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Inhibition of nitric oxide by reactive oxygen species

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A thesis submitted for the degree
of Doctor of Philosophy

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Kousar B. Mian

SUMMARY

1. The aim of this study was to determine the role of the antioxidant enzymes, superoxide dismutase and catalase, in the regulation of vascular tone in isolated rings of rat aorta.

2. In the first part of the study a comparison was made of the ability of superoxide anion to destroy the relaxant activity of basal and acetylcholine (ACh)-stimulated NO.

3. Superoxide dismutase (SOD, 1-300 u ml^{-1}) had no effect on endothelium-denuded rings but it produced a concentration-dependent relaxation of phenylephrine (PE)-induced tone in endothelium-containing rings which was blocked by the NO synthase inhibitor, N^G-nitro-L-arginine (L-NOARG, 30 μM). In contrast, SOD (50 u ml^{-1}) had no effect on ACh-induced relaxation. These results demonstrated a selective potentiation of basal but not ACh-stimulated activity of NO by SOD.

4. Superoxide anion generation using hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}) augmented PE-induced tone in endothelium-containing but not endothelium-denuded rings. This was likely to have resulted from removal of the tonic vasodilator actions of basally-produced NO by superoxide anion, since it was blocked in tissues treated with SOD (250 u ml^{-1}), N^G-mono-methyl-L-arginine (L-NMMA, 30 μM) or L-NOARG (30 μM). Pyrogallol (0.1 mM) had a similar action to HX/XO, but produced an additional augmentation of tone by an endothelium-independent mechanism which was unaffected by SOD (250 u ml^{-1}). In contrast to their ability to almost completely destroy basal activity of NO, HX (0.1 mM)/XO (16 mu ml^{-1}) and pyrogallol (0.1 mM) had no effect on ACh-induced relaxation. Increasing the concentration of HX to 1 mM (keeping XO at 16 mu ml^{-1}) or pyrogallol to 0.3 mM, however, profoundly blocked ACh-induced relaxation and this was prevented by treatment with SOD (250 u ml^{-1}).

5. Treatment with diethyldithiocarbamate (DETCA, 0.1 mM, 1h, followed by washout) to irreversibly inhibit endogenous Cu-Zn SOD, augmented PE-induced tone in endothelium-containing, but not endothelium-denuded rings, and abolished the ability of HX (0.1 mM)/XO (16 mu ml^{-1}) and L-NMMA (30 μM) to augment tone. It was likely that DETCA had led to destruction of basal NO by increasing superoxide anion levels since its actions were reversed by SOD (10-300 u ml^{-1}).

6. In contrast to its ability to completely destroy basal activity of NO, DETCA (0.1 mM) produced only a slight blockade of ACh-induced relaxation. Furthermore, DETCA potentiated the ability of HX (0.1 mM)/XO (16 mu ml^{-1}) or pyrogallol (0.1 mM) to block ACh-induced relaxation and this was prevented by pretreatment with SOD (250 u ml^{-1}).

7. The data suggest that basal activity of NO is more sensitive to inactivation by superoxide anion than ACh-stimulated activity and that endogenous Cu-Zn SOD is vital for the protection of endothelial NO.

8. In the second part of the study the role of catalase in the relaxation induced by sodium azide, hydroxylamine, glyceryl trinitrate and hydrogen peroxide was investigated in isolated rings of rat aorta.

9. Hydrogen peroxide (100 nM-1 mM) induced concentration-dependent relaxation of PE-induced tone in endothelium-containing rings. It also relaxed endothelium-denuded rings but higher concentrations (0.1-1 mM) were required. The endothelium-dependent component of hydrogen peroxide-induced relaxation was abolished with N^G-nitro-L-arginine methyl ester (L-NAME, 30 μM), and thus was due to enhanced production of NO. In contrast, methylene blue (MeB, 30 μM) inhibited both the endothelium-dependent and endothelium-independent components of hydrogen peroxide-induced

relaxation, suggesting that both were dependent upon the stimulation of soluble guanylate cyclase.

10. Pretreatment of endothelium-denuded rings with 3-amino-1,2,4-triazole (AT, 1-50 mM, 90 min) to inhibit endogenous catalase blocked relaxation induced by sodium azide (1-300 nM) and hydroxylamine (1-300 nM), but had no effect on relaxation induced by hydrogen peroxide (100 nM-1 mM) or glyceryl trinitrate (1-100 nM).

11. In a cell free system, incubation of sodium azide (10 μM-3 mM) or hydroxylamine (10 μM-30 mM) but not glyceryl trinitrate (10 μM-1 mM) with catalase (1000 u ml^{-1}) in the presence of either hydrogen peroxide (1 mM) or the glucose (5 mM)/ glucose oxidase (300 mu ml^{-1}) system led to production of nitrite, the major breakdown product of NO. Furthermore, AT (0.1-100 mM) inhibited, in a concentration-dependent manner, the catalase-dependent formation of nitrite from sodium azide (0.3 mM) in the presence of hydrogen peroxide.

12. These data thus suggest that metabolism by catalase plays an important role in the relaxation induced by sodium azide and hydroxylamine in isolated rings of rat aorta. The relaxation appears to be due to formation of NO and activation of soluble guanylate cyclase. In contrast, metabolism by catalase does not appear to be involved in the relaxant actions of hydrogen peroxide or glyceryl trinitrate.

13. In the final part of the study the damage induced by hydrogen peroxide to vascular reactivity and the role of endogenous catalase in protection against this damage was assessed in isolated rings of rat aorta.

14. Incubation with hydrogen peroxide at 1 mM, but not at 0.1 mM, for 15, 30 or 60 min followed by washout depressed, in a time-dependent manner, the ability of the tissue to contract to PE (10 nM) and relax to ACh, suggesting subsequent impairment of vascular reactivity. Furthermore, incubation with hydrogen peroxide at 1 mM (30 min, followed by washout) also inhibited relaxation induced by glyceryl trinitrate or isoprenaline.

