduced form of cytochrome c has a greater absorbance at 550 nm than the oxidised form (McCord & Fridovich, 1968; Halliwell & Gutteridge, 1989; Laight *et al.*, 1997).

$$\text{ferricytochrome c } (Fe^{3+}) + O_2^{\cdot -} \rightarrow O_2 + \text{ferrocytochrome c } (Fe^{2+})$$

The complete assay system consisted of (final concentrations in parenthesis): 10 µl of 90 mu ml^{-1} XO (3 mu ml^{-1}), 10 µl of 3 mM HX (0.1 mM), 10 µl of 900 µM cytochrome c (30 µM), 10 µl of 3000 u ml^{-1} catalase (100 u ml^{-1}) and was taken to a total volume of 300 µl with Tris buffer (tris[hydroxymethyl]-aminomethane hydrochloride; 50 mM, pH 7.6). Catalase was present to prevent the re-oxidation of cytochrome c resulting from the build up of hydrogen peroxide, formed from the dismutation of superoxide anion. SOD activity was assessed by the addition of 10 µl of a range of concentrations of SOD or a SOD mimetic to the assay system at the cost of 10 µl of Tris buffer. As with the nitro blue tetrazolium assay, each plate included two separate blanks to correct for the intrinsic absorbance of the reagents used. The first consisted of all the reagents except XO and SOD or SOD mimetic. The second, consisted of all the reagents along with the highest concentration of SOD or SOD mimetic without XO. Each protocol had n = 8 replicates on each plate. The reaction was initiated with the addition of XO. Following incubation for 30 min the absorbance of the solution was measured at 550 nm.

For both assays, concentration-effect curves, calculation of pEC_{50} values and statistical analyses were calculated using a computer-based programme (Graph Pad, Prism).

3.2.3 Drugs, reagents and solvents

Drugs and reagents used in these experiments, but not referred to in Chapter 2, were:

Cytochrome c, 4,5-dihydroxy-1,3-benzene-disulfonic acid (tiron), 4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl (tempol), β-nicotinamide adenine dinucleotide (reduced form; NADH), nitro blue tetrazolium (NBT) and phenazine methosulfate (PMS) were obtained from Sigma (Poole, UK). Cu (II)-[diisopropylsalicylate]$_2$ (CuDIPS) and 4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide (PTIYO) were obtained from Aldrich (Dorset, UK). Mn (III) tetrakis [1-methyl-4-pyridyl] porphyrin (MnTMPyP) was obtained from Alexis (Nottingham, UK), whilst $CuSO_4$ and $MnCl_2$ were obtained from Hopkin & Williams (Essex, UK).

All drugs were dissolved in normal saline (0.9 %), except CuDIPS (3 mM stock) which was dissolved in 50% ethanol/ 50% Tris buffer (50 mM, pH 7.4). Control experiments demonstrated that the solvent ethanol/Tris buffer did not account for the effects observed with CuDIPS. All dilutions were made in the buffer appropriate to the particular assay. Concentrations of drugs are expressed as the final molar concentration in the plate well.

3.3 RESULTS

3.3.1 Time-dependent reduction of nitro blue tetrazolium and cytochrome c

The reduction of nitro blue tetrazolium to formazan by NADH/phenazine methosulphate proceeds rapidly, before reaching completion within 10 min or so (Figure 3.1a). Reduction of ferricytochrome c to ferrocytochrome c by the xanthine oxidase/hypoxanthine (XO/HX) superoxide generating system proceeds at a slower rate, reaching completion after around 60 min (Figure 3.1b).

Incubation periods corresponding approximately to the time to half maximum reduction, i.e. 5 and 30 min for nitro blue tetrazolium and cytochrome c, respectively, were used in all subsequent experiments.

3.3.2 Inhibition of reduction of nitro blue tetrazolium and cytochrome c by superoxide dismutase

When present during the incubations, authentic Cu/Zn SOD (0.03 - 300 u ml^{-1}) produced concentration-dependent inhibition of the reduction both of nitro blue tetrazolium by the NADH/phenazine methosulphate system and of cytochrome c by the XO/HX system (Figure 3.2): the inhibition of reduction was virtually complete in each case (Table 3.1). The ability of authentic SOD to produce powerful inhibition of reduction of both nitro blue tetrazolium and cytochrome c provides validation of these methods for the assessment of SOD-like activity.

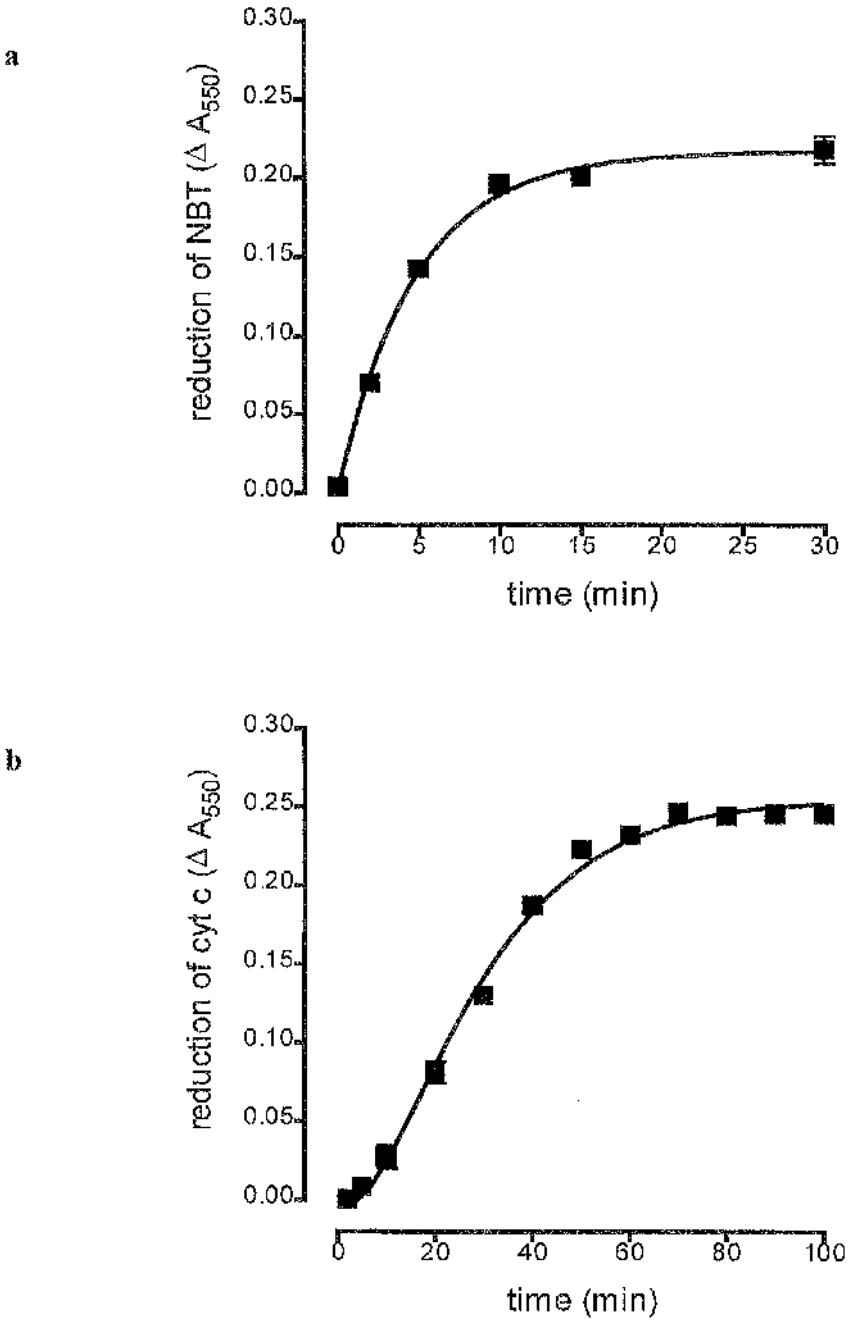


FIGURE 3.1 Time-dependent reduction of (**a**) nitro blue tetrazolium and (**b**) cytochrome c by the NADH/phenazine methosulphate and the xanthine oxidase/hypoxanthine superoxide anion generating systems, respectively, measured as changes in absorbance at 550 nm (Δ A_{550}). Each point is the mean ± s.e. mean of 8-15 observations.

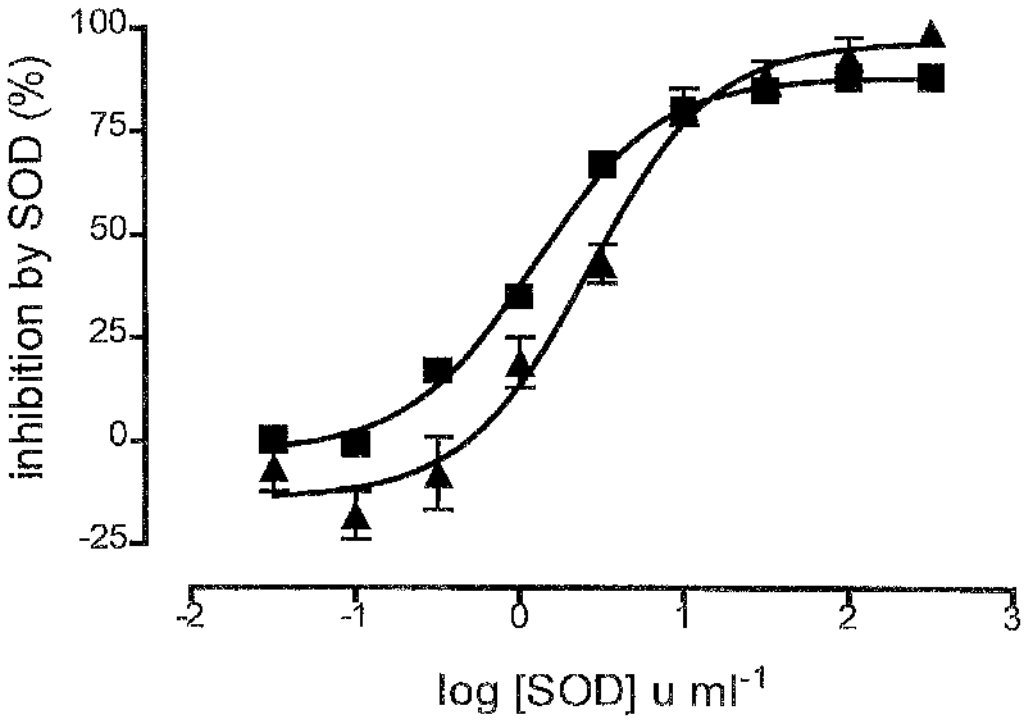


FIGURE 3.2 The ability of authentic Cu/Zn superoxide dismutase (SOD) to inhibit in a concentration-dependent manner the reduction both of nitro blue tetrazolium (■) by NADH/phenazine methosulphate and of cytochrome c (▲) by xanthine oxidase/hypoxanthine. Each point is the mean ± s.e. mean of 8-16 observations.

	nitro blue tetrazolium reduction		**cytochrome c reduction**	
compound	*pEC_{50}*	*maximum inhibition (%)*	*pEC_{50}*	*maximum inhibition (%)*
Cu/Zn SOD	-0.02 ± 0.02	88.2 ± 1.3	-0.41 ± 0.09	99.1 ± 3.5
PTIYO	3.38 ± 0.07	95.6 ± 1.1	2.59 ± 0.23	88.2 ± 5.2
tempol	3.88 ± 0.08	98.9 ± 2.1	3.36 ± 0.13	94.5 ± 4.6
tiron	4.57 ± 0.16	62.5 ± 0.7**	3.11 ± 0.10	51.3 ± 5.1***
$CuSO_4$	4.04 ± 0.01	81.4 ± 10.7	6.70 ± 0.04	75.7 ± 4.8
CuDIPS	5.72 ± 1.04	6.7 ± 3.1***	6.27 ± 0.11	69.7 ± 1.6*
$MnCl_2$	4.28 ± 0.04	74.7 ± 2.4	6.46 ± 0.21	93.3 ± 4.0
MnTMPyP	7.08 ± 0.30	95.9 ± 2.2	6.05 ± 0.17	40.0 ± 13.9***

Table 3.1 The ability of authentic Cu/Zn SOD and SOD mimetics to inhibit the reduction of nitro blue tetrazolium and cytochrome c. Data are the mean ± s.e. mean of 8-16 observations. *P<0.05, **P<0.01 and ***P<0.001 indicate a significant difference from maximal inhibition by Cu/Zn SOD. $_pEC_{50}$ values are expressed in molar units except for Cu/Zn SOD which are in u ml^{-1}.

3.3.2 Inhibition of reduction of nitro blue tetrazolium and cytochrome c by superoxide dismutase mimetics

All of the putative SOD mimetics investigated exhibited some degree of SOD-like activity by inhibiting the reduction of either nitro blue tetrazolium or cytochrome c, although the range of effective concentrations and maximum inhibitions varied widely (Figures 3.3 and 3.4, and Table 3.1).

The metal-based agent, MnTMPyP, was clearly the most potent compound at inhibiting the reduction of nitro blue tetrazolium, and the inhibition it produced was nearly complete (Figure 3.3 and Table 3.1). The other metal-based agents, $CuSO_4$, CuDIPS and $MnCl_2$, the nitroxide spin traps, PTIYO and tempol, and the superoxide scavenger, tiron, were all considerably less potent. Nevertheless, of all the agents tested, only tiron and CuDIPS failed to reach near complete inhibition of reduction. In fact CuDIPS had virtually no inhibitory activity even at the highest concentration tested (30 μM; Figure 3.3 and Table 3.1).

All of the SOD mimetics tested produced a concentration-dependent inhibition of reduction of cytochrome c (Figure 3.4). The metal-based agents, $CuSO_4$, CuDIPS, $MnCl_2$ and MnTMPyP, were clearly more potent than the spin traps, PTIYO, tempol and tiron, in inhibiting the reduction of cytochrome c: of these, only $MnCl_2$, PTIYO and tempol produced near complete inhibition of reduction. MnTMPyP was considerably (greater than 10-fold) less potent in inhibiting the reduction of cytochrome c than of nitro blue tetrazolium but PTIYO and tiron also displayed a lower potency.

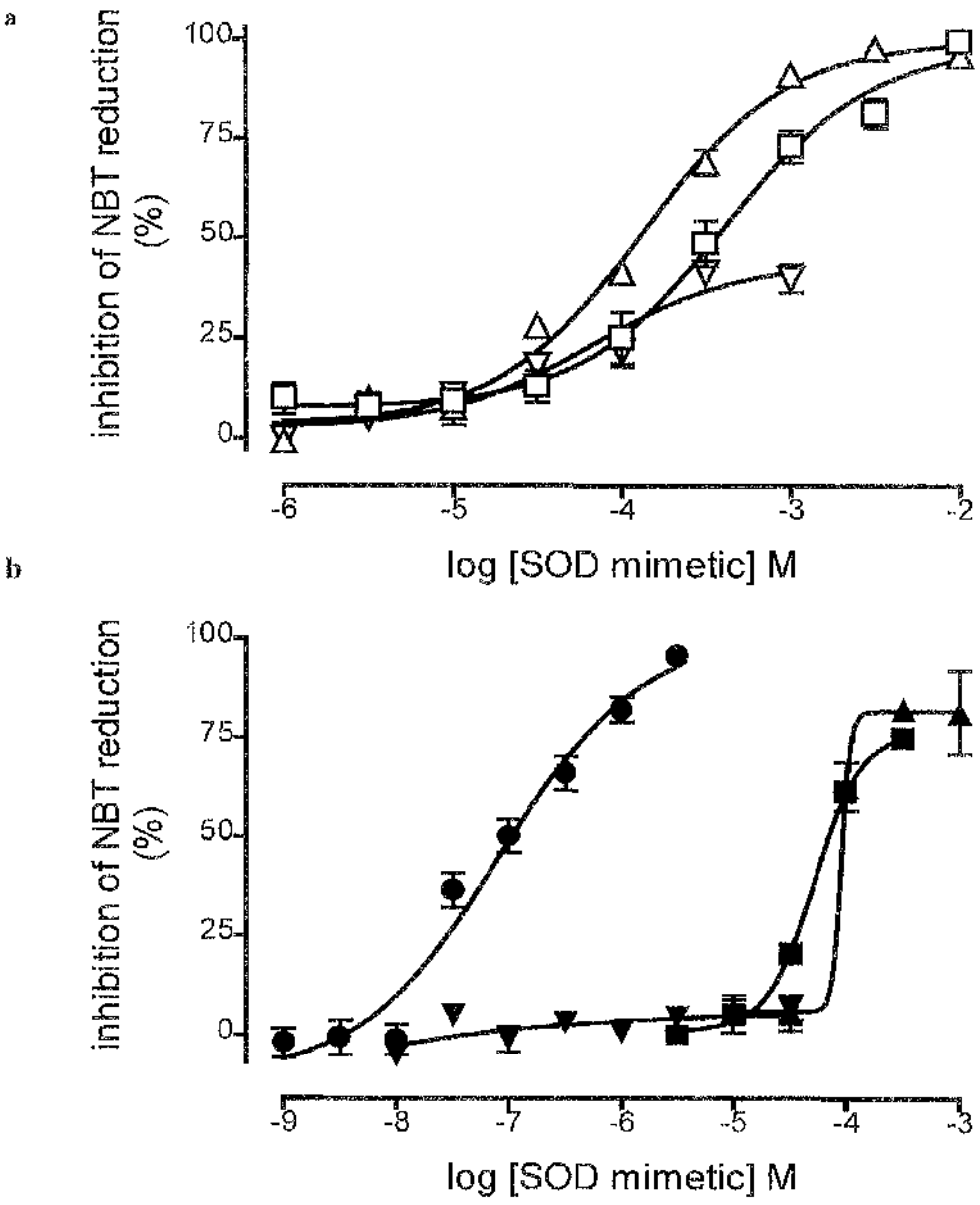


FIGURE 3.3 The ability of (a) PTIYO (□), tempol (Δ) and tiron (▽) and (b) $CuSO_4$ (▲), CuDIPS (▼), $MnCl_2$ (■) and MnTMPyP (●) to inhibit the reduction of nitro blue tetrazolium (NBT) by the NADH/phenazine methosulphate superoxide anion generating system. Each point is the mean ± s.e. mean of 8-16 observations.

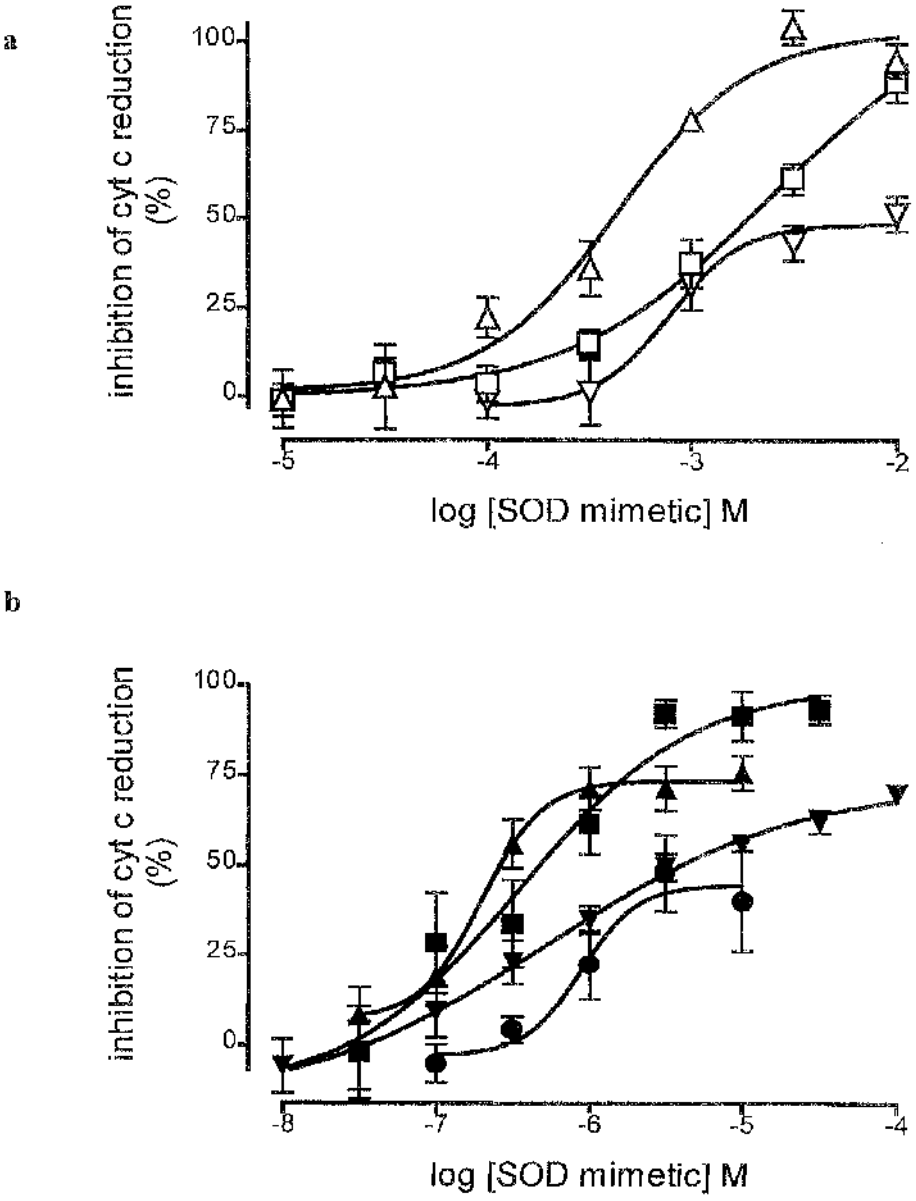


FIGURE 3.4. The ability of (**a**) PTIYO (□), tempol (Δ) and tiron (▽) and (**b**) $CuSO_4$ (▲), CuDIPS (▼), $MnCl_2$ (■) and MnTMPyP (●) to inhibit the reduction of cytochrome c (cyt c) by the xanthine oxidase/hypoxanthine superoxide anion generating system. Each point is the mean ± s.e. mean of 8-16 observations.