15. Incubation with AT (50 mM, 90 min) to inhibit endogenous catalase led to a profound enhancement of the ability of hydrogen peroxide (1 mM) to depress PE (10 nM)-induced contraction and to block relaxation to ACh, glyceryl trinitrate or isoprenaline.

16. It is likely therefore that endogenous catalase plays an important role in protection of vascular reactivity against oxidant damage induced by high (1 mM) concentrations of hydrogen peroxide. The data are consistent with hydrogen peroxide inducing non-selective damage to the vascular smooth muscle, but endothelial damage may also be sustained.

17. In conclusion, the data from this study suggest a vital role for endogenous Cu-Zn SOD and catalase in the regulation of normal vascular reactivity in rat aorta.

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ABBREVIATIONS

ACh	acetylcholine
ATP	adenosine triphosphate
NOS	nitric oxide synthase
cNOS	constitutive nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
eNOS	endothelial nitric oxide synthase
DNA	deoxyribonucleic acid
mRNA	messenger ribonucleic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
GTP	guanosine triphosphate
cGMP	cyclic guanosine monophosphate
CaM	calmodulin
Ca^{2+}	calcium ion
H_4B	tetrahydrobiopterin
L-arg	L-arginine
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
L-NOARG	N^G-nitro-L-arginine
L-NAA	N^G-amino-L-arginine
LPS	lipopolysaccharide
IFN-γ	interferon-γ
Hb	haemoglobin
MeB	methylene blue
NO	nitric oxide
EDRF	endothelium-derived relaxing factor

+EC	endothelium-containing
-EC	endothelium-denuded
PE	phenylephrine
DETCA	diethyldithiocarbamate
AT	3-amino-1,2,4-triazole
NANC	non-adrenergic non-cholinergic
MLCK	myosin light chain kinase
PGI_2	prostacyclin
NaN_3	sodium azide
NH_2OH	hydroxylamine
GTN	glyceryl trinitrate
ISO	isoprenaline
HX	hypoxanthine
XO	xanthine oxidase
GLU	glucose
GO	glucose oxidase
CAT	catalase
SOD	superoxide dismutase
Cu-Zn SOD	copper-zinc superoxide dismutase
Mn-SOD	manganese superoxide dismutase
EC-SOD	extracellular superoxide dismutase
Fe-SOD	iron superoxide dismutase
GSH	glutathione
Gpx	glutathione peroxidase
Se-Gpx	selenium-glutathione peroxidase
GSSG	reduced glutathione disulphide
compound I	catalase-hydrogen peroxide complex
H_2O_2	hydrogen peroxide

$ONOO^-$	peroxynitrite
$^{\bullet}OH$	hydroxyl radical
$^{\bullet}O_2^-$	superoxide anion
Fe^{2+}	ferrous iron
Cu^+	cuprous iron
LDL	low density lipoproteins
ROOH	organic hydroperoxides
TNF-α	tumour necrosis factor-α
NO_2^-	nitrite
NO_3^-	nitrate
L-cys	L-cysteine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
AMP	adenosine monophosphate

INTRODUCTION

Chapter 1

1.1 The vascular endothelium

1.1.1 Role of the endothelium in the regulation of homeostasis

'Endothelial cells.....[are]..... more than a sheat of nucleated cellophane.....'

Lord Florey, 1966

The original concept of the vascular endothelium as a physical and biochemical barrier between the circulating blood and the interstitium, with no other functional properties, has changed dramatically over the past three decades. In the adult human, the net mass of the endothelium is equivalent to approximately 1 % of the body mass and has a surface area of approximately 5000 m^2 (Jaffe, 1984). In certain aspects, the endothelium fulfils the definitions of an "organ" and perhaps today, as our knowledge of its biological significance develops, it should be considered as such.

It has been widely demonstrated that the endothelium plays an important role in both short- and long-term regulation of vascular homeostasis as well as the modulation of several physiological functions, such as inflammation, platelet aggregation, fibrinolysis, angiogenesis and mechanoreception (Vane *et al.*, 1990; Marin & Rodriguez-Martinez, 1995).

Endothelial cells synthesise and release a great variety of paracrine substances. The first endothelium-derived vasoactive substance was discovered in 1976, when it was demonstrated that endothelial cells synthesise prostacyclin (PGI_2), a metabolite of arachidonic acid, in response

to chemical or mechanical stimuli (Moncada *et al.*, 1976). Another potent vasodilator substance produced by the endothelium was originally termed "endothelium-derived relaxing factor" (EDRF; Furchgott & Zawadzki, 1980a) but is now believed to be nitric oxide (NO; Palmer *et al.*, 1987). PGI_2 and NO are both potent anti-aggregating substances (Moncada *et al.*, 1976; Moncada, 1982; Azuma *et al.*, 1986; Radomski *et al.*, 1987a,b). NO has additionally been shown to inhibit adhesion of platelets to endothelial monolayers (Sneddon & Vane, 1988) whereas PGI_2 has been shown to inhibit adhesion only of activated platelets (Fry *et al.*, 1980).

1.2 Endothelium-derived relaxing factor (EDRF)

1.2.1 Requirement for endothelial cells

In 1914, Dale found that intravenous injection of acetylcholine (ACh) at doses that had no effect upon the strength of the heart led to transient, but profound falls in blood pressure and he attributed this to dilatation of blood vessels (Dale, 1914). Similarly, Hunt (1915) demonstrated a fall in blood pressure following injection of ACh into the femoral vein of rabbit leg, but in one particular experiment he observed an unexpected vasoconstriction when he injected ACh into the perfusion cannula in rabbit ear (the outflow fell from 17 to 2 drops according to the perfusion pressure). Subsequently, Burn and Robinson noted a biphasic action (Burn & Robinson, 1951); they demonstrated that ACh when injected repeatedly into the circulation of the perfused rabbit ear, initially caused dilatation which, over a period of few hours, changed into a constriction. In isolated blood vessels, however, there seemed to be consensus on the actions of ACh: a review of the literature by Furchgott (1955) indicated that all reports showed constriction to ACh. One subsequent report (Jellife, 1962), however, using a chain of rabbit aortic rings

precontracted with 5-HT, reported that ACh produced relaxation at low concentrations and contraction at higher concentrations. Several hypotheses were put forward to explain the biphasic activity of ACh, including the ability of ACh to release the vasoconstrictor agent, noradrenaline (Jellife, 1962).

The paradox regarding the *in vivo/in vitro* action of ACh was resolved with a report in 1980 (Furchgott & Zawadzki, 1980a), demonstrating the obligatory role of endothelial cells in relaxation of vascular tissue. For many years, workers in Furchgott's laboratory had been using strips of helically cut rabbit aorta and had consistently obtained contractions to ACh. However, when they began to use aortic rings, they sometimes found that ACh relaxed the preparation. In investigating this apparent discrepancy Furchgott's group discovered that the loss of relaxation to ACh in the case of helical strips had resulted from the unintentional rubbing of the intimal surface during the preparation. The strict dependence of the relaxation on endothelial cells was confirmed using both scanning electron microscopy and *en face* microscopy after silver staining (Furchgott & Zawadzki, 1980a). They suggested that endothelial cells on the intimal surface of the aorta were activated by ACh acting on muscarinic receptors, leading to the release of a relaxing substance (Furchgott & Zawadzki, 1980b). Furchgott called this relaxing substance endothelium-derived relaxing factor (EDRF).

1.2.2 Release of EDRF

1.2.2.1 Agonist-stimulated release of EDRF

Direct evidence for EDRF release from endothelial cells by ACh was obtained in rabbit aortic rings by the use of a so-called "sandwich" mount (Furchgott & Zawadzki, 1980a; Furchgott, 1981). In this procedure, an endothelium-

denuded transverse aortic strip (the recipient strip) whose contractile activity was recorded was mounted with an endothelium-containing longitudinal strip (the donor strip) whose contractile state did not register on the recorder. It was demonstrated that following precontraction with phenylephrine (PE), ACh acting on the endothelium of the longitudinal strip induced release of a substance which relaxed the transverse strip.

Subsequent reports demonstrated that many other substances including ATP, substance P, bradykinin, the calcium ionophore A23187, histamine, 5-HT and thrombin (Furchgott, 1984; Stork & Cocks, 1994; Drummond & Cocks, 1995) in many preparations including arteries, veins and microvessels require the presence of endothelium to produce relaxation. Table 1.1 lists some of these substances together with the tissues in which they have been shown to act.

1.2.2.2 Flow-induced release of EDRF

Endothelium-dependent relaxation occurs not only in response to a wide variety of endothelial agonists, but also to an increase in flow rate. The strict dependence of flow-induced dilatation on the endothelium was confirmed when its mechanical removal by means of a balloon catheter abolished both flow-dependent and local ACh-induced dilatation (Pohl *et al.*, 1986). As the blood flow rate in a vessel increases so does the shear force on the endothelium. Since blood flow rate depends on the vessel size and shape, the shear stress experienced by the endothelial surface varies considerably throughout the vasculature. The ability of methylene blue (MeB), an inhibitor of soluble guanylate cyclase (Gruetter *et al.*, 1979; Holzmann, 1982), and of haemoglobin (Hb, binds and inactivates EDRF, Martin *et al.*, 1985a,b) to inhibit flow-dependent vasodilatation in canine femoral arteries (Pohl *et al.*, 1986) and the perfused rabbit ear (Griffith *et al.*, 1987), suggested that

Stimulus	Vessel	Species	Reference
ACh	Femoral artery	Rabbit	Griffith *et al.*, 1984
	Coronary artery	Rabbit	Griffith *et al.*, 1984
	Femoral vein	Dog	De Mey & Vanhoutte, 1982
	Saphenous vein	Dog	De Mey & Vanhoutte, 1982
Bradykinin	Intrapulmonary artery	Dog	Chand & Altura, 1981
	Aorta	Pig	Gordan & Martin, 1983
	Epicardial coronary artery	Human	Stork & Cocks, 1994
Substance P	Epicardial coronary artery	Human	Stork & Cocks, 1994
	Coronary artery	Dog	Cocks & Angus, 1983
ATP	Aorta	Rabbit	Furchgott, 1981
	Aorta	Pig	Gordan & Martin, 1983
	Saphenous vein	Dog	De Mey & Vanhoutte, 1982
Thrombin	Femoral artery	Dog	De Mey & Vanhoutte, 1982
	Coronary artery	Dog	Cohen *et al.*, 1984
Histamine	Aorta	Rat	Van de Voorde & Leusen, 1983
A23187	Aorta	Rabbit	Furchgott, 1983
	Aorta	Rat	Rapoport & Murad, 1983
	Epicardial coronary artery	Human	Stork & Cocks, 1994

Table 1.1 Vasorelaxant agents classified as "endothelium-dependent" and some of the tissues in which they have been shown to act.

flow-dependent dilatation was mediated by release of the same EDRF as was released by ACh. In addition, pulsatile flow which increases the shear stress in arteries was shown to stimulate the release of EDRF from the endothelium of perfused canine arteries (Rubanyi *et al.*, 1986; Rubanyi, 1993).

1.2.2.3 Hypoxia-induced release of EDRF

Endothelium has been shown to play a functional role in vasodilatation of isolated arteries at low levels of oxygen. It was demonstrated that endothelial cells release vasoactive autacoids such as EDRF and PGI_2 in response to hypoxia (Pohl & Busse, 1989). Endothelium can, therefore, sense reductions in oxygen levels and subsequently instigate a rapid vasodilator response to counter such decreases (Busse *et al.*, 1993).

1.2.2.4 Spontaneous release of EDRF

Several lines of evidence suggest that, in addition to release following stimulation by agonists or shear stress, there is a continuous basal release of EDRF from the endothelium. Cascade bioassay experiments provided direct evidence for this spontaneous release of EDRF from endothelial cells. In these experiments an endothelium-denuded vessel was used as a bioassay tissue for the detection of EDRF released from an endothelium-containing blood vessel. Using such cascade systems, basal release of EDRF has been detected from various preparations, including rabbit aorta (Griffith *et al.*, 1984), rat aorta (Long & Stone, 1985), and femoral artery of the dog (Rubanyi *et al.*, 1985). In these experiments, however, the extent to which flow-induced release contributed to "basal" activity was not determined.