3.4 DISCUSSION

A number of biochemical assays can be employed to detect superoxide dismutase (SOD)-like activity. The majority of these are based on the ability of SOD to inhibit superoxide anion-mediated reactions. These assays, therefore, do not measure dismutation of superoxide directly. As a consequence, caution must be observed since the ability of a compound to interfere with the generation of superoxide anion could be interpreted incorrectly as SOD-like activity. Consequently, the use of two quite independent assays was regarded as a prudent step to insure against an agent being falsely identified as having SOD-like activity. The first assay was based on the ability of superoxide anion, generated using NADH and phenazine methosulphate, to reduce nitro blue tetrazolium (Halliwell & Gutteridge, 1989; Ewing and Janero, 1995), and the second on the ability of xanthine oxidase/hypoxanthine to reduce cytochrome c (McCord & Fridovich, 1968; Halliwell & Gutteridge, 1989; Laight *et al.*, 1997). The use of these assays was fully validated by the ability of authentic Cu/Zn SOD to inhibit virtually completely the reduction both of nitro blue tetrazolium and cytochrome c.

The presence of EDTA is necessary component in the nitro blue tetrazolium assay in order to chelate contaminating metals such as free copper of iron ions which could otherwise cause the dismutation of superoxide anion and therefore interfere with the assay of SOD activity. Consequently, assessment of the SOD-like activity of the metal-based agents that exist in an ionic state ($CuSO_4$ and $MnCl_2$) or as a weak co-ordination complex (CuDIPS; Huber *et al.*, 1987), was compromised in this assay. When the concentration of $CuSO_4$ or $MnCl_2$ was increased beyond the chelating

capacity of EDTA, i.e. above 0.1 mM, then both these agents exhibited substantial SOD-like activity. The insolubility of CuDIPS, however, limited the highest concentration of this agent to 30 μM, and this was well within the chelation capacity of EDTA. Consequently, CuDIPS demonstrated virtually no SOD-like activity in this assay. In contrast, the manganese centre of MnTMPyP is firmly located within the porphyrin ring (Faulkner *et al.*, 1994), and is not chelated by EDTA. Consequently, MnTMPyP demonstrated considerable SOD-like activity in this assay. For those agents which could easily be assessed in the nitro blue tetrazolium assay, the rank order of potency was MnTMPyP > tiron > tempol > PTIYO.

In contrast to the nitro blue tetrazolium assay, the cytochrome c assay does not utilise EDTA and, therefore, proved more versatile, permitting the activity of all of the SOD mimetics to be assessed. In this assay, the rank order of potency was $CuSO_4$ > $MnCl_2$ > CuDIPS > MnTMPyP > tiron ≥ tempol > PTIYO. Encouragingly, this rank order of potency was essentially similar to that of the nitro blue tetrazolium assay (excluding the metal-based agents inhibited by EDTA). Of the metal-based agents, only $MnCl_2$ produced near maximal inhibition of the reduction of cytochrome c. The remaining metal based agents, $CuSO_4$, CuDIPS and MnTMPyP, produced substantially lower inhibition. Amongst the metal-based agents, only $MnCl_2$ has its metal in its lower valence state, i.e. $Mn(II)Cl_2$, while the others all have the metal in their higher valence state, i.e. $Cu(II)SO_4$, Cu(II)DIPS and Mn(III)TMPyP. The lower maximal inhibition by $CuSO_4$, CuDIPS and MnTMPyP may result from an additional superoxide-independent component of reduction of cytochrome c. Specifically, during the dismutation of superoxide anion, the metal centres Cu(II) and

Mn(III) are reduced to Cu(I) and Mn(II), respectively (Step 1; de Alvare *et al.*, 1976; Huber *et al.*, 1987; Halliwell & Gutteridge, 1989; Faulkner *et al.*, 1994) and during subsequent normal redox cycling with superoxide anion (O_2^-), the Cu(I) and Mn(II) are re-oxidised, with hydrogen peroxide (H_2O_2) being produced (Step 2; Halliwell & Gutteridge, 1989):

Step 1: $\quad Cu(II)$ or $Mn(III) + O_2^- \rightarrow Cu(I)$ or $Mn(II) + O_2$

Step 2: $\quad Cu(I)$ or $Mn(II) + O_2^- + 2H^+ \rightarrow Cu(II)$ or $Mn(III) + H_2O_2$

From the electrochemical series, however, it is likely that the Cu(I) or Mn(II) formed in Step 1 will have the additional opportunity to participate in a redox reaction with the Fe^{3+} iron of cytochrome c, resulting in its reduction. Thus, the inhibition of superoxide-catalysed reduction of cytochrome c achieved by these compounds will be offset by the ability of their respective lower valency states to directly produce reduction. In contrast, the metal-free agents, PTIYO and tempol, produce near complete inhibition of reduction of cytochrome c. The metal-free agent, tiron, on the other hand produced only about half maximal inhibition of reduction of cytochrome c. This is likely to be due to the ability of tiron to cause direct reduction of cytochrome c by itself (Mok *et al.*, 1998).

Most of the compounds, including SOD itself, were more potent in the nitro blue tetrazolium assay than in the cytochrome c assay. This could suggest that less superoxide is being produced in the former assay than in the latter. The difference in potency is particularly striking for MnTMPyP (greater than 10-fold). As explained

above, MnTMPyP will dismutate superoxide anion by undergoing redox cycling from Mn(III) to Mn(II). The reaction rates for superoxide dismutation by Mn(II)TMPyP and Mn(III)TMPyP are, however, strikingly different, i.e. 4×10^9 M^{-1} s^{-1} and 3.9×10^7 M^{-1} s^{-1}, respectively (Faulkner *et al.*, 1994). Clearly, the optimum valency state of MnTMPyP for superoxide scavenging is the Mn(II) form. Reduction of the native state of Mn(III)TMPyP to the more active Mn(II)TMPyP form by the superoxide dismutation process is relatively slow, but can proceed faster, at least in *E. coli*, by reduction at the hand of cellular elements such as NADPH and glutathione (Faulkner *et al.*, 1994). This is likely to explain the reportedly greater superoxide removing potency of MnTMPyP in *E. coli* than in *in vitro* biochemical assays of SOD-like activity where these additional means of reduction are absent (Faulkner *et al.*, 1994; Gardner *et al.*, 1996). The nitro blue tetrazolium assay uses the NADH/phenazine methosulphate reducing system as the means of generating superoxide anion (Halliwell & Gutteridge, 1989; Ewing and Janero, 1995) and this is likely to reduce at least some of the Mn(III)TMPyP to the more active Mn(II)TMPyP. HX/XO has no such reducing ability, however, and this probably explains the lower potency of MnTMPyP in the cytochrome c assay.

Having assessed the rank-order of SOD-like activity of the different SOD mimetics in two independent biochemical assays, the path was clear to investigate how this compared with the ability of these agents to protect nitric oxide from destruction by superoxide anion in the models of oxidant stress developed in rabbit aorta (Chapter 2).

CHAPTER 4

PROTECTION OF NITRIC OXIDE BY SUPEROXIDE DISMUTASE MIMETICS

4.1 INTRODUCTION

The loss of vasodilator function following the interaction of nitric oxide with superoxide (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986b; Kaustic & Vanhoutte, 1989; Ohlstein & Nichols, 1989, Mian & Martin, 1995a) has led to the suggestion that this process is involved in a number of cardiovascular pathologies. Therapeutic intervention with the scavenger of superoxide anion, superoxide dismutase (SOD), would seem to be a logical approach to redress the superoxide/nitric oxide imbalance. The large size of this enzyme, however, prevents its entry into cells to protect nitric oxide from an intracellular oxidant stress (Gardner *et al.*, 1996). There is considerable interest, therefore, in the therapeutic potential of membrane permeant superoxide dismutase (SOD) mimetics in the treatment of pathologies associated with oxidant stress. A diverse range of these has been described, including the metal-based compounds CuDIPS (Cu [II]-[diisopropylsalicylate]$_2$; Huber *et al.*, 1987; Sorenson, 1995) and MnTMPyP (Mn [III] tetrakis [1-methyl-4-pyridyl] porphyrin; Faulkner *et al.*, 1994; Gardner *et al.*, 1996) which dismutate superoxide in a manner similar to that of authentic Cu/Zn SOD and Mn SOD, respectively. Simple metal salts of Cu and Mn also possess SOD-like activity (Huber *et al.*, 1987; Beyer & Fridovich, 1990). Other compounds, such as the nitroxide spin traps PTIYO (4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide) and tempol (4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl) (Mitchell *et al.*, 1990; Ewing & Jancro, 1995), and the superoxide scavenger tiron (4,5-dihydroxy-1,3-benzene-disulfonic acid; Ledenev *et al.*, 1986) are also known to relieve oxidant stress in isolated cell studies.

In the previous chapter, the rank order of potency of the above SOD mimetics to scavenging superoxide anion was assessed using two independent biochemical assays. The central aim of this chapter was to investigate the potential ability of these SOD mimetics to restore nitric oxide-dependent vasodilator function following its inhibition by oxidant stress. As described in Chapter 2, two distinct models of oxidant stress were created in isolated rings of rabbit aorta in an attempt to mimic the impaired endothelial-dependent relaxation associated with vascular disease. One model involved treatment with a low concentration of the inactivator of endogenous Cu/Zn SOD, diethyldithiocarbamate (DETCA; 0.3 mM). This powerfully potentiated the ability of xanthine oxidase/hypoxanthine, which generates superoxide only in the extracellular space, to impair both basal and acetylcholine (ACh)-stimulated activity of nitric oxide. These impairments were reversed following treatment with exogenous SOD suggesting that in this form of oxidant stress, nitric oxide was destroyed by superoxide in the extracellular space. The other model of oxidant stress involved treatment with a high concentration of DETCA (3 mM) alone to inhibit extracellular and intracellular isoforms of Cu/Zn SOD. Although this had no effect on basal nitric oxide activity, it resulted in blockade of ACh-induced relaxation in a manner which was not reversed by membrane-impermeant, exogenously applied SOD. The destruction of ACh-induced nitric oxide activity under these conditions is therefore likely to have resulted from inhibition of intracellular Cu/Zn SOD leading to intracellular destruction of nitric oxide by superoxide anion

In this chapter, these models of oxidant stress were used to assess the ability of the SOD mimetics to protect nitric oxide from destruction *in vitro*.

4.2 METHODS

4.2.1 Preparation of tissues and assessment of nitric oxide activity

Methods for preparation of rabbit aortic rings for tension recording are detailed in Chapter 2. Basal activity of nitric oxide was assessed indirectly by measuring the endothelium-dependent depression of phenylephrine (PE)-induced contraction in aortic rings. This endothelium-dependent depression of vasoconstriction would be expected to be blocked by agents which inhibit the activity of nitric oxide. Specifically, submaximal tone was generated in aortic rings using PE (0.1 μM) and basal nitric oxide activity was assessed by the augmentation of tone seen following addition of the inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), or the superoxide-generating system, xanthine oxidase (4.8 mu ml^{-1})/ hypoxanthine (0.1 mM). Agonist-stimulated activity of nitric oxide was determined by assessing acetylcholine (ACh)-induced relaxation. Specifically, cumulative concentration response curves to ACh (10 nM - 3 μM) were constructed on endothelium containing rings following induction and stabilisation of submaximal PE (30 - 300 nM)-induced tone.

4.2.2 Effect of superoxide dismutase mimetics

The effects of superoxide dismutase (SOD) mimetics were assessed on basal and ACh-stimulated activity of nitric oxide. Specifically, following PE (30 – 300 nM)-induced contraction and a 20 min treatment with PTIYO (100 μM), tempol (3 mM), tiron (10 mM), $CuSO_4$ (3 μM), CuDIPS (3 μM), $MnCl_2$ (100 μM) or MnTMPyP

(100 μM), basal nitric oxide activity was assessed from the augmentation of tone observed following the addition of L-NAME (100 μM). The effects of the mimetics on agonist-induced activity of nitric oxide was assessed by construction of concentration-response curves to ACh (10 nM – 3 μM) on PE (30 – 300 nM)-contracted rings pretreated for 20 min with PTIYO (10 - 300 μM), tempol (0.1 - 3 mM), tiron (1 - 10 mM), $CuSO_4$ (1 - 100 μM), CuDIPS (1 - 100 μM), $MnCl_2$ (1 - 100 μM) or MnTMPyP (10 μM - 1 mM).

4.2.3 Generation of oxidant stress by inactivation of endogenous Cu/Zn superoxide dismutase together with superoxide anion production

Oxidant stress was generated by inactivating endogenous Cu/Zn SOD with a low concentration of the copper chelator, diethyldithiocarbamate (DETCA) in combination with superoxide anion generation using the xanthine oxidase (XO)/hypoxanthine (HX) system. In these experiments, following a 60 min incubation period with DETCA (0.3 mM; followed by washout), aortic rings were submaximally contracted with PE (30 – 300 nM). XO (4.8 mu ml^{-1}) was then added at least 20 min before the addition of its substrate, HX (0.1 mM). As indicated in Chapter 2, this treatment resulted in an inhibition of both basal and ACh-stimulated activity of nitric oxide.

An examination was made of the ability of exogenously applied authentic Cu/Zn SOD and of the SOD mimetics to prevent this inhibition of nitric oxide activity. In these experiments, SOD (250 u ml^{-1}), PTIYO (10 - 300 μM), tempol (30 μM - 1 mM), tiron (0.3 - 10 mM), $CuSO_4$ (0.3 - 3 μM), CuDIPS (1 - 100 μM), $MnCl_2$ (1 -

100 μM) or MnTMPyP (100 - 600 μM) were added to the XO (4.8 mu ml^{-1})-treated tissue 20 min before addition of HX (0.1 mM). The ability of SOD and the SOD mimetics to protect basal activity of nitric oxide was assessed indirectly from the depression of the increase in tone observed upon the addition of HX. Furthermore, their ability to protect agonist-stimulated activity of nitric oxide was assessed from cumulative concentration-response curves to ACh (10 nM – 3 μM).

4.2.4 Generation of oxidant stress by inactivation of endogenous Cu/Zn superoxide dismutase

The other condition of oxidant stress described in Chapter 2 involved a 60 min incubation with a high concentration of DETCA (3 mM; followed by washout). This had no effect on basal nitric oxide activity but resulted in blockade of ACh-induced relaxation which was likely to be due to intracellular destruction of nitric oxide by superoxide anion. An examination was made of the ability of the SOD mimetics to reverse this inhibition of ACh-induced relaxation. In these experiments, after incubation and washout of DETCA (3 mM; 60 min) and induction of submaximal PE (30 – 300 nM) tone, PTIYO (10 μM), tempol (0.1 mM), tiron (1 mM), $CuSO_4$ (1 μM), CuDIPS (1 μM), $MnCl_2$ (100 μM) or MnTMPyP (600 μM and 1 mM) were added 20 min before the generation of concentration response curves to ACh (10 nM – 3 μM).

All experiments involving DETCA or XO/HX were conducted in the presence of catalase (1000 u ml^{-1}) to prevent the accumulation of hydrogen peroxide.

4.3 RESULTS

4.3.1 Effects of superoxide dismutase mimetics on acetylcholine-induced relaxation

Before examining the ability of the SOD mimetics to protect nitric oxide under conditions of oxidant stress, experiments were conducted to determine if they had any effects by themselves on acetylcholine (ACh)-induced relaxation.

Following induction of phenylephrine (PE; 30 - 300 nM)-induced tone in endothelium-containing rings of rabbit aorta, exogenously applied Cu/Zn superoxide dismutase (SOD; 250 u ml^{-1}) had no effect on ACh-induced relaxation (Chapter 2; Figure 2.12). As with SOD, treatment for 20 min with the superoxide dismutase (SOD) mimetics, tiron (1 - 30 mM), MnTMPyP (10 μM – 1 mM), or $MnCl_2$ (1 - 100 μM) failed to affect ACh (10 nM - 3 μM)-induced relaxation (Figures 4.2 and 4.4). Relaxation was, however, impaired slightly following treatment with tempol at 3 mM but not at 0.1 or 1 mM (Figure 4.2) and to a greater extent by PTIYO at 300 μM but not at 10 or 100 μM (Figure 4.1). Both CuDIPS and $CuSO_4$ caused a significant impairment of relaxation at 10 and 100 μM but not at 1 μM (Figure 4.3). Thus, although some SOD mimetics have no detrimental action on ACh-induced relaxation, the ability of others to produce impairment of relaxation by themselves will clearly compromise their ability to protect nitric oxide in conditions of oxidant stress.

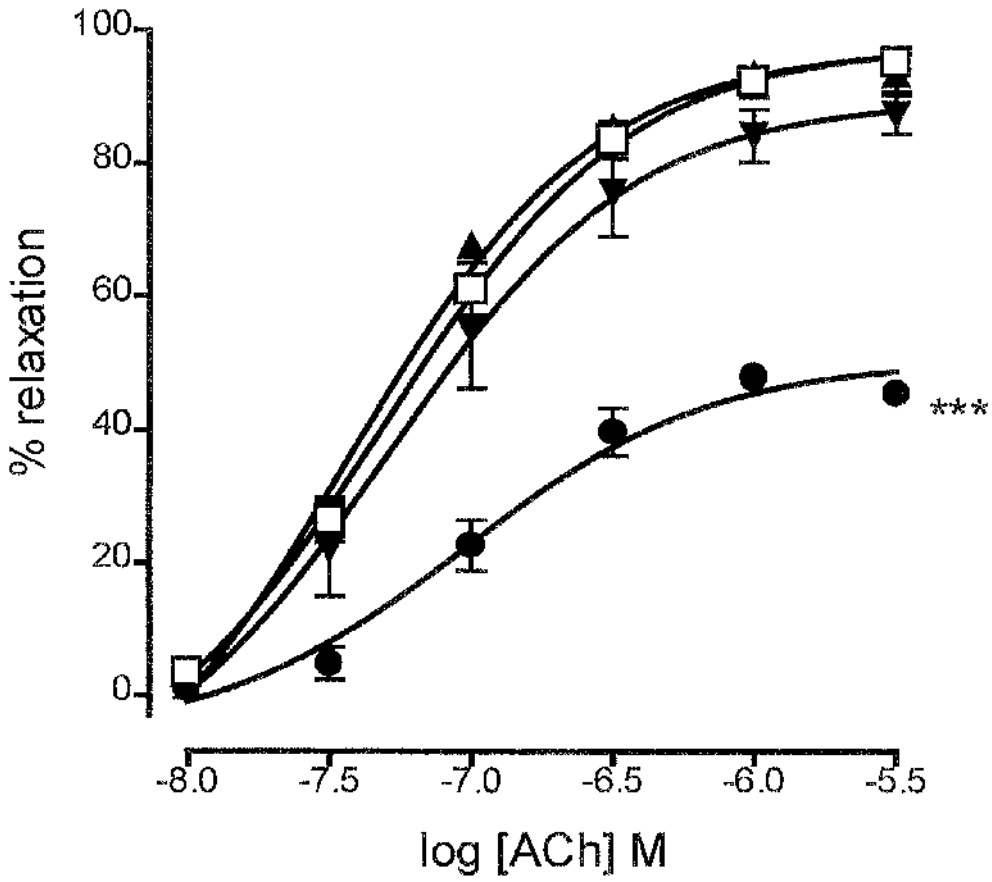


FIGURE 4.1 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and the effects of PTIYO at 10 (▲), 100 (▼) and 300 μM (●) on this relaxation. Each point is the mean ± s.e. mean of 5-11 observations. ***P<0.001 indicates a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

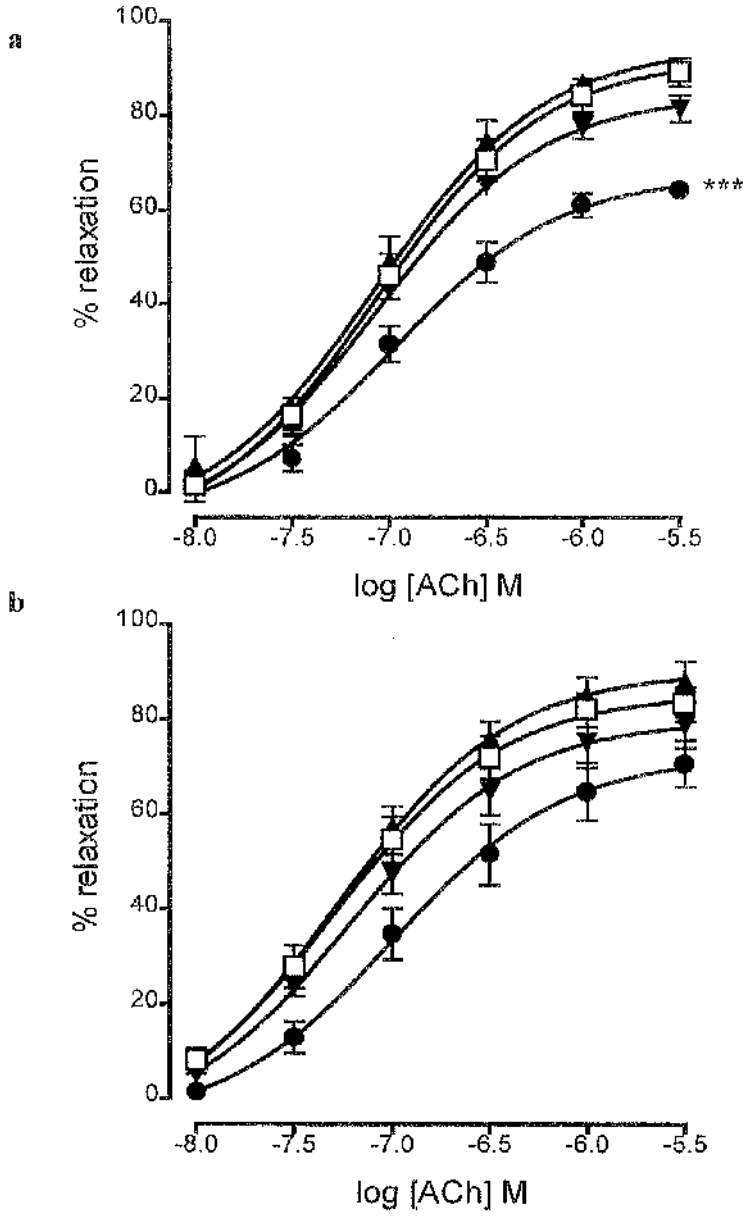


FIGURE 4.2 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and the effects of (**a**) tempol 0.1 (▲), 1 (▼) and 3 mM (●) and (**b**) tiron at 1 (▲), 10 (▼) and 30 mM (●) on this relaxation. Each point is the mean ± s.e. mean of 5-11 observations. ***P<0.001 indicates a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

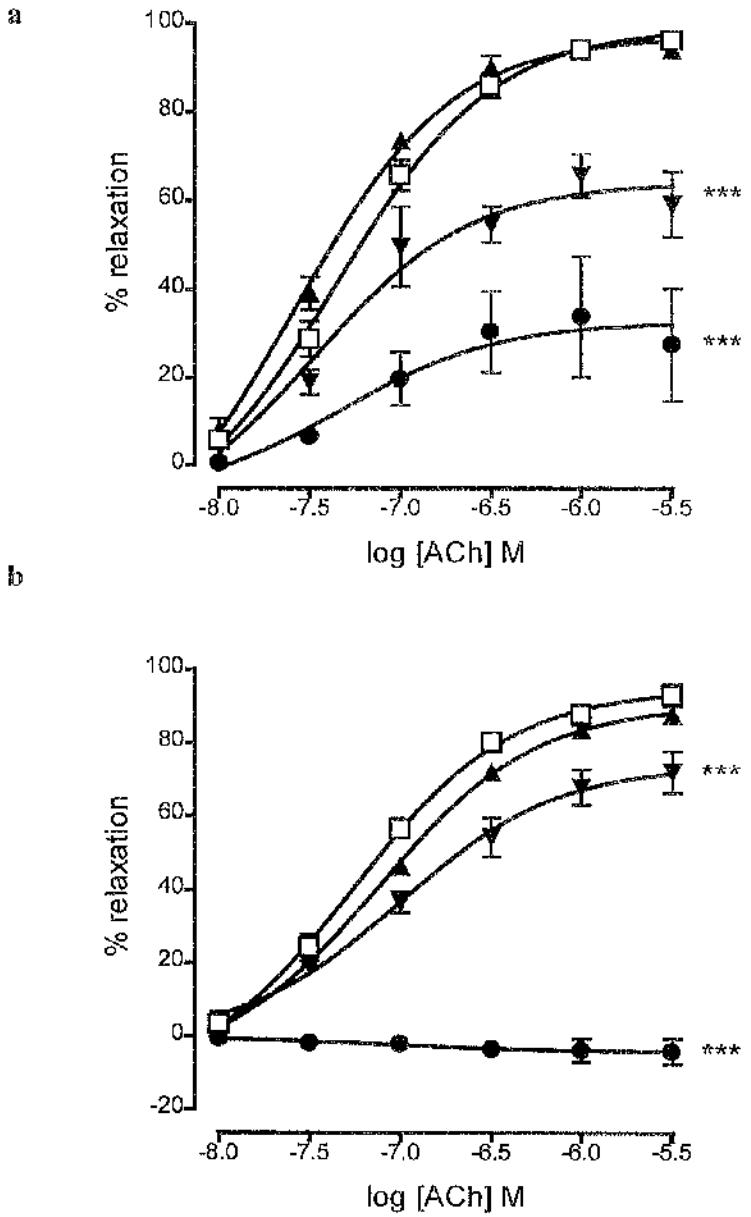


FIGURE 4.3 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and the effects of (a) $CuSO_4$ at 1 (▲), 10 (▼) and 100 μM (●) and (b) CuDIPS at 1 (▲), 10 (▼) and 100 μM (●) on this relaxation. Each point is the mean ± s.e. mean of 7-10 observations. ***P<0.001 indicates a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

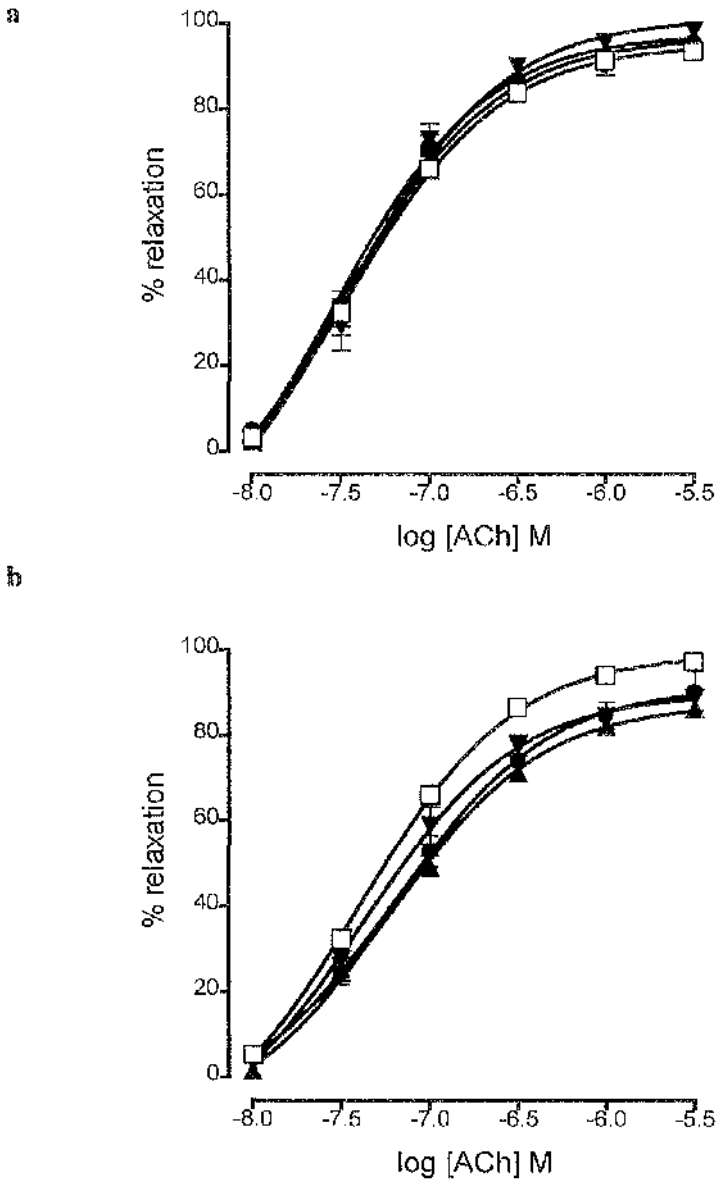


FIGURE 4.4 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and the effects of (a) $MnCl_2$ at 1 (▲), 10 (▼) and 100 μM (●) and (b) MnTMPyP at 10 (▲) and 100 μM (▼) and 1 mM (●) on this relaxation. Each point is the mean ± s.e. mean of 5-12 observations.

4.3.2 Ability of superoxide dismutase mimetics to inhibit the endothelium-dependent rise in tone induced by xanthine oxidase/hypoxanthine in diethyldithiocarbamate (0.3 mM)-treated tissues

In Chapter 2, evidence was provided that the augmentation of tone seen in endothelium-containing tissues upon addition of xanthine oxidase/hypoxanthine (XO/HX) was due to destruction of basal nitric oxide by superoxide anion. Exogenously applied authentic membrane-impermeant SOD prevented this augmentation (Chapter 2; Figures 2.6 and 2.7). This suggests that the destruction of nitric oxide occurred in the extracellular space. The ability of SOD mimetics to inhibit this augmentation was therefore examined as an additional means of assessing their SOD-like action.

As expected, the endothelium-dependent rise in PE (30 – 300 nM)-induced tone produced by XO (4.8 mu ml^{-1})/HX (0.1 mM) in DETCA (0.3 mM, 60 min. washout)-treated tissues was inhibited by a 20 min pretreatment with SOD (250 u ml^{-1}; Figure 4.5 - 4.8). It was also inhibited in a concentration-dependent manner by a 20 min pretreatment with PTIYO (10 - 300 μM), tempol (0.1 - 3mM), tiron (1 - 30 mM), CuDIPS (0.1 - 3 μM), $CuSO_4$ (0.1 - 3 μM), MnTMPyP (100 - 600 μM) and $MnCl_2$ (1 - 100 μM) (Figures 4.5 - 4.8).

Thus, all of the SOD-mimetics were able to prevent the XO/HX-mediated augmentation of PE-induced tone. However, based upon these results alone it would be unwise to conclude that all of the SOD mimetics had indeed protected basal nitric oxide from destruction by superoxide anion. An alternative explanation needed to be

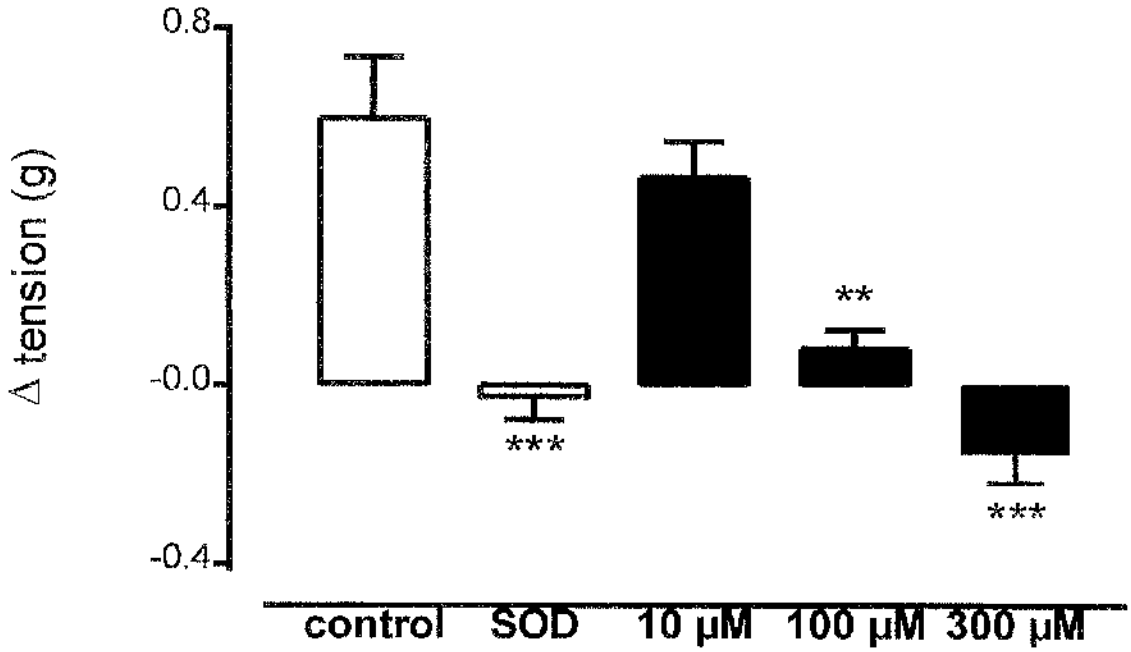


FIGURE 4.5 The enhancement of phenylephrine (0.1 µM)-induced tone resulting from treatment with xanthine oxidase (4.8 mu ml^{-1})/hypoxanthine (0.1 mM) in endothelium-containing rings of rabbit incubated with diethyldithiocarbamate (0.3 mM) (control) was abolished by pretreatment with superoxide dismutase (SOD; 250 u ml^{-1}), and inhibited in a concentration-dependent manner by PTIYO (10, 100 and 300 µM) (filled columns). Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each column is the mean ± s.e. mean of 6 observations. **P<0.01 and ***P<0.001 indicate significant differences from the change in tone of control tissues, respectively.

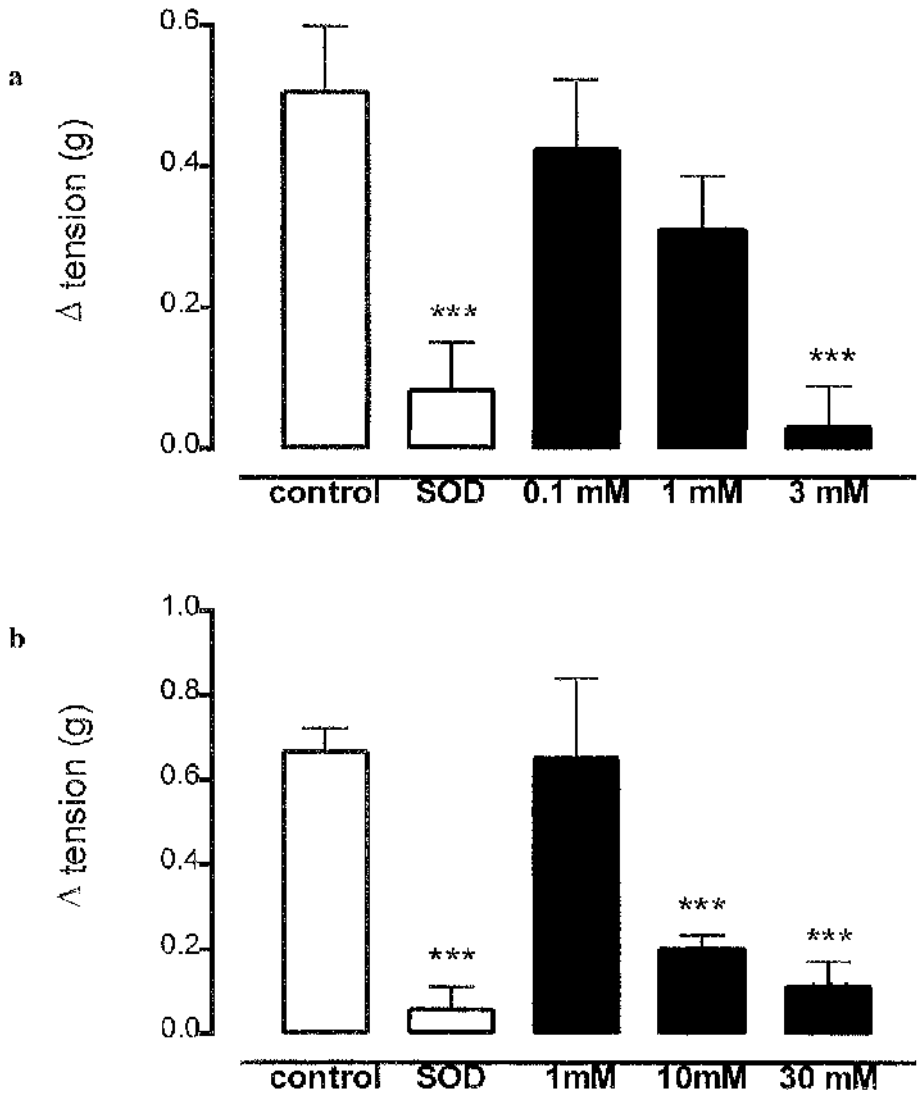


FIGURE 4.6 The enhancement of phenylephrine (0.1 μM)-induced tone resulting from treatment with xanthine oxidase (4.8 mu ml^{-1})/hypoxanthine (0.1 mM) in endothelium-containing rings of rabbit incubated with diethyldithiocarbamate (0.3 mM) (control) was abolished by pretreatment with superoxide dismutase (SOD; 250 u ml^{-1}), and inhibited in a concentration-dependent manner by (**a**) tempol (0.1, 1 and 3 mM) and (**b**) tiron (1, 10 and 30 mM) (filled columns). Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each column is the mean ± s.e. mean of 5-10 observations. ***P<0.001 indicates a significant differences from the change in tone of control tissues.

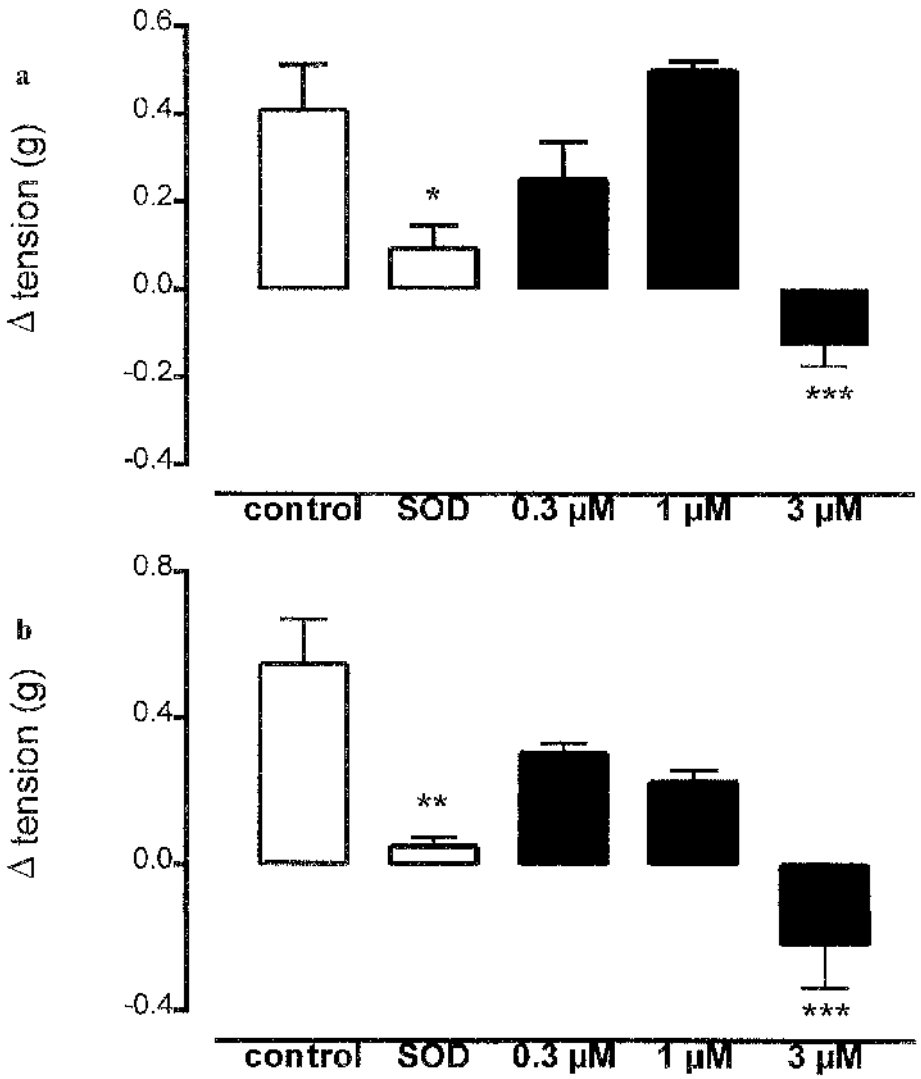


FIGURE 4.7 The enhancement of phenylephrine (0.1 μM)-induced tone resulting from treatment with xanthine oxidase (4.8 mu ml^{-1})/hypoxanthine (0.1 mM) in endothelium-containing rings of rabbit incubated with diethyldithiocarbamate (0.3 mM) (control) was abolished by pretreatment with superoxide dismutase (SOD; 250 u ml^{-1}), and inhibited in a concentration-dependent manner by (**a**) $CuSO_4$ (0.3, 1 and 3 μM) and (**b**) CuDIPS (0.3, 1 and 3 μM) (filled columns). Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each column is the mean ± s.e. mean of 5-10 observations. *P<0.05, **P<0.01 and ***P<0.001 indicate significant differences from the change in tone of control tissues, respectively.