The most compelling evidence for the basal release of EDRF came from experiments conducted in isolated preparations demonstrating the endothelium-dependent depression of vasoconstriction i.e. endothelium-containing vessels were less sensitive to constrictors than endothelium-denuded vessels (Egléme *et al.*, 1984; Martin *et al.*, 1986; Mian & Martin, 1995a). Furthermore, the inhibitors of EDRF, Hb and MeB, were found to augment drug-induced tone in endothelium-containing but not in endothelium-denuded rings (Martin *et al.*, 1985a,b).

Further evidence for basal release of EDRF has come from the measurements of cyclic GMP (cGMP) levels. EDRF-induced relaxation was shown to be associated with increased levels of cGMP in vascular smooth muscle cells (Holzmann, 1982; Rapoport & Murad, 1983), resulting from activation of soluble guanylate cyclase (this will be discussed in detail later). Resting levels of cGMP were found to be higher in endothelium-containing than in endothelium-denuded preparations of rat aorta (Rapoport & Murad, 1983; Martin *et al.*, 1986), rabbit aorta (Martin *et al.*, 1985a) and bovine coronary artery (Holzmann, 1982). Moreover, in the presence of the inhibitors of EDRF, Hb and MeB, the resting levels of cGMP were lowered in endothelium-containing but not in endothelium-denuded blood vessels (Holzmann, 1982; Martin *et al.*, 1985a), suggesting that higher levels of cGMP in endothelium-containing arteries are produced by the tonic action of basally released EDRF. Thus, basal release of EDRF exerts a tonic vasodilator influence on the vasculature.

1.2.3 Characterisation of EDRF

Early experiments conducted on rabbit aorta in attempts to characterise EDRF ruled out the possibility that it was a product of the cyclooxygenase pathway

since indomethacin, an inhibitor of cyclooxygenase, had no effect on relaxation (Furchgott & Zawadzki, 1980a; Furchgott, 1981). There was speculation that EDRF was a product of arachidonic acid metabolism by the lipoxygenase pathway. This was based on the findings that inhibitors of lipoxygenase, nordihydroguaiaretic acid and 5,8,11,14-eicosatetraynoic acid, or phospholipase A_2, quinacrine and p-bromophenacylbromide (Flower, 1974; Flower & Blackwell, 1976; Roberts *et al.*, 1977) were able to inhibit ACh-induced relaxation in rabbit aorta (Furchgott & Zawadzki, 1980a) and rat aorta (Van de Voorde & Leusen, 1983). Furthermore, arachidonic acid itself was shown to relax arteries in an endothelium-dependent manner (Singer & Peach, 1983). However, other findings appeared inconsistent with the role for lipoxygenase. For example, BW 755C, another inhibitor of lipoxygenase, failed to inhibit ACh-induced relaxation of rabbit aorta (Furchgott, 1984). As an alternative, it was proposed that EDRF was a cytochrome P_{450} monoxygenase product of arachidonic acid (Förstermann & Neufang, 1984). However, the inconsistent blockade of endothelium-dependent relaxation obtained with the inhibitor of cytochrome P_{450}, SKF 525A (Rees *et al.*, 1988) questioned this speculation.

The possibility that EDRF was a free radical intermediate produced by the oxidation of arachidonic acid (or some other unsaturated fatty acid) also found some favour, since various free radical scavengers including hydroquinone were shown to inhibit relaxations induced by ACh, A23187 and histamine (Furchgott, 1981; Van de Voorde & Leusen, 1983). In contrast, Griffith *et al.* (1984) using spin trap reagents and specific oxygen-centred radical scavengers proposed that EDRF was not a free radical.

Up until the mid 1980's, there was no clear idea as to the identity of EDRF. Following on from the "sandwich" preparation described by Furchgott &

Zawadzki (1980a), cascade bioassay techniques were used to confirm the humoral nature of EDRF. For example, Griffith *et al.* (1984) and Rubanyi *et al.* (1985) perfused the isolated endothelium-intact aorta and passed the effluent over endothelium-denuded vascular rings, and thereby demonstrated release of a vasodilator factor. Although, this perfused vascular donor/detector system allowed the labile characteristics of EDRF to be studied, they provided only small amounts of EDRF for chemical analysis and did not provide a separation of endothelial cells from the underlying smooth muscle (Griffith *et al.*, 1984; Rubanyi *et al.*, 1985). Subsequently, however, Cocks *et al.* (1985) modified the technique and perfused vascular endothelial cells cultured on microcarrier beads packed in the barrel of a syringe. This technique allowed the generation of greater amounts of EDRF in a relatively small volume and therefore facilitated detection by the assay tissue. This technique was improved yet further by Gryglewski *et al.* (1986a) who used the effluent from microcarrier cultures of endothelial cells in a modified jacketed chromatographic column to superfuse a sequential series of endothelium-denuded detector strips.

Using such techniques it was established that EDRF was a labile substance with a half-life between 4 and 50 seconds, depending on the experimental procedure, in oxygenated physiological salt solutions (Griffith *et al.*, 1984; Cocks *et al.*, 1985; Rubanyi *et al.*, 1985; Gryglewski *et al.*, 1986a,b).