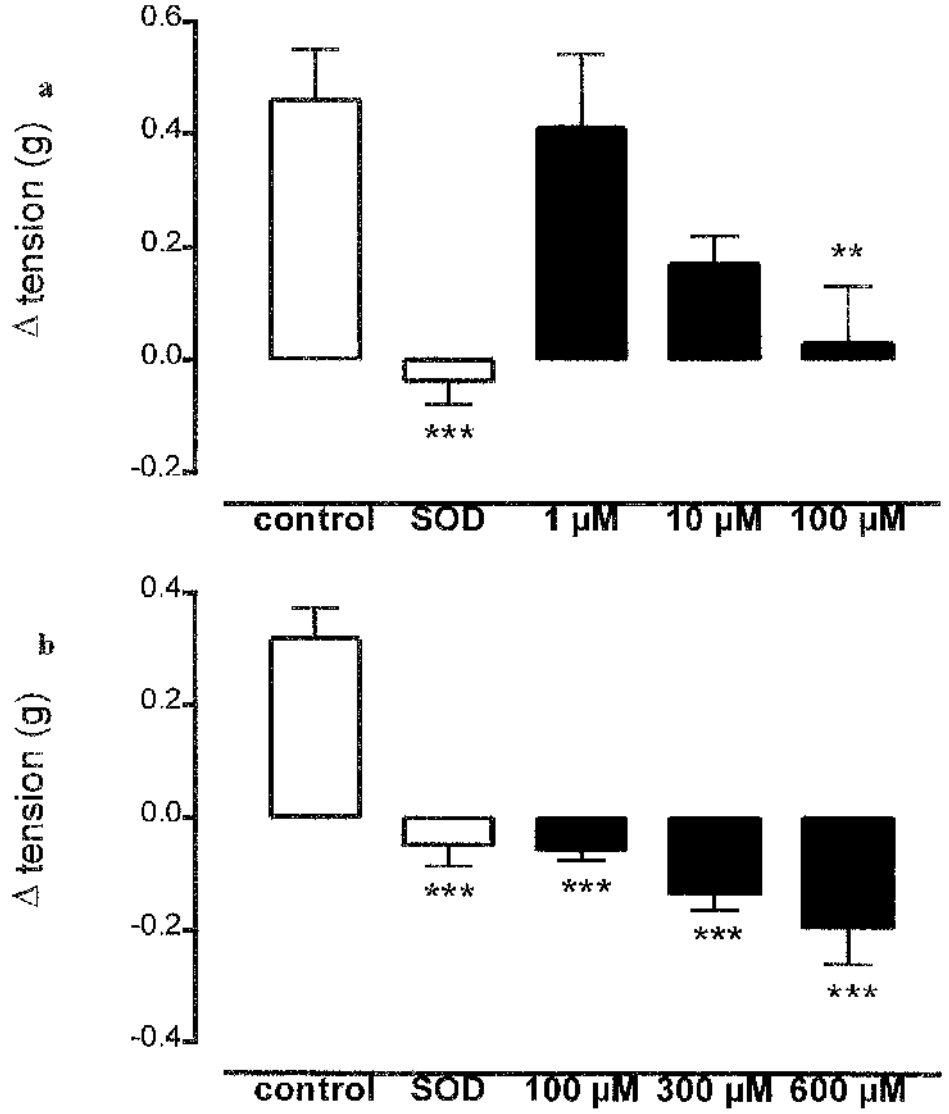


FIGURE 4.8 The enhancement of phenylephrine (0.1 μM)-induced tone resulting from treatment with xanthine oxidase (4.8 mu ml^{-1})/hypoxanthine (0.1 mM) in endothelium-containing rings of rabbit incubated with diethyldithiocarbamate (0.3 mM) (control) was abolished by pretreatment with superoxide dismutase (SOD; 250 u ml^{-1}), and inhibited in a concentration-dependent manner by (**a**) $MnCl_2$ (1, 10 and 100 μM) and (**b**) MnTMPyP (100, 300 and 600 μM) (filled columns). Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each column is the mean ± s.e. mean of 5-11 observations. **P<0.01 and ***P<0.001 indicate significant differences from the change in tone of control tissues, respectively.

considered first, i.e. that the SOD mimetics had inactivated basal nitric oxide themselves and this action, and not removal of superoxide had led to blockade of the XO/HX-induced augmentation of tone. To test this possibility, each of the SOD mimetics was added to rings of rabbit aorta to determine if they affected the endothelium-dependent augmentation of PE-induced tone seen upon the addition of the inhibitor of nitric oxide synthase, L-NAME (100 μM). The augmentation of tone by L-NAME does not involve superoxide anion and would therefore be unaffected by an agent possessing SOD-like activity only. A single high concentration of each SOD mimetic was used in this section of the study.

The L-NAME (100 μM)-induced rise in tone of PE (0.1 μM)-contracted endothelium-containing rings was unaffected following treatment with exogenously applied authentic Cu/Zn SOD (Chapter 2; Figure 2.5a). As with SOD, the L-NAME-induced rise in tone was unaffected following a 20 min pretreatment with tiron (10 mM), CuDIPS (3 μM), $CuSO_4$ (3 μM) or $MnCl_2$ (100 μM) (Figure 4.9). In contrast, the rise in tone was impaired by around 50 % following treatment with PTIYO (100 μM) and tempol (3 mM), and by around 25 % by MnTMPyP (100 μM).

Thus, CuDIPS, $CuSO_4$ and $MnCl_2$ do not appear to impair basal nitric oxide activity and their ability to inhibit the augmentation of PE-induced tone produced by XO/HX probably reflects their activity in removing superoxide anion. For PTIYO, tempol and to a lesser extent MnTMPyP, a major component of their ability to impair the augmentation of PE-induced tone will have resulted from inhibition of the action of basal nitric oxide, with the remainder occurring from the scavenging of superoxide.

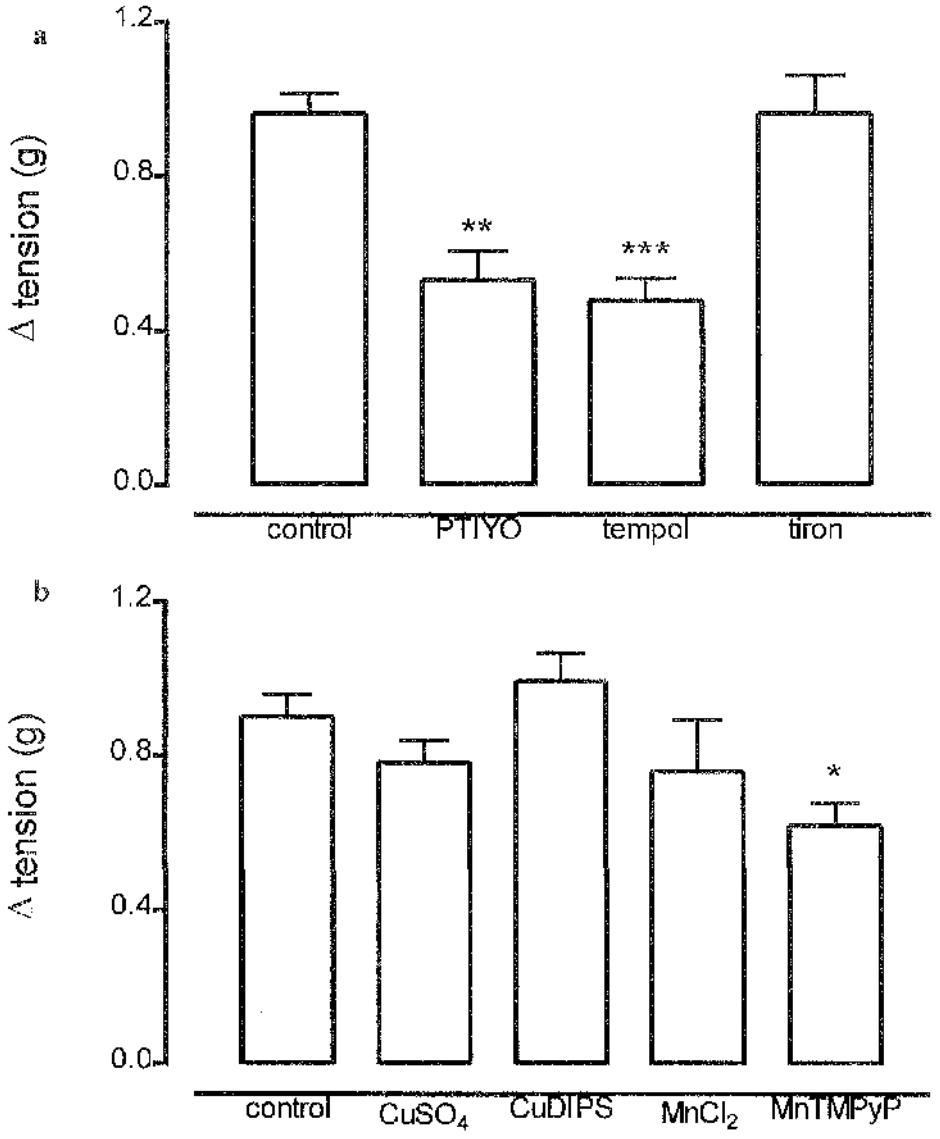


FIGURE 4.9 The enhancement of phenylephrine (0.1 μM)-induced tone resulting from treatment with L-NAME (100 μM; control) in endothelium-containing rings of rabbit aorta was impaired by treatment with (**a**) PTIYO (100 μM) and tempol (3 mM) but not tiron (3 mM) and (**b**) MnTMPyP (100 μM) but not $CuSO_4$ (3 μM), CuDIPS (3 μM) or $MnCl_2$ (100 μM). Each column is the mean ± s.e. mean of 6-12 observations. *P<0.05, **P<0.01 and **P<0.001 indicate significant differences from the change in tone of control tissues, respectively.

4.3.3 Ability of superoxide dismutase mimetics to protect acetylcholine-induced relaxation from blockade by xanthine oxidase/hypoxanthine in diethyldithiocarbamate (0.3 mM)-treated tissues

The blockade of ACh-induced relaxation seen with by xanthine oxidase (XO; 4.8 mu ml^{-1})/hypoxanthine (HX; 0.1 mM) in DETCA (0.3 mM, 60 min, washout)-treated tissues was completely prevented by pretreatment with authentic SOD (250 u ml^{-1}) (Chapter 2; Figure 2.17). The blockade was also prevented, in a concentration-dependent manner, by a 20 min pretreatment with MnTMPyP (100 - 600 μM) and $MnCl_2$ (1 - 100 μM) (Figure 4.13). Substantial prevention of blockade was also observed following pretreatment with CuDIPS at 1 and 3 μM, but not at 0.1 mM, and with $CuSO_4$ (0.3 - 3 μM) (Figure 4.12). There was, however, a tendency for both of these, at the highest concentration used (3 μM), to be less effective at concentrations of ACh of 1 μM and above. Weak prevention of blockade of ACh-induced relaxation was observed following pretreatment with tiron at 1 mM but none was seen at 0.3 or 10 mM (Figure 4.11). Tempol produced weak prevention of blockade at 0.1 mM but none was seen at 30 μM or 1 mM (Figure 4.11). No reversal of blockade was observed, however, following pretreatment with PTIYO at 10 and 100 μM, and at 300 μM the blockade was intensified (Figure 4.10).

Thus, the metal-based SOD mimetics seem more effective than the spin traps in protecting nitric oxide from destruction by this extracellular oxidant stress.

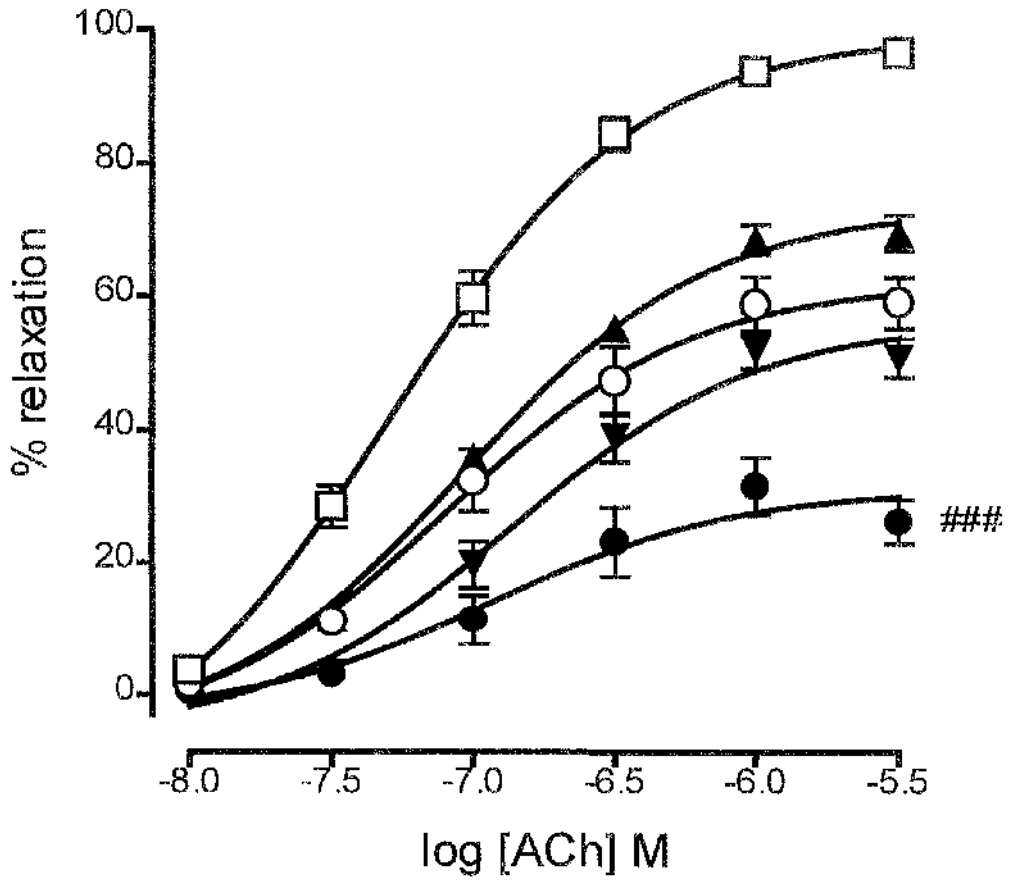


FIGURE 4.10 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and the blockade of this relaxation following combined treatment with diethyldithiocarbamate (0.3 mM, 60 min, washout) and xanthine oxidase (4.8 mu ml^{-1})/hypoxanthine (0.1 mM) (○). The effects of 20 min pretreatment with PTIYO at 10 (▲), 100 (▼) and 300 μM (●) on this blockade are also shown. Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 6-12 observations. ###P<0.001 indicates that the SOD mimetic had a significant effect on the relaxation induced by the maximal concentration of ACh in rings treated with diethyldithiocarbamate and xanthine oxidase/ hypoxanthine.

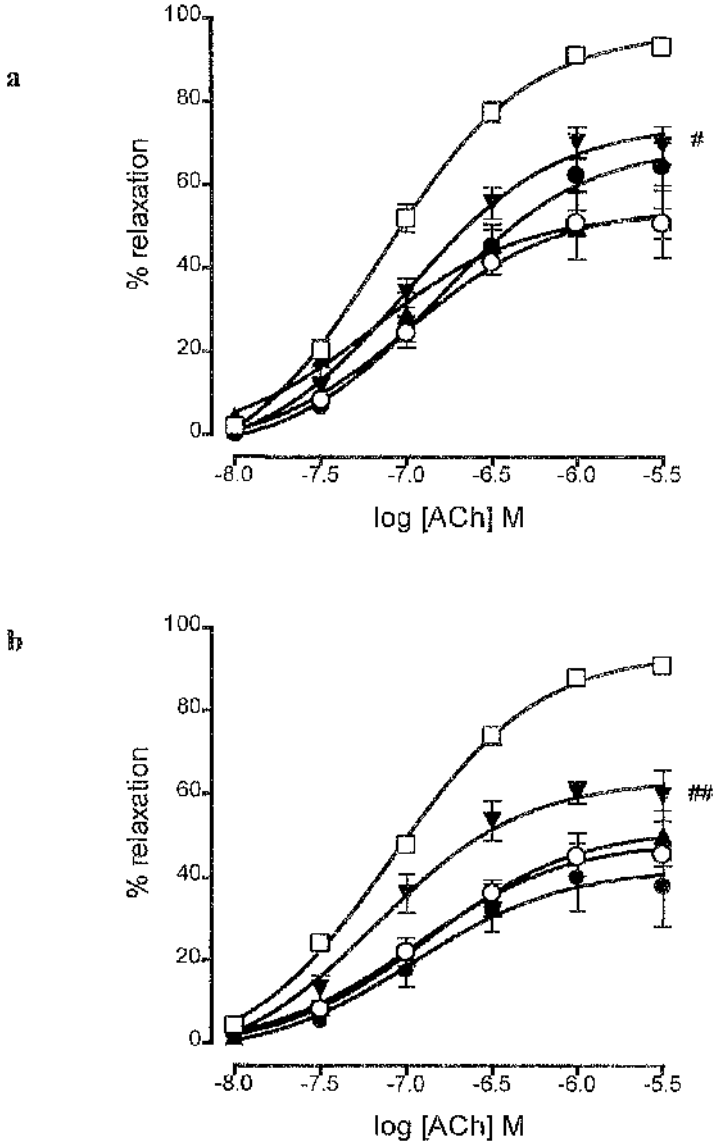


FIGURE 4.11 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and the blockade of this relaxation following combined treatment with diethyldithiocarbamate (0.3 mM, 60 min, washout) and xanthine oxidase (4.8 mu ml^{-1})/hypoxanthine (0.1 mM) (○). The effects of a 20 min pretreatment with (**a**) tempol at 30 μM (▲), 0.1 (▼) and 1 mM (●) and (**b**) tiron at 0.3 (▲), 1 (▼) and 10 mM (●) on this blockade are also shown. Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 5-15 observations. #P<0.05 and ##P<0.01 indicate that the SOD mimetic had a significant effect on the relaxation induced by the maximal concentration of ACh in rings treated with diethyldithiocarbamate and xanthine oxidase/ hypoxanthine.

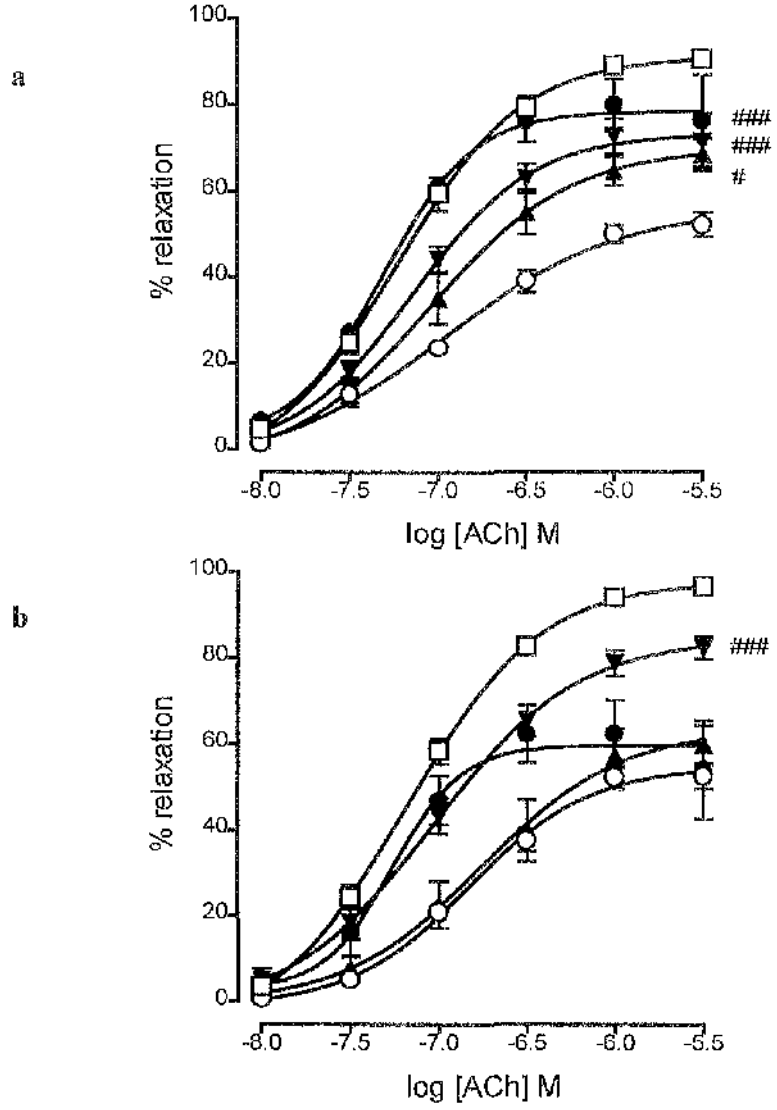


FIGURE 4.12 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and the blockade of this relaxation following combined treatment with diethyldithiocarbamate (0.3 mM, 60 min, washout) and xanthine oxidase (4.8 mu ml^{-1})/hypoxanthine (0.1 mM) (○). The effects of a 20 min pretreatment with (**a**) $CuSO_4$ at 0.3 (▲), 1 (▼) and 3 μM (●) and (**b**) CuDIPS at 0.1 (▲), 1 (▼) and 3 μM (●) on this blockade are also shown. Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 5-15 observations. #P<0.05 and ###P<0.001 indicate that the SOD mimetic had a significant effect on the relaxation induced by the maximal concentration of ACh in rings treated with diethyldithiocarbamate and xanthine oxidase/ hypoxanthine.

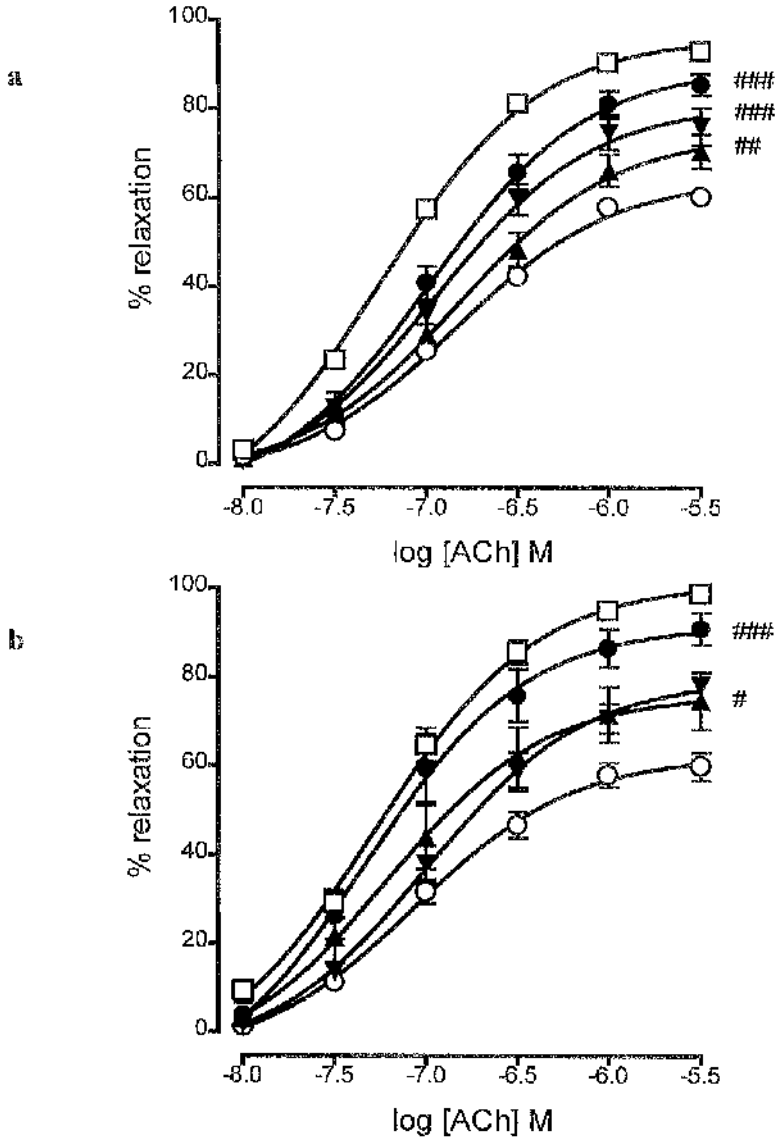


FIGURE 4.13 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and the blockade of this relaxation following combined treatment with diethyldithiocarbamate (0.3 mM, 60 min, washout) and xanthine oxidase (4.8 mu ml^{-1})/hypoxanthine (0.1 mM) (○). The effects of a 20 min pretreatment with (a) $MnCl_2$ at 1 (▲), 10 (▼) and 100 μM (●) and (b) MnTMPyP at 100 (▲), 300 (▼) and 600 μM (●) on this blockade are also shown. Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 5-15 observations. #P<0.05, ##P<0.01 and ###P<0.001 indicate that the SOD mimetic had a significant effect on the relaxation induced by the maximal concentration of ACh in rings treated with diethyldithiocarbamate and xanthine oxidase/ hypoxanthine.

4.3.4 Effects of superoxide dismutase mimetics on acetylcholine-induced relaxation in diethyldithiocarbamate (3 mM)-treated tissues

The blockade of ACh-induced relaxation resulting from treatment with a high concentration of DETCA (3 mM) alone is unaffected by treatment with membrane-impermeant exogenous SOD (250 u ml^{-1}) (Chapter 2; Figure 2.12). This blockade may therefore result from the inactivation of endogenous intracellular Cu/Zn SOD leading to an increase in the background levels of superoxide which in turn would destroy nitric oxide inside cells, i.e. it is likely therefore that DETCA exerts an intracellular oxidant stress. If this is indeed the case, then, in contrast to the ineffectiveness of SOD itself, the membrane-permeant SOD mimetics would be expected to reverse this blockade. Consequently, experiments were designed to test this possibility. The concentrations of the SOD mimetics used in this section of the study were those which proved most effective at reversing the blockade induced by the extracellular oxidant stress (or for PTIYO, the highest concentration that did not enhance the blockade induced by the extracellular oxidant stress).

The blockade of ACh (10 nM – 3 μM)-induced relaxation resulting from treatment with DETCA (3 mM; 60 min; washout) was not reversed to any degree by treatment for 20 min with PTIYO (10 μM), tempol (0.1 mM) or tiron (1 mM) (Figure 4.14) or with CuDIPS or $CuSO_4$ (both at 1 μM; Figure 4.15). In fact, treatment with PTIYO and CuDIPS actually enhanced the blockade seen. In contrast, significant, although incomplete reversal of blockade was observed following treatment with MnTMPyP (600 μM and 1 mM) and with $MnCl_2$ (100 μM) (Figure 4.16).

Thus, a major component of the actions of DETCA is likely to result from inhibition of intracellular Cu/Zn SOD, with the remainder perhaps reflecting non-selective effects of DETCA.

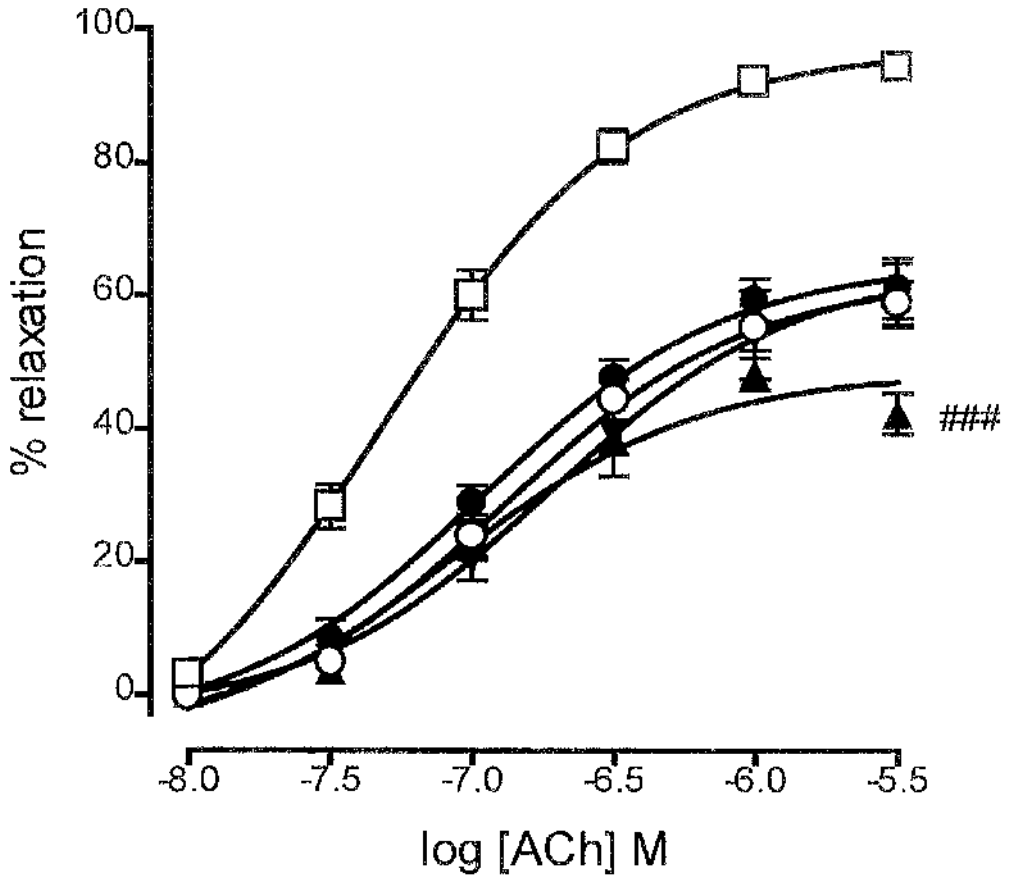


FIGURE 4.14 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and the blockade of this relaxation following treatment with diethyldithiocarbamate (3 mM; 60 min; washout; ○). The effects of PTIYO (10 μM; ▲), tempol (0.1 mM; ▼) and tiron (1 mM; ●) on this blockade are also shown. Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 5-15 observations. ###P<0.001 indicates that the SOD mimetic had a significant effect on the relaxation induced by the maximal concentration of ACh in diethyldithiocarbamate-treated rings.

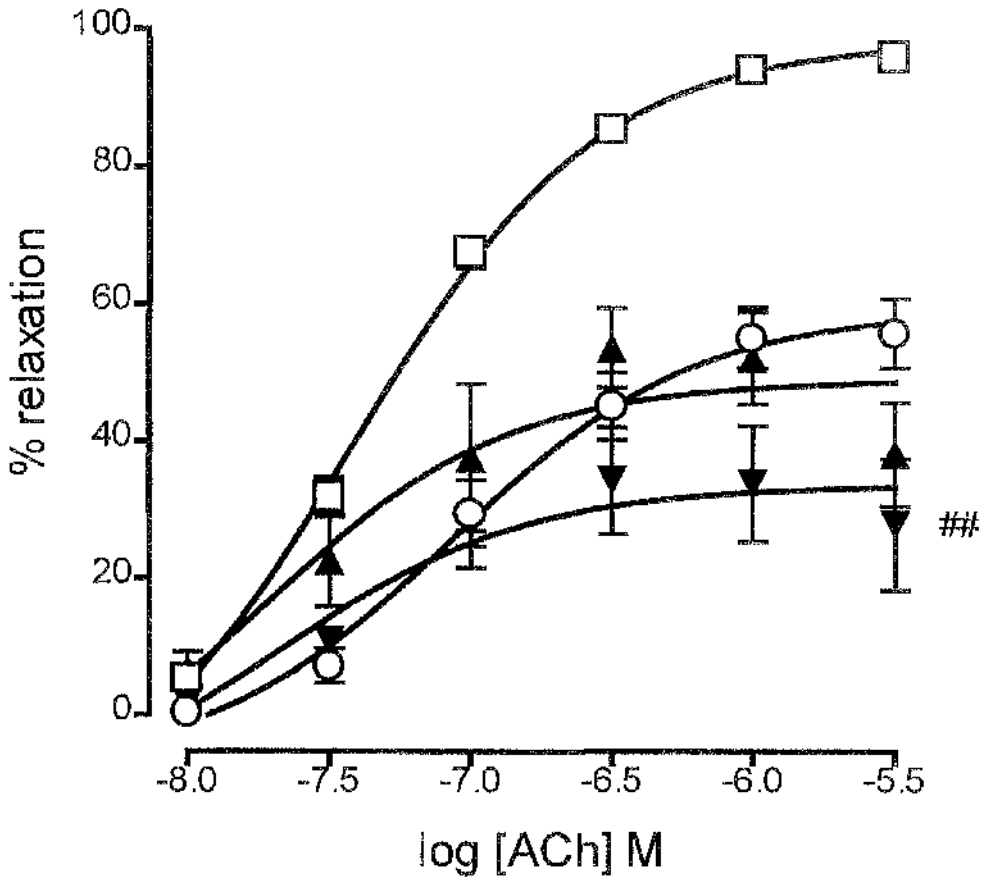


FIGURE 4.15 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and the blockade of this relaxation following treatment with diethyldithiocarbamate (3 mM; 60 min; washout; ○). The effects of $CuSO_4$ (1 μM; ▲) and CuDIPS (1 μM; ▼) on this blockade are also shown. Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 5-11 observations. ##P<0.01 indicates that the SOD mimetic had a significant effect on the relaxation induced by the maximal concentration of ACh in diethyldithiocarbamate-treated rings.

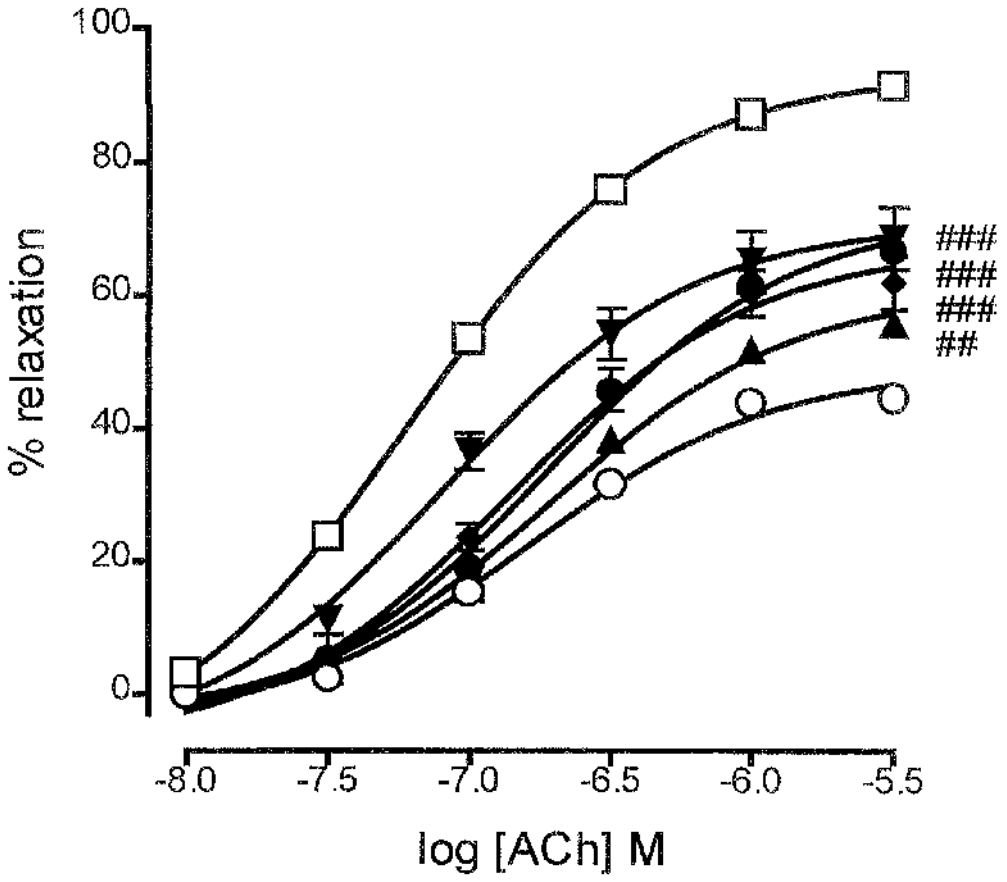


FIGURE 4.16 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine-contracted endothelium-containing rings of rabbit aorta and the blockade of this relaxation following treatment with diethyldithiocarbamate (3 mM; 60 min; washout; ○). The effects of MnTMPyP at 100 μM (▲), 600 μM (▼) and 1mM (●) and $MnCl_2$ at 100 μM (◆) on this blockade are also shown. Experiments were conducted in the presence of catalase (1000 u ml^{-1}) to prevent accumulation of hydrogen peroxide. Each point is the mean ± s.e. mean of 5-15 observations. ##P<0.01 and ###P<0.001 indicate that the SOD mimetic had a significant effect on the relaxation induced by the maximal concentration of ACh in diethyldithiocarbamate-treated rings, respectively.

4.4 DISCUSSION

The vital vasodepressor role played by endothelium-derived nitric oxide is impaired following its destruction by superoxide anion (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986b; Kaustic & Vanhoutte, 1989; Ohlstein & Nichols, 1989, Mian & Martin, 1995a). The enhanced destruction of nitric oxide by superoxide anion forms a common defect in a number of pathological conditions including diabetes (Hattori *et al.*, 1991; Kamata & Kobayashi, 1996), hypertension (Nakazono *et al.*, 1991; Grunfeld *et al.*, 1995; Bouloumie *et al.*, 1997), ischaemia-reperfusion injury (Downey, 1990), heart failure (McMurray *et al.*, 1990; Katz *et al.*, 1993) and atherosclerosis (Sharma *et al.*, 1992; Ohara *et al.*, 1993). Nitric oxide is protected from destruction, however, by the endogenous scavenger of superoxide anion, superoxide dismutase (SOD). Three isoform of SOD are expressed in eukaryotic cells: an Cu/Zn containing form found both in the intracellular and extracellular space; and a Mn containing form found in the mitochondria (see Fridovich, 1989).

Results from Chapter 2 confirmed the findings of others by showing that inhibition of both the intracellular and extracellular isoforms of Cu/Zn SOD, using the copper chelator diethyldithiocarbamate (DETCA), leads to loss of nitric oxide activity in the vasculature (Cherry *et al.*, 1990; Mügge *et al.*, 1991a; Omar *et al.*, 1991; Mian & Martin, 1995a). Such findings demonstrate that Cu/Zn SOD provides a vital role in protecting endothelium-derived nitric oxide from destruction by superoxide anion. In view of this vital protective role of Cu/Zn SOD, it seems, logical in pathologies associated with oxidant stress to attempt to restore impaired nitric oxide function by

augmenting superoxide anion scavenging activity. SOD itself, however, is likely to be of limited therapeutic potential because it cannot enter cells to protect nitric oxide intracellularly. Greater therapeutic potential lies therefore in the development of low molecular weight, membrane-permeant SOD mimetics. A number of structurally distinct classes of SOD mimetic have been described including the spin traps PTIYO, tempol, and tiron, and the metal-based agents $CuSO_4$, CuDIPS, $MnCl_2$ and MnTMPyP.

As described in Chapter 2, two distinct models of oxidant stress were generated in isolated rings of rabbit aorta in an attempt to mimic the impaired endothelium-dependent relaxation associated with many vascular diseases. One model, involving treatment with a low concentration of the inhibitor of endogenous SOD, DETCA (0.3 mM), together with the extracellular generation of superoxide by xanthine oxidase/hypoxanthine (XO/HX), resulted in impairment both of basal and acetylcholine (ACh)-induced activity of nitric oxide. This impairment could be prevented by exogenous application of authentic membrane-impermeant SOD and is, therefore, likely to be due to extracellular destruction of nitric oxide by superoxide anion. The second model, involving treatment with a high concentration of DETCA (3 mM) alone, resulted in an impairment of ACh-induced nitric oxide activity which was not reversed by application of SOD and, therefore, is likely to involve intracellular destruction of nitric oxide by superoxide anion.

The aim of this section of the study was to assay the efficacy of the putative SOD mimetics to restore nitric oxide dependent relaxation in isolated rings of rabbit aorta following inhibition by extracellular and intracellular oxidant stress.

4.4.1 Actions of non metal-based superoxide dismutase mimetics

Despite possessing SOD-like activity in biochemical assays (Chapter 3), the spin trap, PTIYO failed to restore convincingly ACh-induced relaxation following its inhibition by either the extracellular (DETCA 0.3 mM and XO/HX) or the intracellular (DETCA 3mM) oxidant stress. In fact, PTIYO actually intensified the blockade in both conditions. Tempol (0.1 mM), however, produced a weak reversal of ACh-induced relaxation following its inactivation by the extracellular oxidant stress. Nevertheless, both agents did prevent the superoxide-mediated enhancement of tone in endothelium-containing tissues consistent with protection of basal nitric oxide activity. This action, however, was found to be due in part to the inactivation of basal nitric oxide activity since the augmentation of tone induced by the inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methyl ester (L-NAME), was impaired. Furthermore, both agents impaired ACh-induced relaxation by themselves.

The inability of these stable nitroxides to restore relaxation in conditions of oxidant stress is presumably related to the demonstrated ability of this class of agent to inhibit the activity of nitric oxide by a direct chemical reaction (Akaike *et al.*, 1993). Considering that spin traps bind free radicals and that nitric oxide itself is a free radical then this is perhaps not too surprising. The reaction, as described by Akaike

et al (1993), is that stable nitroxides (R-N-O; where R symbolises the rest of molecule) can react with nitric oxide (NO) to produce nitrogen dioxide (NO_2) (Step 1) which can then form, via a reaction with water (H_2O), nitrite (NO_2^-) and nitrate (NO_3^-) and the hydrogen ion (H^+) (Step 2).

Step 1: $$R\text{-}N\text{-}O + NO \rightarrow R\text{-}N + NO_2$$

Step 2: $$2NO_2 \leftrightarrow N_2O_4 \xrightarrow{H_2O} NO_2^- + NO_3^- + 2H^+$$

Stable nitroxides are known to scavenge nitric oxide with different degrees of potency (Akaike *et al.,* 1993). Indeed, in our study PTIYO was considerably more potent than tempol in its ability to impair both basal and ACh-induced nitric oxide activity. PTIYO thus appears to have a stronger affinity for nitric oxide than tempol. On the other hand, tempol, appears to have a greater affinity than PTIYO for superoxide anion since it produced a partial recovery of ACh-induced relaxation. It would appear, therefore, to have a greater affinity with superoxide anion than nitric oxide. In fact, tempol proved to be a more effective scavenger of superoxide than PTIYO in the two independent biochemical assays (Chapter 3).