Bioassay studies proved valuable since they allowed the study of the effects of physical or chemical manipulation on the generation, stability, and action of EDRF. Using such techniques, it was found that superoxide anion ($^{\bullet}O_2^-$) contributed to the instability of EDRF, since its activity was prolonged by the addition of the superoxide anion scavengers, superoxide dismutase (SOD; Gryglewski *et al.*, 1986b; Moncada *et al.*, 1986; Rubanyi & Vanhoutte, 1986a,b) and Cu^{2+} (Gryglewski *et al.*, 1986b). The activity of EDRF was also

inhibited by Fe^{2+} (Gryglewski *et al.*, 1986b) and by hyperoxia (Rubanyi & Vanhoutte, 1986a), both of which lead to enhanced production of $^{\bullet}O_2^-$. Furthermore, a number of redox compounds described as inhibitors of EDRF, such as pyrogallol and hydroquinone, were shown to act by generating $^{\bullet}O_2^-$ in solution (Hutchinson *et al.*, 1987; Palmer *et al.*, 1987; Radomski *et al.*, 1987a). Indeed, SOD attenuated their inhibitory effects on EDRF (Moncada *et al.*, 1986). Moreover, another substance capable of removing $^{\bullet}O_2^-$, i.e. oxidised cytochrome C, was shown to inhibit the actions of these redox compounds on EDRF (Moncada *et al.*, 1986). Pyrogallol has been widely used by a number of authors to investigate the biological properties of EDRF (Ohlstein & Nichols, 1989; Abrahamsson *et al.*, 1992; Mian & Martin, 1995a). The wide variations in the half-life reported for EDRF by different groups, might be explained in terms of different concentrations of $^{\bullet}O_2^-$ present. Different experimental conditions in different laboratories will result in different oxygen tensions and, consequently, different concentrations of intracellular and extracellular $^{\bullet}O_2^-$ will be produced.

1.2.4 Mechanism by which EDRF induces relaxation

1.2.4.1 Nitrovasodilators and cGMP

Prior to the discovery of endothelium-dependent relaxation, other workers had reported that in certain smooth muscles there was a positive relationship between an increase in cGMP and relaxation (Schultz *et al.*, 1979; Murad *et al.*, 1979). The mechanisms of humoral regulation of cGMP synthesis and possible biochemical or physiological functions of cGMP, however, were not known.

Initial experiments conducted to characterise the enzyme guanylate cyclase which synthesised cGMP from GTP (Kimura & Murad, 1974), revealed that there were two isoforms in the smooth muscle; soluble (cytosolic) and particulate (membrane-bound). Kimura *et al.* (1975), while performing experiments to define the kinetic properties of the enzyme, accidentally found that sodium azide activated preparations of soluble guanylate cyclase. This was an exciting turn of events and provided a valuable insight into the regulation of the enzyme. In addition to sodium azide, many other nitrocompounds including glyceryl trinitrate, isosorbide dinitrate, amyl nitrite, sodium nitrite, sodium nitroprusside, nitrosamines, hydroxylamine, and some hydrazines were also shown to activate the enzyme and increase cGMP levels in preparations from many tissues (Katsuki *et al.*, 1977). Atrial natriuretic factors, which also relax vascular smooth muscle, were subsequently shown to activate the particulate but not the soluble form of guanylate cyclase (Waldman *et al.*, 1984). The finding that 8-bromo cyclic GMP, a membrane permeant analogue of cGMP, induced vasorelaxation (Schultz *et al.*, 1979), suggested that increases in cGMP levels were closely associated with relaxation. Moreover, the ability of a specific inhibitor of cGMP-phosphodiesterase, M & B 22948 (zaprinast) to potentiate relaxations by activators of soluble and particulate guanylate cyclase, further supported a role for cGMP in vascular relaxation (Kukovetz *et al.*, 1979; Martin *et al.*, 1986a).

It has been suggested that the broad class of guanylate cyclase activators mediate their effects by the formation of nitric oxide (NO; Katsuki *et al.*, 1977; Arnold *et al.*, 1977, Murad, 1994). Murad coined the term "nitrovasodilator" to categorise these compounds (Murad *et al.*, 1978). Thus, it appeared that sodium azide, hydroxylamine, glyceryl trinitrate and isosorbide could be converted enzymatically or non-enzymatically, depending on the prodrug

used, to the active intermediate, NO (Katsuki *et al.*, 1977; Feelisch, 1991; Murad, 1994, also see Chapter 2). It has been shown that nitrovasodilators such as sodium azide and hydroxylamine are metabolised to NO by the peroxidase activity of the enzyme, catalase (Klebanoff & Nathan, 1993; Mian & Martin, 1995b), whereas glyceryl trinitrate is metabolised via glutathione-S-transferase (Armstrong *et al.*, 1980), cytochrome P_{450} (Schröder, 1992) or by a direct reduction by tissue thiols (Feelisch, 1991).

The precise mechanism of activation of soluble guanylate cyclase by NO is not clear. However, it has been proposed that NO binds to the haem group of soluble guanylate cyclase to form nitrosyl-haem-enzyme ternary complex which represents the activated state of the enzyme (Ignarro, 1991). Binding of NO produces an immediate increase in catalytic activity of 50-200-fold. NO activates the enzyme by pulling the iron molecule away from the enzyme and out of plane from the planar porphyrin ring configuration (Ignarro *et al.*, 1984), thus enabling the conversion of GTP to cGMP.

1.2.4.2 EDRF and cGMP

Endothelium-dependent relaxation induced by ACh and other agents was subsequently shown to correlate with increases in cGMP, which preceded the relaxation of the smooth muscle (Holzmann, 1982; Rapoport & Murad, 1983). In addition, EDRF generated by ACh in isolated arteries was shown to directly activate soluble guanylate cyclase in cell homogenates (Förstermann *et al.*, 1986), suggesting that within the vascular system EDRF could act as an "endogenous nitrovasodilator". Furthermore, the vascular relaxation and concomitant increase in cGMP induced by both EDRF and nitrovasodilators was found to be inhibited by Hb (Martin *et al.*, 1985a,b) and MeB (Gruetter *et al.*, 1979; Holzmann, 1982; Garcia-Pascual *et al.*, 1995).

1.2.4.3 Mechanism by which cGMP induces relaxation

In vascular smooth muscle the cycle of contraction-relaxation is mainly regulated by changes in the free intracellular Ca^{2+} concentration. Cytosolic Ca^{2+} binds to calmodulin and the Ca^{2+}-complex subsequently activates myosin light chain kinase (MLCK; Dabrowska *et al.*, 1977), which phosphorylates myosin light chains leading to an increase in actin-myosin Mg^{2+}-ATPase activity and hence, muscle contraction. Inhibition of excitation-contraction coupling, i.e. smooth muscle relaxation, can result from withdrawal of the contractile stimulus leading to a decrease in intracellular Ca^{2+} or can be mediated by an inhibitor of contraction in the continued presence of the contractile agonist. The latter mechanism involves the generation of an intracellular second messenger. cGMP is one of the major second messengers mediating relaxation in vascular smooth muscle (Murad, 1994).