Overall, in addition to their ability to scavenge superoxide, stable nitroxides also react with nitric oxide and impair the ability of the endothelium to cause relaxation. It is therefore unlikely that this class of SOD mimetic could be developed as therapeutic agents with which to restore nitric oxide-dependent relaxation in pathologies associated with oxidant stress.

The superoxide anion scavenger, tiron, provided only weak protection to ACh-induced relaxation against the blockade induced by an extracellular oxidant stress, and was ineffective against the intracellular stress. Tiron did, however, fully protect basal nitric oxide against the extracellular oxidant stress. Despite tiron demonstrating poor SOD-like action against oxidant stress, it had no inhibitory effect on basal or ACh-induced nitric oxide activity in control preparations even when used at concentrations up to 30 mM. Therefore, unlike the stable nitroxides which inactivate nitric oxide, spin traps like tiron have potential to be developed into an effective therapeutic agent.

4.4.2 Actions of copper-based superoxide dismutase mimetics

The copper-based agents, $CuSO_4$ and CuDIPS, both powerfully protected basal and ACh-induced nitric oxide activity, in a concentration-dependent manner, against inhibition by the extracellular (DETCA 0.3 mM and XO/HX) oxidant stress. Neither agent, however, restored nitric oxide-dependent relaxation following its inactivation by the intracellular (DETCA 3mM) oxidant stress. In light of their effective SOD-like actions against the extracellular oxidant stress, the inability to restore relaxation in the intracellular oxidant stress probably reflects the inability of these agents to penetrate cell membranes in this tissue. $CuSO_4$ has, however, previously been reported to restore endothelium-dependent and nitrovasodilator-mediated relaxation following inhibition by DETCA in rat aorta (Plane *et al.*, 1997). We found no such

restoration in rabbit aorta, however, perhaps reflecting a reduced ability of copper ions to enter cells in this tissue.

Neither $CuSO_4$ or CuDIPS impaired ACh-induced or basal activity of nitric oxide when used at low concentrations (1 - 3 μM). However, at higher concentrations (10 - 100 μM) both agents powerfully impaired ACh-induced relaxation; the effect of the higher concentrations was not investigated on basal nitric oxide activity. Inhibition of relaxation by these copper-based agents may occur by at least two pathways. One pathway derives from the finding that CuDIPS directly inactivates nitric oxide synthase and therefore inhibits nitric oxide production (Baquial & Sorenson; 1995). Specifically, according to these authors, CuDIPS acts as an electron acceptor and disrupts the five-electron oxidation of the guanidino nitrogen atom of L-arginine during the formation nitric oxide. The second pathway derives from the basis that both $CuSO_4$ and CuDIPS can form a highly reactive oxidant, hydroxyl radical, which could potentially impair relaxation (Halliwell & Gutteridge, 1989). Both $CuSO_4$ (existing in an ionic complex) and CuDIPS (existing in weak co-ordination complex; Huber et al., 1987) will generate free copper ions in solution. Free copper can lead to the generation of hydroxyl radical by a process known as the superoxide-assisted Fenton reaction or the copper-catalysed Haber-Weiss reaction. Here, free native copper (Cu^{2+}) is reduced by superoxide anion ($O_2^{\cdot}$) to Cu^{+} (Step 1). The reduced copper is then oxidised by hydrogen peroxide (H_2O_2) to form hydroxyl radical ($OH^{\cdot}$) and hydroxide anion (OH^{-}) (Step 2). (Halliwell & Gutteridge, 1989).

Step 1: $Cu^{2+} + O_2^{\cdot-} \rightarrow Cu^{+} + O_2$

Step 2: $Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{\cdot} + OH^{-}$

Net: $O_2^{\cdot-} + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + O_2$

Hydroxyl radical can react with virtually all biological molecules and may initiate a propagating chain of reactions that can lead to lipid peroxidation, DNA alteration and damage to vital proteins (Marin & Rodriguez-Martinez, 1997 and see Chapter 1 Section 1.2.4). The potential production of such a highly reactive agent by $CuSO_4$ or CuDIPS may contribute to the impaired relaxant ability of ACh. Indeed, this could have been investigated using hydroxyl radical scavengers such as mannitol or dimethyl sulfoxide. The greater potency of CuDIPS than $CuSO_4$ to impair ACh-induced relaxation, perhaps reflects the ability of the former agent to be involved in both of the above described pathways.

The concentration-dependent SOD-like effects of $CuSO_4$ and CuDIPS described in this chapter are almost identical. Since both complexes will generate free copper ions in solution, it is possible that it is the free copper, rather than the $CuSO_4$ and CuDIPS complexes *per se*, that accounts for the SOD-like actions. Compounds that lead to elevation of free copper ions *in vivo* are likely to be too toxic to be considered as therapeutic agents.

4.4.3 Actions of manganese-based superoxide dismutase mimetics

The manganese-based agents, $MnCl_2$ and MnTMPyP, both fully protected ACh-induced activity of nitric oxide from destruction by the extracellular oxidant stress (DETCA 0.3 mM and XO/HX). A major finding of this study, however, is that, in sharp contrast to the effects of authentic SOD or the other SOD mimetics investigated, both $MnCl_2$ and MnTMPyP reversed the inhibition of ACh-induced relaxation generated by the intracellular oxidant stress i.e. treatment with a high concentration of DETCA (3 mM) alone. Neither agent, however, fully reversed this DETCA-induced inhibition; MnTMPyP produced no greater restoration when used at 1 mM than at 600 μM, while $MnCl_2$ precipitation in Krebs solution limited its use to concentrations of 100 μM or less. Thus, a major component of the actions of DETCA (3 mM) is likely to result from inhibition of intracellular Cu/Zn SOD, with a lesser component reflecting non-selective effects of DETCA, such as depletion of intracellular glutathione and generation of lipid peroxides (Kelner *et al.*, 1989). In view of the membrane permeance of MnTMPyP (Faulkner *et al.*, 1994; Gardner *et al.*, 1996) it may not be surprising that this agent can reverse the effects of inhibition of intracellular Cu/Zn SOD. In contrast, manganese ions resulting from addition of $MnCl_2$ would not be expected to cross cell membranes readily to relieve an intracellular oxidant stress. It is possible, however, that this ion gained entry to cells through non-selective divalent cation channels (Fasolato *et al.*, 1993; Somasundaram & Mahaut-Smith, 1994).

The potency of $MnCl_2$ in protecting nitric oxide-dependent relaxation against both an extracellular and intracellular oxidant stress was considerably greater than for MnTMPyP. This may be related to the valency state of native $MnCl_2$ with respect to MnTMPyP, i.e. Mn(II)Cl_2 and Mn(III)TMPyP. As was demonstrated by Faulkner *et al.* (1994), the rate constant for removal of superoxide anion by MnTMPyP was substantially different depending on the valency state of the metal i.e. the Mn(II) form was 100 fold more active than the native Mn(III) form (rate constants of 4×10^9 M^{-1} S^{-1} and 3.9×10^7 M^{-1} S^{-1}, respectively). Reduction of the native state of Mn(III)TMPyP to the more active Mn(II)TMPyP form by the superoxide dismutation process is relatively slow but can proceed faster, at least in *E. coli*, by reduction at the hand of cellular elements such as NADPH and glutathione (Faulkner *et al.*, 1994). This is likely to explain the reportedly greater superoxide removing potency of MnTMPyP in *E. coli* than in *in vitro* biochemical studies (Faulkner *et al.*, 1994). The higher affinity of Mn(II) over Mn(III) for superoxide anion may therefore explain the greater potency of Mn(II)Cl_2 than of Mn(III)TMPyP in restoring ACh-induced relaxation following its inhibition by both extracellular and intracellular oxidant stress. SOD mimetics based on a Mn(II) metal centre may therefore have greater therapeutic potential than those based on the Mn(III) form. The simple metal salt, $MnCl_2$, is, however, too toxic to be considered as a potential therapeutic agent. High levels of free manganese have been linked to decreases in brain dopamine levels leading to Parkinson's disease-like symptoms (Halliwell & Gutteridge, 1989).

As expected for SOD mimetics, both $MnCl_2$ and MnTMPyP prevented the superoxide-mediated enhancement of tone in endothelium-containing rings induced

by the extracellular oxidant stress. For MnTMPyP, this action, however, was found to be due in part to the inactivation of basal nitric oxide activity rather than to the scavenging of superoxide anion. Surprisingly, the concentration of MnTMPyP (100 μM) that impaired basal nitric oxide activity had no effect on ACh-induced relaxation. An explanation for this is not instinctively obvious and a more detailed examination of the actions of MnTMPyP on basal activity of nitric oxide is detailed in the next chapter.

CHAPTER 5

MANGANESE BASED SUPEROXIDE DISMUTASE MIMETICS AND BASAL NITRIC OXIDE ACTIVITY

5.1 INTRODUCTION

In Chapter 4, a series of structurally distinct superoxide dismutase (SOD) mimetics was assayed for the ability to protect endothelium-derived nitric oxide from destruction by superoxide anion. Amongst these compounds, we found that two, i.e. Mn [III] tetrakis [1-methyl-4-pyridyl] porphyrin (MnTMPyP; Faulkner *et al.*, 1994; Gardner *et al.*, 1996) and the metal salt, $MnCl_2$ (Huber *et al.*, 1987; Beyer & Fridovich, 1990), were unique in that they were able to restore acetylcholine (ACh)-induced relaxation in rings of rabbit aorta following inhibition with an extracellular (treatment with the inactivator of Cu/Zn SOD diethyldithiocarbamate, DETCA 0.3 mM, together with the superoxide generating system xanthine oxidase/hypoxanthine) and an intracellular (DETCA 3mM alone) oxidant stress. Moreover, the ability of these compounds to restore ACh-induced relaxation by an intracellular oxidant stress was not shared with authentic, membrane-impermeant SOD. These compounds could, therefore, provide a lead in the development of agents with greater therapeutic potential than SOD itself in the treatment of pathologies associated with oxidant stress. Surprisingly, one of these, i.e. MnTMPyP appeared to inhibit basal but not ACh-induced, activity of nitric oxide in rabbit aorta. This ability to inhibit basal nitric oxide activity was completely unexpected since previous work in rat aorta showed that authentic SOD (Ohlstein & Nichols, 1989; Mian & Martin, 1995a) and certain Mn-based SOD mimetics, i.e. the porphyrinic compound SC50268 (Kasten *et al.*, 1995) and $MnCl_2$ (Kasten *et al.*, 1994) actually produced endothelium-dependent

relaxation by protecting basal nitric oxide from destruction by superoxide anion. These relaxant actions are believed to result from protection of basal nitric oxide from destruction by endogenous superoxide.

The aim of this section of the study was to explore further the effects of MnTMPyP on basal activity of nitric oxide. These experiments were conducted on rat aorta since it exhibits much greater basal activity of nitric oxide that rabbit aorta (Martin *et al.*, 1986a and see Chapter 2). The effects of authentic SOD and $MnCl_2$ on basal activity of nitric oxide were also studied for comparison with MnTMPyP on this tissue.

5.2 METHODS

5.2.1 Preparation of tissues

Methods for preparation of aortic rings from female Wistar rat (200 – 250 g) for tension recording are identical to those detailed for rabbit aorta in Chapter 2 with the exceptions that: rats were killed by stunning and exanguation; all tissues were used fresh on the day of preparation; when removing the endothelium, the aortic ring was weighted with a 1 g rather than 2 g weight; and aortic rings were mounted under 1 g rather than 2 g resting tension within tissue baths.

5.2.2 Assessment of effects of superoxide dismutase, $MnCl_2$ and MnTMPyP on basal nitric oxide activity

The effects of authentic superoxide dismutase (SOD) and the SOD mimetics, MnTMPyP and $MnCl_2$, on basal nitric oxide activity were examined in rat aortic rings. This was achieved by constructing cumulative concentration-response curves to SOD (0.1 - 300 u ml^{-1}), MnTMPyP (10 nM - 300 μM) or $MnCl_2$ (10 nM - 100 μM) on endothelium-containing rings following induction of submaximal (40 – 50 % of maximal) PE (0.1 - 0.3 μM)-induced tone: enhancement of basal activity was assessed as a fall in tone and inhibition as an augmentation of tone. The effects of prior inhibition of nitric oxide synthase with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) or of endothelial removal were examined on responses to SOD, MnTMPyP and $MnCl_2$. In addition, the effects of pretreatment with SOD (250 u ml^{-1}) and the scavenger of hydrogen peroxide, catalase (1000 u ml^{-1}), were also examined on MnTMPyP-induced changes in tone.

5.2.3 Assessment of effects of MnTMPyP on acetylcholine-induced relaxation

The effects of MnTMPyP were also examined on acetylcholine (ACh)-induced relaxation. In order to achieve this, cumulative concentration-response curves to ACh (10 nM - 3 μM) were constructed on endothelium-containing rings of rat aorta following a 20 min pretreatment with MnTMPyP (3 and 300 μM). The relaxation obtained was compared with that of control rings. As will be seen from the Results, MnTMPyP produced an endothelium-dependent rise in PE-induced tone in rat aortic

rings. Therefore when MnTMPyP was employed, the concentration of PE was reduced such that the level of tone obtained was similar to that of control rings.

5.2.4 Presentation of data

The ability of authentic SOD, $MnCl_2$ and MnTMPyP to affect basal activity of nitric oxide on rat aortic rings was expressed as a percentage (%) change in the pre-existing level of PE-induced tone. Results are expressed as the mean ± s.e. mean of n separate experiments. Statistical comparisons were made by one-way analysis of variance followed by the Bonferroni post-test. A value of $P<0.05$ was considered significant. Concentration-response curves and statistical analyse were performed using a computer-based programme (Graph Pad, Prism).

5.3 RESULTS

5.3.1 Effects of superoxide dismutase, $MnCl_2$ and MnTMPyP on phenylephrine-induced tone on rat aorta

Following induction of submaximal tone with phenylephrine (PE, 0.1 - 0.3 μM) in endothelium-containing rings of rat aorta, authentic Cu/Zn superoxide dismutase (SOD, 0.1 - 300 u ml^{-1}) and $MnCl_2$ (10 nM - 100 μM) each produced concentration-dependent relaxation (Figures 5.1 and 5.2). If relaxation was mediated by nitric oxide then it would be expected to be blocked by inhibition of nitric oxide synthase or endothelial denudation. Indeed, pretreatment of endothelium-containing rings with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) and endothelial denudation almost completely blocked SOD- and $MnCl_2$–induced relaxation. The relaxant effects of SOD and $MnCl_2$ are, therefore, likely to have arisen from the scavenging of superoxide anion leading to enhanced activity of basal nitric oxide activity.

In contrast to the action of SOD and $MnCl_2$, the change in tone observed following treatment of endothelium-containing rings with MnTMPyP (10 nM - 300 μM) comprised two components: an initial enhancement of PE-induced tone (from 10 nM - 30 μM, to a maximum of 156.6 ± 9.2 % of initial tone), followed by a relaxant component (from 100 - 300 μM, to a maximum relaxation of 28.8 ± 7.6 % of initial tone; Figure 5.3). Pretreatment with the L-NAME (100 μM) or endothelial removal abolished the enhancement of PE-induced tone seen with MnTMPyP (Figure 5.3; maximum levels attained were 113.7 ± 4.5 and 106.2 ± 4.7 % of initial tone,

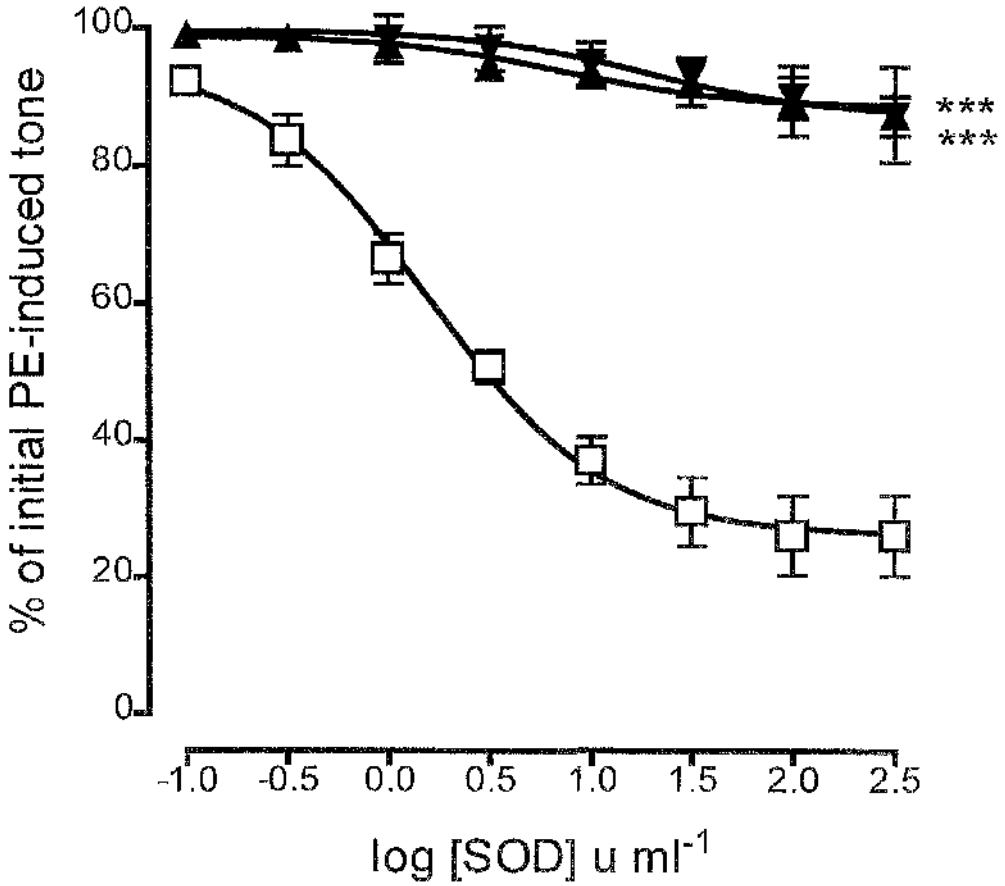


FIGURE 5.1 Concentration-response curves showing relaxation to authentic Cu/Zn superoxide dismutase (SOD, □) on phenylephrine (PE)-contracted endothelium-containing rings of rat aorta and the impairment of this relaxation following treatment with L-NAME (100 μM; ▲) or endothelial removal (▼). Each point is the mean ± s.e. mean of 8 observations. ***P<0.001 indicates a significant difference from the relaxation induced by the maximal concentration of SOD in untreated rings.

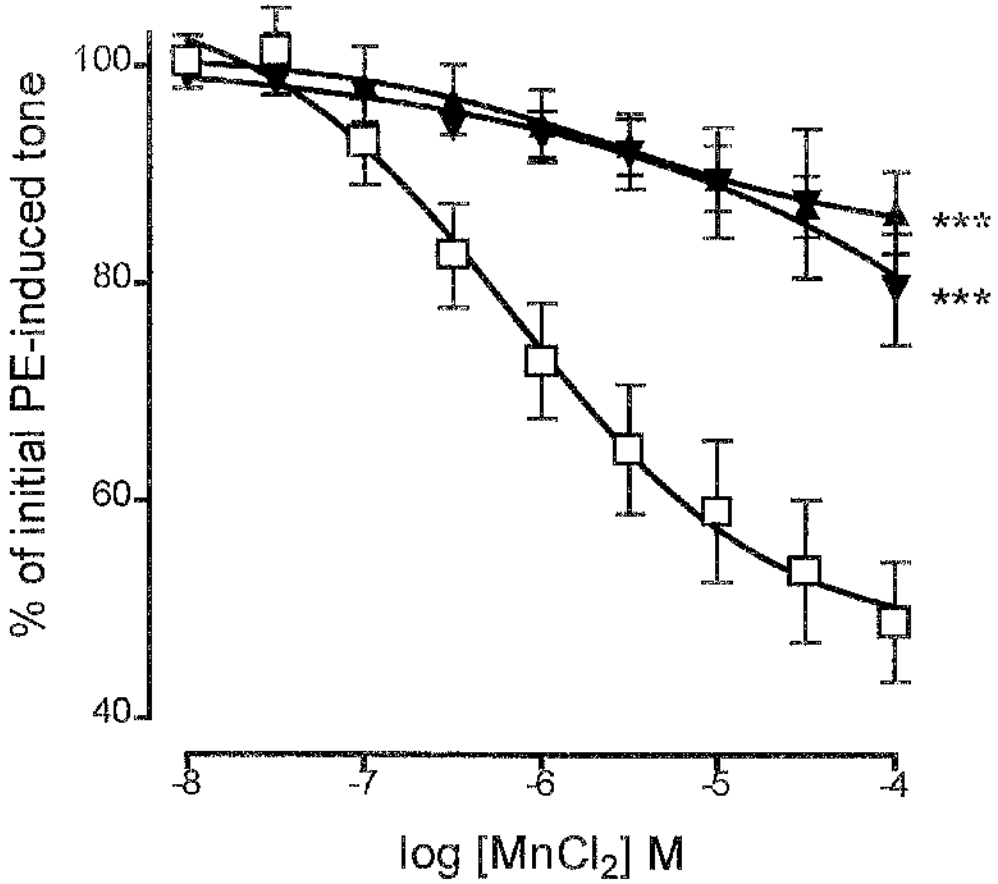


FIGURE 5.2 Concentration-response curves showing relaxation to $MnCl_2$ (□) on phenylephrine (PE)-contracted endothelium-containing rings of rat aorta and the impairment of this relaxation following treatment with L-NAME (100 μM; ▲) or endothelial removal (▼). Each point is the mean ± s.e. mean of 5-14 observations. ***P<0.001 indicates a significant difference from the relaxation induced by the maximal concentration of $MnCl_2$ in untreated rings.

respectively). Pretreatment for 20 min with SOD (250 u ml^{-1}; Figure 5.3) also resulted in blockade of the MnTMPyP-induced enhancement of PE-induced tone: it produced a greater than 10-fold rightward shift in the concentration response curve but did not depress the maximum enhancement observed (137.6 ± 9.9 % of initial tone).

The inhibition of the MnTMPyP-induced augmentation of the following endothelial denudation or treatment with L-NAME or SOD, suggests that it may be due to a superoxide-mediated destruction of basal nitric oxide.

The relaxant action of high concentrations of MnTMPyP (100 – 300 μM) was unaffected by endothelial removal or treatment with L-NAME (100 μM; Figure 5.3), suggesting that this relaxation does not involve nitric oxide. Furthermore, the relaxant actions of MnTMPyP in endothelium-containing or endothelium-denuded tissues was unaffected by pretreatment for 20 min with either SOD (250 u ml^{-1}) or, the scavenger of hydrogen peroxide, catalase (1000 u ml^{-1}; Figure 5.4). The fall in tone is unlikely, therefore to be mediated by superoxide anion or hydrogen peroxide.

5.3.2 Effects of MnTMPyP on acetylcholine-induced relaxation in rat aorta

From the section 5.3.1, MnTMPyP appears to inactivate basal nitric oxide by a superoxide-mediated action. MnTMPyP also impaired basal but not ACh-induced activity of nitric oxide in rabbit aorta (Chapter 4). The effects of MnTMPyP on ACh-induced relaxation in rat aorta were, therefore, assessed.

ACh (10 nM - 3 μM) induced a concentration-dependent relaxation of rat endothelium-containing aortic rings following induction of submaximal PE-induced tone. Pretreatment for 20 min with MnTMPyP at 3 and 300 μM (concentrations of MnTMPyP that had maximal contractile and relaxant effects, respectively) had no effect on this relaxation (Figure 5.5).

Thus, in rat aorta, as with rabbit aorta, MnTMPyP inactivates basal but not ACh-induced activity of nitric oxide.

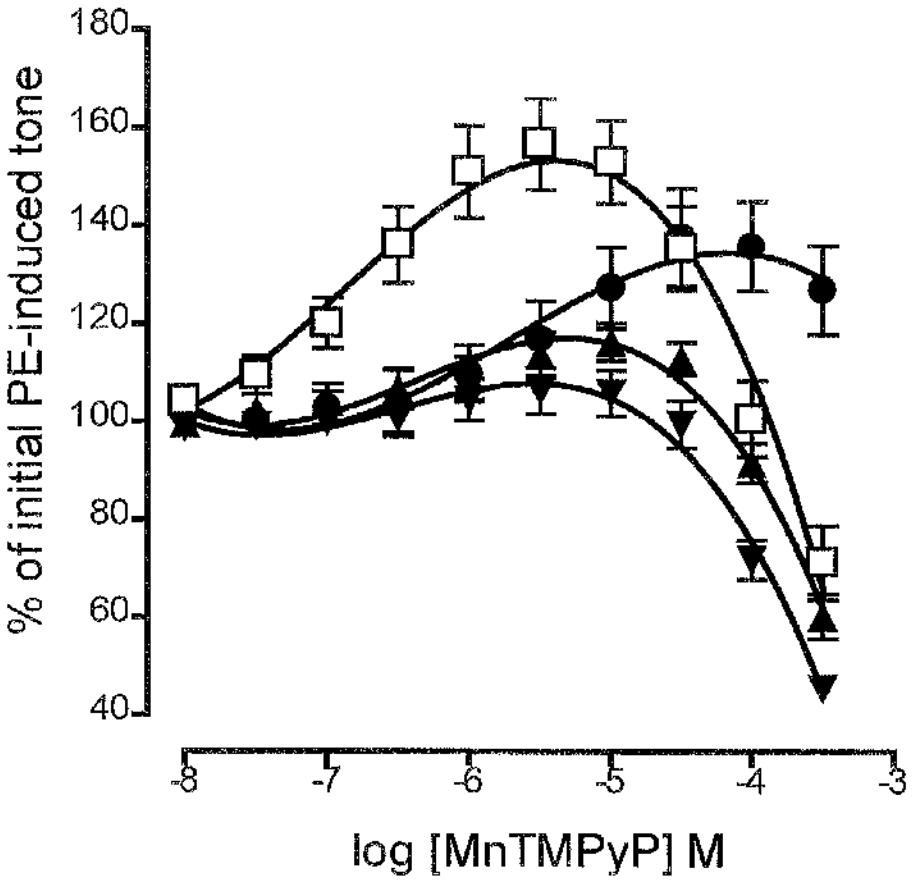


FIGURE 5.3 Concentration-response curves showing changes in tone induced by MnTMPyP (□) on phenylephrine (PE)-contracted endothelium-containing rings of rat aorta and the effects of superoxide dismutase (250 u ml^{-1}; ●) , L-NAME (100 μM; ▲) or endothelial removal (▼) on these changes. Each point in the mean ± s.e. mean of 5 - 15 observations.

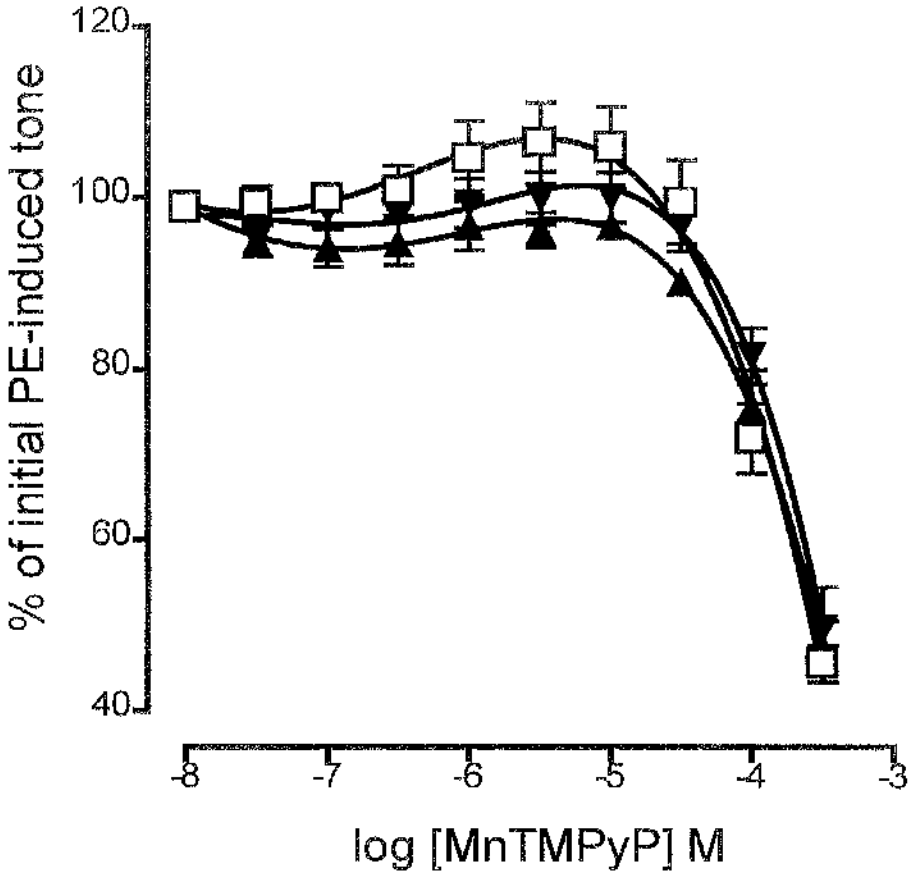


FIGURE 5.4 Concentration-response curves showing changes in tone induced by MnTMPyP (□) on phenylephrine (PE)-contracted, endothelium-denuded rings of rat aorta and the effects of superoxide dismutase (250 u ml^{-1}; ▲) or catalase (1000 u ml^{-1}; ▼) on these changes. Each point in the mean ± s.e. mean of 5 observations.

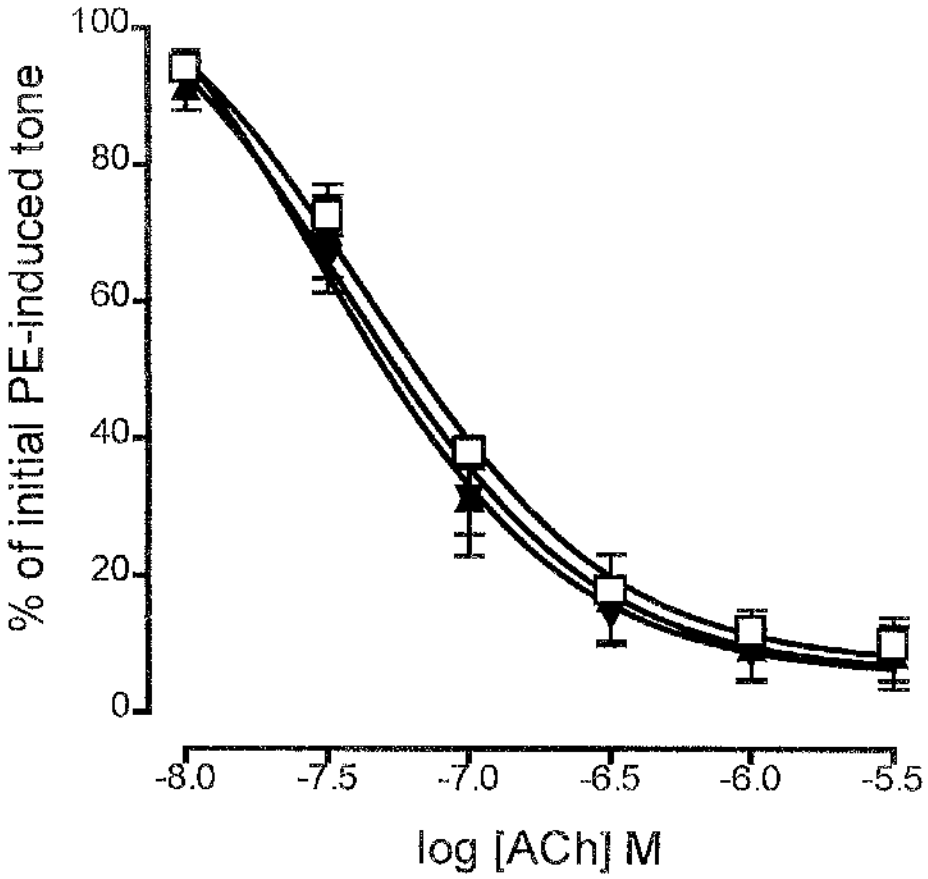


FIGURE 5.5 Concentration-response curves showing relaxation to acetylcholine (ACh, □) on phenylephrine (PE)-contracted, endothelium-containing rings of rat aorta and the effects of MnTMPyP at 3 (▲) and 300 μM (▼) on this relaxation. Each point is the mean ± s.e. mean of 6-11 observations.

5.4 DISCUSSION

The Mn-based SOD mimetics, MnTMPyP and $MnCl_2$, were able to restore agonist-stimulated nitric oxide-dependent vasodilator function following inhibition by oxidant stress in rabbit aorta and, therefore, may have therapeutic potential in pathologies associated with oxidant stress (Chapter 4). Surprisingly, MnTMPyP appeared to inhibit basal nitric oxide in rings of rabbit aorta. In an attempt to investigate this action further, the effects of MnTMPyP were compared with those of SOD and $MnCl_2$ in rings of rat aorta where basal nitric oxide is much more developed (Martin *et al.*, 1986a and see Chapter 2), and therefore easier to study.

5.4.1 Superoxide dismutase and $MnCl_2$ potentiate basal nitric oxide activity on rat aorta

Exogenously applied authentic Cu/Zn SOD produced powerful concentration-dependent relaxations of endothelium-containing rings of rat aorta in confirmation of previous reports (Ohlstein & Nichols, 1989; Mian & Martin, 1995a). The relaxation was abolished by endothelial removal or treatment with L-NAME, an inhibitor of nitric oxide synthesis. It is therefore likely to have arisen from removal of superoxide anion, generated either within the tissue or in the oxygenated Krebs solution, which was continuously destroying some of the basally produced nitric oxide. $MnCl_2$, like authentic SOD, produced concentration-dependent relaxation of rat aortic rings and this too was abolished by treatment with the nitric oxide synthase inhibitor, L-NAME, or endothelial removal.

Similar findings were reported by Kasten *et al.* (1994) who showed additionally that $MnCl_2$-induced relaxation was associated with a rise in cyclic guanosine monophosphate levels in the tissue. They too concluded that this relaxation was likely to have occurred as a consequence of protection of basal nitric oxide from destruction by superoxide anion.

Studies by Rubanyi & Vanhoutte (1986a) and Laight *et al.* (1998) reported that SOD-induced relaxation of endothelium-containing preparations of dog coronary artery and rat aorta, respectively, was impaired by pretreatment with catalase. This would suggest that the relaxation obtained is not completely due to potentiation of basal nitric oxide activity but also due to the SOD-mediated elevation of the end product of superoxide dismutation, i.e. hydrogen peroxide. Hydrogen peroxide has been reported to cause vasorelaxation by endothelium-dependent and -independent mechanisms (Furchgott, 1991; Zembowicz *et al.*, 1993; Mian & Martin, 1995a; Mian & Martin, 1995b). Unpublished data from our laboratory has demonstrated, however, that catalase has no effect on SOD-induced relaxation in rat aorta and therefore rules out the involvement of hydrogen peroxide.

5.4.2 MnTMPyP inhibits basal but not agonist-stimulated nitric oxide activity on rat aorta

In contrast to the actions of SOD and $MnCl_2$, the SOD mimetic, MnTMPyP, induced a biphasic effect on PE-precontracted rings of rat aorta. This comprised an initial

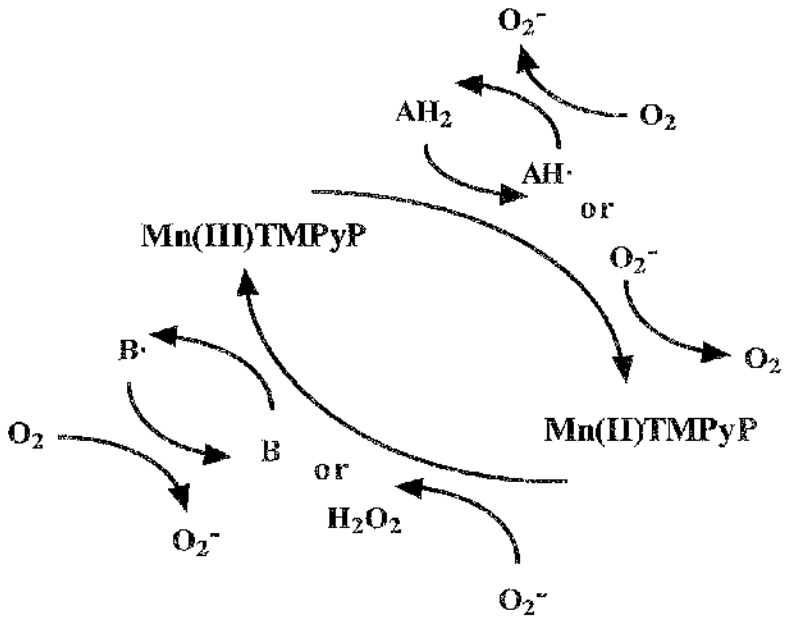
enhancement of PE-induced tone followed by a secondary relaxant component. The abolition of the initial enhancement of tone by treatment with L-NAME or by endothelial removal suggests that this results from destruction of the action of basal nitric oxide. Furthermore, as determined by its ability to impair the L-NAME-induced rise in PE-induced tone, MnTMPyP was also shown to impair the action of basal nitric oxide in rabbit aorta (Chapter 4; Figure 4.9).

Pretreatment with SOD also produced blockade of the MnTMPyP-induced enhancement of tone in rat aorta, although the characteristics of this were different from the simple inhibition by L-NAME and endothelium-denudation. In this case, the MnTMPyP concentration-response curve was shifted to the right by greater than 10-fold, although SOD did not depress the maximum enhancement of tone obtained. Quite paradoxically, therefore, the destruction of basal nitric oxide induced by MnTMPyP, a putative SOD mimetic, would appear to be due to generation of superoxide anion. The parallel shift suggests that in the presence of SOD, higher concentrations of MnTMPyP can still produce sufficient superoxide to destroy basal nitric oxide. On the basis that the high molecular weight of SOD prevents its entry into cells it is likely that the superoxide-mediated destruction of basal nitric oxide produced by MnTMPyP occurs largely in the extracellular space. An alternative explanation, however, is that higher concentrations of MnTMPyP may penetrate cells to generate superoxide and thereby inactivate basal nitric oxide intracellularly, where exogenously applied SOD is not active.

If, indeed, MnTMPyP destroys basal nitric oxide by producing superoxide anion then it would be expected that this compound would similarly impair agonist-induced nitric oxide-dependent relaxation. This, however, was not the case. When used at concentrations that impaired the action of basal nitric oxide both in rabbit and rat aorta, MnTMPyP had no effect on ACh-induced relaxation in either tissue (Chapter 4 and this chapter). There is, therefore, a clear difference in the susceptibly of basal and agonist-stimulated activity of nitric oxide to destruction by MnTMPyP-generated superoxide anion. This could suggest that the agonist-stimulated endothelium-derived relaxing factor (EDRF) is not nitric oxide *per se* but a different, superoxide anion resistant compound. Indeed, some workers have proposed that basal EDRF is free nitric oxide, while that released in response to agonists comes from a pre-formed store (Ignarro, 1991; Cocks & Angus, 1991) of a stable nitric oxide-releasing molecule such as an S-nitrosothiol (Myres *et al.*, 1990). However, this explanation seems unlikely since there is overwhelming evidence that agonist-stimulated EDRF/nitric oxide is indeed susceptible to destruction by superoxide anion (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986b; Moncada *et al.*, 1986; Ohlstein & Nichols, 1989; Lawson *et al.*, 1990; Furchgott *et al.*, 1994; Mian & Martin, 1995a; this study).

Thus, in contrast to its ability to protect ACh-induced relaxation from inactivation by an applied oxidant stress (Chapter 4), MnTMPyP paradoxically destroys basal nitric oxide through the generation of superoxide anion. MnTMPyP, therefore, can be either a net scavenger or generator of superoxide anion. These dual effects maybe explained on the basis of differences in the prevailing redox environment (Gardner *et al.*, 1996).

Specifically, these authors found that MnTMPyP blocked the superoxide-dependent inhibition of growth of human lung carcinoma cells and murine fibrosarcoma cells seen following addition of the superoxide-generator phenezine pyocyanine. In contrast, MnTMPyP enhanced the ability of the naphthoquinone menadione to inhibit cell growth by a superoxide-dependent mechanism. They suggest that redox cycling of MnTMPyP occurs during dismutation of superoxide, in a manner similar to that of Mn SOD, and that both valency states i.e. Mn(III)TMPyP and Mn(II)TMPyP can remove the free radical (see below). They provide evidence, however, of an alternative pathway for reduction of native Mn(III)TMPyP to Mn(II)TMPyP by cellular reductants (AH_2) which produces radical intermediates ($AH^{\bullet}$) that then may react with molecular oxygen (O_2) to produced superoxide ($O_2^{\bullet-}$). Furthermore, although, the oxidation of Mn(II)TMPyP back to Mn(III)TMPyP can be catalysed by superoxide, leading to its dismutation to hydrogen peroxide (H_2O_2), it may, alternatively, be catalysed by other cellular electron acceptors (B), forming partially reduced intermediates ($B^{\bullet}$), which may then also produce superoxide anion. Although both Mn(III)TMPyP and Mn(II)TMPyP can scavenge superoxide anion, Faulkner *et al.* (1994) provided evidence that the reaction rates are quite different 3.9×10^7 M^{-1} s^{-1} and 4×10^9 M^{-1} s^{-1}, respectively. Therefore, superoxide dismutation is more likely to occur during the oxidation of Mn(II)TMPyP than the reduction of Mn(III)TMPyP. This suggests that the $AH_2 \rightarrow AH^{\bullet}$ pathway is more likely to produce superoxide than the $B \rightarrow B^{\bullet}$ pathway.