As discussed previously, cGMP synthesis is catalysed from GTP by soluble guanylate cyclase which is activated by a number of compounds including NO (Arnold *et al.*, 1977; Murad, 1994). The primary mechanism by which cGMP causes smooth muscle relaxation is by lowering intracellular Ca^{2+} (Collins *et al.*, 1986). cGMP exerts its action through the activation of a protein kinase, termed cGMP-dependent protein kinase. The mechanisms by which cGMP-dependent protein kinase decreases intracellular Ca^{2+} include: (i) inhibition of phopholipase C, presumably through the phosphorylation of a regulatory protein, resulting in the inhibition of inositol 1,4,5-trisphosphate (IP_3) formation (Hirata *et al.*, 1990); (ii) stimulation of Ca^{2+}-activated K^+ channels leading to hyperpolarisation with consequent inhibition of Ca^{2+} entry through voltage-operated Ca^{2+} channels (Robertson *et al.*, 1993); and (iii) stimulation of Ca^{2+}-ATPase activity leading to augmentation of Ca^{2+} sequestration into the sarcoplasmic reticulum (Amrani *et al.*, 1995).

1.2.5 Identification of EDRF as NO

Although a clear picture had emerged on the mechanisms by which EDRF induced relaxation, its chemical identity remained to be determined. NO had long been suspected to account for the vasorelaxant activity of nitrovasodilators (Katsuki *et al.*, 1977; Arnold *et al.*, 1977). The similarities in the known properties, i.e. stability, biological activity and susceptibility to inhibitors and potentiators, of EDRF and NO, led to the suggestion that EDRF may be NO or a closely related species.

The identification of EDRF as NO was further supported by their indistinguishable action on vascular and non-vascular smooth muscles (Gillespie & Sheng, 1988). An additional similarity in the pharmacological properties became evident when both EDRF and NO were reported to inhibit platelet aggregation (Radomski *et al.*, 1987a). Moreover, their biological half-lives as inhibitors of platelet aggregation were similar (Radomski *et al.*, 1987a).

The major breakthrough came when EDRF released from cultured porcine aortic endothelial cells was shown both to mimic the biological actions of authentic NO in a cascade bioassay and be indistinguishable from it, as assessed by a chemical assay based upon the formation of a luminescent product by the reaction of NO with ozone (see section 1.3.3) (Palmer *et al.*, 1987). Moreover, the amount of NO released by the endothelial cells was sufficient to account for the vascular relaxation attributed to EDRF (Palmer *et al.*, 1987). Furthermore, EDRF released from the perfused intact bovine pulmonary artery and vein was shown to be identical to authentic NO, as assessed by a chemical assay based on diazotization, where NO combines with sulphanilic acid at acid pH and the resulting diazo product couples with N-

(1-naphthyl)-ethylenediamine to produce an intense colour (Ignarro *et al.*, 1987a,b). In addition, a spectrophotometric assay based on the reaction between NO and Hb (see section 1.3.4) was used to compare the release of NO from vascular endothelial cells in culture with the vasorelaxant effect of EDRF and NO determined by bioassay (Kelm *et al.*, 1988). These authors provided further evidence that endothelial cells release NO in amounts sufficient to account for the vasorelaxant activity of EDRF.

More recently, Rand & Li (1995) demonstrated the ability of carboxy-PTIO, an NO-trapping agent, to inhibit relaxations mediated by both EDRF and authentic NO in rat aorta. Furthermore, La *et al.* (1996) by comparing the effects of hydroxocobalamin and oxyhaemoglobin on the relaxant responses to NO and EDRF in rat aorta showed that these compounds could not discriminate between NO and EDRF.

1.2.6 Controversy over the chemical identity of EDRF

Although the evidence for EDRF being NO is compelling, several lines of research have questioned this conclusion. Early reports that EDRF generated from the endothelium only relaxed vascular smooth muscle whereas authentic NO relaxed vascular, tracheal and taenia coli smooth muscles (Shikano *et al.*, 1987) might be due to low concentrations of EDRF generated since tracheal and taenia coli smooth muscle was shown to be less sensitive to NO and EDRF than vascular smooth muscle (Dusting *et al.*, 1988). Moreover, EDRF and NO have clearly been shown to relax vascular and non-vascular smooth muscle preparations in a bioassay system (Gillespie & Sheng, 1988). The stabilisation of EDRF but not NO by acidification (Murray *et al.*, 1986) might be explained by EDRF being an NO-releasing molecule rather than NO itself. Myers *et al.* (1990) compared the vasodilator potencies of NO, S-

nitrosocysteine and EDRF and suggested that EDRF was much more likely to be a nitrosylated compound such as a nitrosothiol than authentic NO. Another group (Kukreja *et al.*, 1993) compared the effects of NO and S-nitrosocysteine on cerebral arteriolar caliber in relation to the associated increment in nitrite concentration in the effluent and concluded that S-nitrosocysteine resembled EDRF more closely than authentic NO. In contrast, Feelisch *et al.* (1994) found that EDRF was more likely to be NO than an S-nitrosothiol as the activities of both EDRF and NO were inhibited by L-cysteine but the actions of S-nitrosothiol were not. It has also been proposed that NO is not released in pure form from endothelial cells but may be bound to a carrier such as in a ferrous iron dithiolate complex (Vanin, 1991). Dinitrosyl ferrous iron complexes have indeed been shown to exhibit similar biological activity and stability to EDRF (Vedernikov *et al.*, 1992). Thus, there is still debate as to the true identity of EDRF.

An alternative hypothesis involves the notion that EDRF is a mixture of NO and S-nitrosothiol. It has been proposed that basal EDRF is free NO whereas the enhanced formation of EDRF elicited by endothelium-dependent relaxants such as ACh may be attributed to Ca^{2+}-dependent exocytosis of recently preformed S-nitrosothiol (Myers *et al.*, 1990; Ignarro, 1991; Cocks & Angus, 1991). The S-nitrosothiol could be synthesised within acidic granules as a result of a simple nitrosation reaction between NO and a thiol (perhaps L-cysteine) and stored temporarily in a relatively stable form. Although S-nitrosocysteine is less lipophilic than NO, some S-nitrosocysteine would be expected to continually leak out of the granules and undergo decomposition to NO and L-cystine (the disulphide of L-cysteine). According to this scheme, exocytotic discharge of S-nitrosocysteine would result in the liberation of NO from the S-nitrosothiol (Ignarro, 1990). The ability of the inhibitors of NO

synthesis to rapidly inhibit actions of both basal and agonist-stimulated EDRF (Mülsch & Busse, 1990; Rees *et al.*, 1990a), however, argues against prior storage.

The general line of evidence suggests that even if EDRF is released from endothelial cells as a NO-containing compound, its biological effects are mediated ultimately by NO.

1.3 Physical and chemical properties and metabolism of NO

NO is a small molecule composed of the two most common atmospheric gases. It is soluble both in water (up to 2 mmol/L at 20 °C and one atmosphere) and lipid (Henry *et al.*, 1993). It is an uncharged molecule with an unpaired electron. Being uncharged, NO can diffuse freely across membranes. Having an unpaired electron, it is a free radical molecule and is highly reactive with a half-life of 4 to 50 seconds (Griffith *et al.*, 1984). NO can both donate and receive electrons, it therefore behaves both as an oxidant and a reductant. Some of the important chemical reactions involving NO are discussed below.

1.3.1 Reactivity with oxygen

NO in the gas phase readily reacts with oxygen (O_2; Ignarro *et al.*, 1993) forming NO_2 and the overall reaction is:

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

This reaction is rapid at high concentrations of NO, but slow at low concentrations. NO in aqueous solution containing O_2 has a different chemical

fate from NO in gaseous mixtures containing O_2. In aqueous solution containing O_2, NO is oxidised mainly to nitrite (NO_2^-) with little or no formation of nitrate (NO_3^-) (Ignarro *et al.*, 1993).

1.3.2 Reactivity with superoxide anion

NO reacts with superoxide anion ($^{\bullet}O_2^-$) resulting in the formation of peroxynitrite anion ($ONOO^-$). $ONOO^-$ is a powerful oxidant (Freeman, 1994). At physiological pH, $ONOO^-$ has a half-life of 1.9 seconds, since when protonated to form ONOOH it undergoes spontaneous homolysis to form the highly reactive hydroxyl radical ($^{\bullet}OH$) and nitrogen dioxide radical ($^{\bullet}NO_2$) (Beckman *et al.*, 1990). NO_3^- is the end-product of this interaction:

$$NO + {}^{\bullet}O_2^- \rightarrow ONOO^- + H^+ \rightarrow ONOOH$$

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

$$\downarrow$$

$$NO_3^-$$

Superoxide anion can rapidly destroy NO and SOD by scavenging superoxide anion protects NO (Gryglewski *et al.*, 1986b; Moncada *et al* ., 1986; Rubanyi & Vanhoutte, 1986a,b; Mian & Martin, 1995a).

1.3.3 Reactivity with ozone

NO reacts with ozone (O_3) leading to the production of activated (high energy state) NO_2. This excited NO_2 is of sufficient energy to dissociate to ground state NO_2 with concomitant generation of light which can easily be detected by chemiluminescence (Zafiriou & McFarland, 1980). This is the technique which was originally used to identify EDRF as NO in biological

systems (Palmer *et al.*, 1987). The reaction is as follows:

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

1.3.4 Reactivity with oxyhaemoglobin

NO reacts with oxyhaemoglobin in the presence of oxygen to yield methamoglobin (metHb) and NO_3^- according to the overall reaction shown below:

$$HbO_2 + NO \rightarrow \text{metHb} + NO_3^-$$

Looking at this reaction in more detail, the component reactions are as follows:

$$Hb^{2+}O_2^- + NO \rightarrow \text{metHb} + ONOO^-$$

and

$$ONOO^- \rightarrow NO_3^-$$

i.e. molecular oxygen binds to the haem iron atom of the protein (Wallace *et al.*, 1974) and this species reacts rapidly with NO to yield $ONOO^-$, which isomerises to NO_3^-. This principle of reacting NO with HbO_2 to yield metHb has been used as a spectrophotometric assay for NO (Kelm *et al.*, 1988).

The reaction of NO with haem-containing proteins such as haemoglobin, myoglobin and soluble guanylate cyclase (Stamler, 1994; Tsai, 1994) is rapid. The reaction of NO with haem groups is thought to be the predominant interaction *in vivo*, and to account both for its activity in smooth muscle cells

and its rapid inactivation in blood (Stamler *et al.*, 1992).

1.3.5 Reactivity with thiols

NO reacts with thiols (R-SH) to form S-nitrosothiols (R-SNO). The reaction is rapid but the resulting S-nitrosothiol is unstable and spontaneously decomposes to NO gas and corresponding disulphides. This process is reported to be enhanced by heat, light, oxygen, and only efficient at acid pH (Ignarro, 1990; Feelisch, 1991). The presence of S-nitrosothiols in human plasma supports the suggestion that these may represent a stabilised form of NO in biological systems (Keaney *et al.*, 1993).

1.3.6 Reactivity with sulphanilic acid

NO reacts with sulphanilic acid at acid pH to yield a diazo product which can be further coupled with N-(1-naphthyl)-ethylenediamine to form an intense chromophore (Green *et al.*, 1982). This reaction is widely used for the spectrophotometric measurement of NO and nitrite (Ignarro *et al.*, 1987a; Mian Martin, 1995b).