Modified from Gardner *et al.* (1996)

From the above scheme, MnTMPyP is likely to be a net scavenger of superoxide if background levels of this free radical are high, i.e. with high levels of superoxide it will be less likely that cellular reductants and electron acceptors will participate in redox reactions with MnTMPyP thereby reducing superoxide generation. For example, in conditions of oxidant stress, where levels of superoxide anion are high, MnTMPyP has been shown to be an effective scavenger of this free radical (Chapter 4). A possible explanation, therefore, of why MnTMPyP inactivates basal but not agonist-stimulated activity of nitric oxide may perhaps be related to background levels of superoxide anion in the different experimental conditions. In Chapter 2 it was suggested that treatment of endothelium-containing rings of rabbit aorta with ACh might enhance production of superoxide anion via stimulation of cyclooxygenase (Kurkreja *et al.*, 1986; Consentino et al., 1994) or protein kinase C

(Matsubara & Ziff, 1986). Therefore levels of superoxide may be lower in conditions where basal nitric oxide is investigated than when ACh is used to elicit relaxation. Consequently, MnTMPyP may have a tendency to be a net scavenger of superoxide during experimental conditions where ACh-induced relaxation is assessed but a net generator of superoxide in its absence, hence the destruction of basal nitric oxide.

Another possible explanation, however, is that low levels of nitric oxide produced under basal conditions are destroyed to a greater extent by superoxide anion than the higher levels produced following agonist stimulation (Mian & Martin, 1995a).

The origin of the relaxation induced by higher concentrations of MnTMPyP is unclear but does not involve nitric oxide since it is unaffected by treatment with L-NAME or endothelial removal. Whether acting as a scavenger or generator of superoxide, high concentrations of MnTMPyP may promote the build up the dismutation product, hydrogen peroxide. Hydrogen peroxide is a well known vasorelaxant (Furchgott, 1991; Zembowicz *et al.*, 1993; Mian & Martin, 1995a; Mian & Martin, 1995b) and this may account for the MnTMPyP-induced relaxation. Pretreatment with the scavenger of hydrogen peroxide, catalase, however did not affect the this relaxation. Such experiments cannot fully rule out the involvement of hydrogen peroxide, since exogenously applied catalase, like SOD, is only active in the extracellular space. The possibility therefore remains that the relaxation produced by high concentrations of MnTMPyP results from the intracellular accumulation of hydrogen peroxide.

CHAPTER 6

GENERAL DISCUSSION

Endothelial cells possess the antioxidant capability to protect themselves from the oxidative stress induced by reactive oxygen species. In normal physiology, a fragile equilibrium exists between free radical production and antioxidant activity. Many pathologies, however, are associated with increased free radical production and/or reduced antioxidant capacity which then shifts this balance towards oxidative stress. Oxidant stress not only damages tissues (Halliwell & Gutteridge, 1989; Berman & Martin, 1993) but can impair many cellular processes, including endothelium-derived nitric oxide-dependent relaxation (Katusic & Vanhoutte, 1989; Ohlstein & Nichols, 1989, Mian & Martin, 1995a). Diseases such as hypertension (Nakazono *et al.*, 1991; Grunfeld *et al.*, 1995; Bouloumie *et al.*, 1997), atherosclerosis (Sharma *et al.*, 1992; Ohara *et al.*, 1993), ischaemia-reperfusion injury (Downey, 1990), diabetes (Hattori *et al.*, 1991; Kamata & Kobayashi, 1996) and heart failure (McMurray *et al.*, 1990; Katz *et al.*, 1993) are all associated with nitric oxide/superoxide anion imbalance.

Therapeutic treatment with authentic superoxide dismutase (SOD) does not usually restore impaired endothelium-dependent relaxation since this enzyme cannot penetrate cell membranes to protect nitric oxide from destruction by superoxide anion intracellularly. Attempts have been made to overcome this problem, however, with the use of modified membrane-permeant forms of SOD. Authentic SOD has been attached to the membrane permeant carrier molecule, polyethylene glycol (PEG; Zalipsky, 1995). This modification also extends the plasma half-life of SOD from minutes to hours. In *in vitro* studies, PEG-SOD has been shown to partially restore endothelium-dependent relaxations that had been impaired in cholesterol fed rabbits (Mügge *et al.*, 1991b). Also, liposome-encapsulated SOD has been shown to

partially restore endothelium-dependent relaxation in angiotensin II-mediated hypertension in rats (Rajagopalan *et al.*, 1996; Laursen *et al.*, 1997). Despite providing greater antioxidant protection than with authentic SOD, these modified forms of SOD did not fully protect endothelium-derived nitric oxide from destruction by superoxide anion in any of these studies. Greater therapeutic potential may lie, however, with membrane permeant compounds that exhibit SOD-like activity. A number of different classes of such SOD mimetic have been described, including the metal-based agents, CuDIPS (Cu [II]-[diisopropylsalicylate]$_2$) and MnTMPyP (Mn [III] tetrakis [1-methyl-4-pyridyl] porphyrin), which dismutate superoxide in a manner similar to authentic Cu/Zn SOD and Mn SOD, respectively. Simple metal salts of Cu and Mn also possess SOD-like activity. Other compounds, such as the nitroxide spin traps, PTIYO (4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide) and tempol (4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl), and the superoxide scavenger tiron (4,5-dihydroxy-1,3-benzene-disulfonic acid) are also known to relieve oxidant stress in a number of test systems.

All of the above agents possess SOD-like activity in biochemical and/or isolated cell and tissue studies. Their ability to protect nitric oxide from destruction by an oxidant stress was, however, unknown prior to the start of this project. The aim of this study, therefore, was to investigate the ability of the above SOD mimetics to protect nitric oxide-dependent relaxation in conditions of oxidant stress. To do this, two separate conditions of oxidant stress were generated in rabbit isolated aortic rings. The first, involved inhibition of endogenous Cu/Zn SOD with a low concentration of diethyldithiocarbamate (DETCA; 0.3 mM) in combination with the generation of

superoxide anion by the xanthine oxidase/hypoxanthine system. This resulted in an inhibition of both basal and acetylcholine (ACh)-stimulated activity of nitric oxide. Exogenously applied membrane-impermeant SOD prevented this inhibition of relaxation suggesting that this oxidant stress was confined to the extracellular space. The second, involved treatment with a high concentration of DETCA (3 mM) alone. The inability of exogenous SOD to reverse the inhibition of ACh-induced relaxation seen with this treatment suggests that this oxidant stress was intracellular, i.e. resulted from inhibition of intracellular Cu/Zn SOD. This chapter discusses the activity of the above SOD mimetics in this study in context with their actions described elsewhere. Other strategies to redress the nitric oxide/superoxide imbalance and speculation regarding the future of the therapeutic potential of SOD mimetics are also discussed.

6.1 Non metal-based superoxide dismutase mimetics

The nitroxide spin traps, PTIYO and tempol, protect mammalian cells from oxidant stress (Mitchell *et al.*, 1990; Krishna *et al.*, 1996) and successfully scavenge superoxide in biochemical assays (Ewing & Janero, 1995; Chapter 3). Of these, only tempol protected nitric oxide-dependent relaxation (albeit weakly) from destruction by the extracellular oxidant stress in this study. In fact, PTIYO actually enhanced the blockade of relaxation resulting from both the extracellular and intracellular oxidant stresses (Chapter 4). It seems unlikely, therefore, that PTIYO could be used as an effective antioxidant in pathologies involving nitric oxide. Indeed, a large number of nitroxide spin traps have previously been shown to oxidise rapidly nitric oxide to nitrite and nitrate (Akaike *et al.*, 1993). Indeed, another member of this group

carboxy-PTIO (2-[4-carbox-yphenyl]-4,4,5,5,-tetramethyl imidazoline-1-oxyl 3-oxide) has been used as an effective scavenger of endothelium-derived nitric oxide (Rand & Li, 1995). Tempol has, however, been used successfully in a number of pathological conditions. In rat, this nitroxide diminished the free radical-induced pancreatic injury produced from cerulin infusion (Sledzinski *et al.*, 1995), retinal degeneration generated from intense light radiation (Wang *et al.*, 1995) and renal dysfunction induced by endotoxic shock (Leach *et al.*, 1998). In addition to its superoxide scavenging activity, tempol is reportedly able to exert a further protective action by inhibiting the catalytic actions of transition metals and, hence, attenuates the formation of hydroxyl radical (Monti *et al.*, 1996). Despite its ability to scavenge nitric oxide (Akaike *et al.*, 1993) and impair endothelium-dependent relaxation (Chapter 4), tempol has been used successfully as a SOD-mimetic in other vascular studies. Administration of tempol normalised blood pressure in spontaneously hypertensive rats (SHR; Schnackenberg *et al.*, 1998). This reduction of blood pressure was nitric oxide-dependent suggesting that tempol had scavenged the enhanced superoxide levels that were destroying nitric oxide in this animal model (Nakazono *et al.*, 1991). Furthermore, tempol is also reported to produce a maintained fall in blood pressure in normotensive rats throughout a sustained infusion (Leach *et al.*, 1998). This suggests that even in normotensive rats superoxide destroys some of the nitric oxide produced. In agreement with this, Zöllner *et al.* (1997) report that tempol enhanced the bioactivity of nitric oxide generated by cultured endothelial cells. It is likely, therefore, that in each case the investigators used an optimum dose of tempol which scavenged superoxide with minimal inactivation of nitric oxide.

The superoxide anion scavenger, tiron, has been shown reduce superoxide levels in lymphocytes (Devlin *et al.*, 1981) and neutrophils (Gyllenhammar, 1987) and in biochemical assays (Laight *et al.*, 1997; Chapter 3). In addition, tiron reduced the enhanced superoxide levels evoked by inhibition of Cu/Zn SOD by treatment with DETCA in rabbit aorta (Pagano *et al.*, 1993). In this study, however, tiron provided only weak protection of nitric oxide-dependent relaxation against impairment by the extracellular oxidant stress. The lack of use of this compound in the recent literature suggests that tiron has become generally unfavoured as a SOD-mimetic of choice.

6.2 Copper-based superoxide dismutase mimetics

The copper-based mimetic, CuDIPS, demonstrated SOD-like activity in biochemical assays (Huber *et al.*, 1987; Chapter 3) and prevented the free radical-induced inhibition of growth of baby hamster cultured kidney fibroblasts (Burdon *et al.*, 1995). CuDIPS also protected powerfully nitric oxide-dependent relaxation from the extracellular oxidant stress in rabbit aorta (Chapter 4). In addition, CuDIPS potentiated relaxation to the nitric oxide donor, 3-morpholinosydnonimine (SIN-1) in rat aorta (Emsley *et al.*, 1998). SIN-1 is known to generate superoxide as well as nitric oxide and the potentiation of relaxation may, therefore, reflect the superoxide scavenging ability of CuDIPS. In contrast, the copper-free carrier molecule 3,5-diisopropylsalicylate$_4$ (DIPS), did not alter the relaxation to SIN-1 (Emsley *et al.*, 1998). This suggests that the DIPS ligand has no SOD-like activity. CuDIPS itself, however, induces nitric oxide-dependent relaxation in rings of rat aorta (Wyatt *et al.*, 1998). This relaxation is, thus, similar to that produced by authentic SOD (Ohlstein

& Nichols, 1989; Mian & Martin, 1995a; Chapter 5). It is likely that this CuDIPS-induced relaxation was due to removal of superoxide anion, generated either within the tissue or in the oxygenated Krebs solution, which was continuously destroying some of the basally produced nitric oxide. All of these results indicate that CuDIPS is a highly effective SOD mimic. In this study, however, high concentrations of CuDIPS (10 – 100 μM) powerfully impaired acetylcholine (ACh)-induced relaxation in rabbit aorta (Chapter 4). CuDIPS also dramatically impaired ACh-induced relaxation in rat aorta (Emsley *et al.*, 1998). In addition, these authors report that the DIPS ligand itself caused a small attenuation of relaxation to ACh (approximately 50 % of the inhibition caused by CuDIPS). Furthermore, Harrison *et al.* (1986) report that the DIPS ligand was just as effective as CuDIPS at preventing the free radical mediated induction of interleukin 2 synthesis in mouse thymoma cell lines. Therefore, in contrast, to the findings of Emsley *et al.* (1998), this suggests that DIPS does indeed posses some SOD-like actions. In this study, however, we found that $CuSO_4$ (which gives free copper in solution) gave almost identical effects to CuDIPS (both as a SOD mimetic and as an inhibitor of ACh-induced relaxation). Since CuDIPS exists in a weak co-ordination complex (Huber *et al.*, 1987), this agent is also likely to give free copper in solution. This suggests that the effects of CuDIPS are perhaps due to free copper and not to DIPS. Since CuDIPS can directly inactivate nitric oxide synthase (Baquial & Sorenson, 1995) and both $CuSO_4$ and CuDIPS give free copper in solution leading to hydroxyl radical formation (see section 1.2.4), and therefore are liable to be toxic, neither agent is likely to be used successfully in the treatment of vascular pathologies.

6.3 Manganese-based superoxide dismutase mimetics

The manganese-based compound, MnTMPyP, is reported to have powerful superoxide scavenging activity in *E.coli* (Faulkner *et al.*, 1994) and cultured mammalian cells (Gardner *et al.*, 1996). In this study, MnTMPyP was shown to be a potent scavenger of superoxide in biochemical assays (Chapter 3) and was the only mimetic investigated, apart from the simple metal salt $MnCl_2$, to protect nitric oxide from destruction by both an extracellular and intracellular oxidant stress (Chapter 4). In another study conducted in this laboratory, MnTMPyP also restored nitrergic neurotransmission following its inactivation by oxidant stress (induced by inactivation of Cu/Zn SOD with DETCA in combination with superoxide generated by LY 83583) in strips of bovine retractor penis muscle (Mok *et al.*, 1998). The SOD-like characteristics of MnTMPyP, however, have been questioned by findings of Pfeiffer *et al.* (1998). These authors propose that MnTMPyP can inhibit directly nitric oxide synthase and soluble guanylate cyclase in cultured porcine endothelial cells. Furthermore, they report that MnTMPyP can even scavenge nitric oxide. No such effect of MnTMPyP was found in this study. Even at millimolar concentrations, MnTMPyP had no inhibitory effect on ACh-induced relaxation (Chapter 4). We did find, however, that MnTMPyP was capable of inactivating basal nitric oxide activity via the generation of superoxide. Therefore, despite its effective SOD-like action in conditions of oxidant stress, MnTMPyP itself is not suitable as a therapeutic agent in the treatment of vascular pathologies. A modified version of MnTMPyP, developed by Batinic-Haberle *et al.* (1997), however, may hold greater therapeutic potential. The reported superoxide scavenging ability of this compound, $Mn(II)OBTMPyP^{4-}$

(5,10,15,20-tetrakis-[N-methylpyridinium-4-yl] porphyrin), is some 60-fold greater than for Mn(III)TMPyP (rate constants 2.2 x 10^8 M^{-1} s^{-1} and 4 x 10^7 M^{-1} s^{-1}, respectively). The ability of this compound to protect nitric oxide from destruction by oxidant stress is likely to be greater than for MnTMPyP, but this remains to be tested. It is also not known if the unwanted side effects of MnTMPyP, i.e. its ability to act as a generator of superoxide, apply additionally to Mn(II)OBTMPyP^{4-}.

Two other potentially interesting manganese-based SOD mimetics are described in the literature. Despite our efforts, however, we were unable to obtain samples of either of these agents to investigate in our models of oxidant stress. The first of these, EUK-8 (salen *N,N'*-bis[salicylideneamino]ethane]-manganese[III]), is a combined SOD and catalase mimetic and is known to prevent free radical-induced damage to hippocampal slices (Musleh *et al.*, 1994) and cultured neuronal cells (Bruce *et al.*, 1996). In addition, EUK-8 protects rat heart from the free radical-induced injury resulting from ischaemia/reperfusion (Pucheu *et al.*, 1996; Tanguy *et al.*, 1996). EUK-8 also protects ACh-induced relaxation from inhibition by superoxide anion and produces a concentration-dependent relaxation of rat aorta (Barandier *et al.*, 1997). This EUK-8-induced relaxation, however, was quite different from that observed from treatment with SOD (Ohlstein & Nichols, 1989; Mian & Martin, 1995a; Chapter 5). Surprisingly, the relaxation was actually enhanced by endothelial removal or treatment with an inhibitor of nitric oxide synthase. The authors conclude that the relaxation was mediated through activation of adenylate cyclase, soluble guanylate cyclase and perhaps potassium channels and not via protection of nitric oxide (Barandier *et al.*, 1997).

The other compound, SC52608 (Mn[II]-dichloro[*trans*-2,3-cyclohexano-1,4,7,10,13-pentaazacyclopentadecane]), has its metal in the more active manganese(II) form. It induces concentration-dependent relaxation of rat aortic rings which is abolished by endothelial removal or treatment with an inhibitor of nitric oxide synthase (Kasten *et al.*, 1995). As with authentic SOD, it is likely that this relaxation was due to removal of superoxide anion, generated either within the tissue or in the oxygenated Krebs solution, which was continuously destroying some of the basally produced nitric oxide. Thus, unlike MnTMPyP which destroys basal nitric oxide through generation of superoxide anion, SC52608 protects basal nitric oxide. This agent also reduces ischaemia/reperfusion damage to rabbit isolated hearts (Kilgore *et al.*, 1994) and skeletal muscle (Deune *et al.*, 1996). There is therefore potential for compounds like SC5608 to be used as therapeutic agents.

6.4 Ascorbic acid as a superoxide dismutase mimetic

In addition to its widespread actions as a vitamin, ascorbate (vitamin C), is an important endogenous scavenger of superoxide anion (Som *et al.*, 1983) and consequently has the potential to protect nitric oxide from this free radical. Indeed, ascorbate exhibits the same two characteristic actions on the vasculature as described for authentic SOD. First, it produces endothelium-dependent, nitric oxide mediated relaxation of rat aorta (Dudgeon *et al.*, 1998). Secondly, ascorbate reverses the blockade of ACh-induced relaxation resulting from the same extracellular oxidant stress used in this study. In addition, reports show that administration of ascorbate

reverses the impaired nitric oxide-mediated vasodilation seen in patients with coronary artery disease (Hattori *et al.*, 1991; Levine *et al.*, 1996), diabetes mellitus (Ceriello *et al.*, 1991; Ting *et al.*, 1996), heart failure (Horning *et al.*, 1998) and hypertension (Ceriello *et al.*, 1991). Overloading with ascorbate may, however, be potentially mutagenic, since amounts of 500 mg per day (compared with the 60 mg per day recommended by the EU) are reported to elevate levels of 8-oxo-adenine, a marker of DNA oxidation (Podmore *et al.*, 1998). Nevertheless, ascorbate has promising potential in the treatment of pathologies associated with oxidant stress and warrants further investigation.

6.5 Gene transfer as a therapeutic intervention

Transfer and expression of genetic material within the vascular endothelium represents an alternative strategy to normalise the nitric oxide/superoxide imbalance associated with oxidant stress. Gene transfer to blood vessels *in vivo* has been accomplished by Von Der Leyden *et al.* (1995). This study used a rat carotid injury model to overexpress the endothelial nitric oxide synthase (eNOS) gene in the neointima using a Sendai virus/liposome *in vivo* gene transfer technique. The eNOS gene transfection restored nitric oxide production to normal levels, increased vascular reactivity and reduced neointima formation significantly (Von Der Leyden *et al.*, 1995). These results show that the eNOS gene is a major modifiable factor in the treatment of vascular disease. Furthermore, genetic transfer of human Cu/Zn SOD to bovine aortic endothelial cells successfully inhibited the oxidation of low density lipoproteins (Rios *et al.*, 1996). To date, however, the transfer of SOD genes

to the vascular endothelium *in vivo* has not been reported. A unified study of the combined transfer of eNOS and SOD genes to animal models of disease remains to be investigated.

6.6 Future of superoxide dismutase mimetics

Ultimately, the genetic transfer of eNOS/SOD may be the most powerful tool in redressing the nitric oxide/superoxide imbalance of vascular disease. Therapeutic intervention with genetic material in human pathologies, however, is not likely to be available for some time. Until then, membrane-permeant SOD mimetics may be the most feasible method to increase antioxidant capacity of cells. Consequently, there is considerable interest in the development of such agents. The initial investigation of novel compounds in biochemical or isolated cell or tissue studies has a role. However, the discovery of harmful side effects of some of these mimetics, as uncovered in this investigation, suggests that more work will be required before an agent can be given safely to patients. The recent literature would suggest that the main focus of activity is with the metal-based SOD mimetics. This is in accord with the findings of this study which show that the metal-based agents were considerably more effective than the spin traps in protecting nitric oxide. In addition to the classes of compound described in this chapter, there are literally hundreds of other antioxidant compounds that could potentially possess SOD-like activity. Many of these, such as poly-phenols and flavanoids, are found in abundance in fruit and wine (Catapano *et al.*, 1997). Screening all of these agents for their ability to protect nitric oxide from oxidant stress would be costly and time consuming. However, a

knowledge of the process of nitric oxide production and of its physiological actions, and of the chemistry involved in superoxide dismutation, could reduce the number of compounds investigated to only those with the pharmacological potential to be effective SOD mimetics.

CHAPTER 7

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