1.4 Biochemical origin of NO

Even prior to the finding that endothelial cells release NO, it was reported that the body produces nitrogen oxides. The first suggestion of endogenous production of NO_3^- in humans was based on nutritional studies reported by Mitchell *et al.* (1916). For a long time, it was believed that this NO_3^- was produced by intestinal microbial metabolism and, therefore, had a little relevance to mammalian biology. It was not until 1978 when Tannenbaum and his colleagues (Tannenbaum *et al.*, 1978) proved that NO_3^- was

biosynthesised mainly outside the intestine that interest in this area was renewed. Subsequent studies of NO_2^- and NO_3^- metabolism revealed that more NO_2^- and NO_3^- were produced than could be accounted for by food intake (Green *et al.*, 1981a). Furthermore, studies in germ-free conventional rats, has demonstrated that NO_3^- biosynthesis is mammalian in origin (Green *et al.*, 1981b). A marked increase in urinary NO_3^- excretion was observed in humans with diarrhoea and fever (Hegesh & Shiloah, 1982). Furthermore, treatment of rats with *Escherichia coli* lipopolysaccharide (LPS) led to a 10-fold increase in the urinary output of NO_3^- which correlated with the degree of fever (Wagner *et al.*, 1983). These results, therefore, demonstrated a correlation between immunostimulation and increased NO_3^- synthesis. Later work by Stuehr and Marletta (1985) demonstrated an increased NO_2^- and NO_3^- production *in vitro* following exposure to lipopolysaccharide (LPS, endotoxin) in LPS-sensitive, but not LPS-resistant, mouse peritoneal macrophages. Thus, the macrophage seemed to be the most likely source of NO_2^- and NO_3^- synthesis *in vivo*.

Further studies on the RAW 264.7 macrophage cell line demonstrated that the synthesis of NO_2^- and NO_3^-, after activation with LPS and interferon-γ (IFN-γ), was dependent upon the presence of the amino acid L-arginine (Iyengar *et al.*, 1987). These experiments with ^{15}N-labelled L-arginine showed that the nitrogens in both NO_2^- and NO_3^- were derived from one of the two equivalent terminal guanidino nitrogen atoms of this amino acid (Iyengar *et al.*, 1987). At about the same time, the cytotoxicity of activated macrophages against tumour target cells was also shown to be dependent upon the presence of L-arginine (Hibbs *et al.*, 1987a). This latter group also demonstrated that activated macrophages synthesised L-citrulline and NO_2^- from L-arginine and that the close structural analogue of L-arginine, N^G-monomethyl-L-arginine (L-NMMA), prevented the formation of both of these products as well as the

expression of cytotoxicity (Hibbs *et al.*, 1987a,b). It was proposed that NO was an intermediate in the formation of NO_2^- and NO_3^- from L-arginine in activated macrophages and that it was responsible for the cytotoxic properties of these cells (Stuehr *et al.*, 1989).

1.4.1 Identification of L-arginine as a precursor of NO in endothelial cells

The above classic experiments demonstrating the synthesis of NO_2^-, NO_3^- and L-citrulline from L-arginine by cytotoxic macrophages led to the discovery that vascular endothelial cells also utilise L-arginine in the biosynthesis of NO (Palmer *et al.*, 1988a). These experiments indicated that when bovine isolated aortic endothelial cells were grown in the absence of L-arginine for the final 24 hours of culture, they failed to generate NO in response to bradykinin and A23187, as assessed by a bioassay cascade technique. Furthermore, infusion with L-arginine enhanced the release of NO by these agents, suggesting the need for free L-arginine (Palmer *et al.*, 1988a). In addition, L-NMMA, an inhibitor of NO_2^- and NO_3^- synthesis in the macrophage (Hibbs *et al.*, 1987a,b), blocked relaxations induced by ACh and histamine in guinea-pig pulmonary artery and this inhibition was rapidly and completely reversed by L-arginine but not D-arginine. These data were consistent with endothelium-derived NO originating by enzymatic action on the guanidino nitrogen(s) of an endogenous pool of L-arginine (Sakuma *et al.*, 1988). Almost simultaneously, Palmer and his colleagues obtained similar results with L-NMMA in cultured porcine aortic endothelial cells (Palmer *et al.*, 1988b).

1.5 Biosynthesis of NO

It is well established that NO is synthesised from one of the guanidino nitrogens of L-arginine, and this is catalysed by the enzyme NO synthase

(NOS, Marletta, 1994). This reaction appears to involve a five-electron oxidation of the guanidino nitrogen atom of L-arginine to NO (Nathan & Xie, 1994). The reaction to form NO occurs in two steps. The first is known to involve an initial N-oxygenation of L-arginine to generate N^G-hydroxy-L-arginine (NOHA) (Leone *et al.*, 1991; Marletta, 1993). The second step consists of an oxidative cleavage of the C = N bond of N^G-hydroxy-L-arginine leading to formation of L-citrulline and NO (Leone *et al.*, 1991; Marletta, 1993; Korth *et al.*, 1994). Both steps consume nicotinamide adenine dinucleotide phosphate (NADPH) and O_2 (Leone *et al.*, 1991; Marletta, 1993; Marin & Rodriguez-Martinez, 1995) (see Figure 1.1).

1.6 Isoforms of NO Synthase

So far, at least, three isoforms of NOS have been isolated, purified and cloned and they appear to be the products of three distinct genes (Nishida *et al.*, 1992; Marsden *et al.*, 1992; Nakane *et al.*, 1993).

Two isoforms: the endothelial-type (eNOS) and neuronal-type (nNOS) are present as normal constituents in certain cell types and are also referred to as constitutive NOS (cNOS; Zhang & Synder, 1995). In contrast, the third isoform (macrophage-type) is not normally present in healthy cells but can be induced after exposure to endotoxin or certain cytokines (Nussler & Billiar, 1993), and is termed inducible NOS (iNOS).

All three isoforms require NADPH, as an electron donor, and contain one molecule each of flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and tetrahydrobiopterin (B_4H) (Mülsch & Busse, 1991, Stuehr *et al.*, 1991a; Bredt *et al.*, 1992; Schmidt *et al.*, 1992) and also contain haem (Förstermann *et al.*, 1994). The activity of cNOS is calmodulin-dependent.

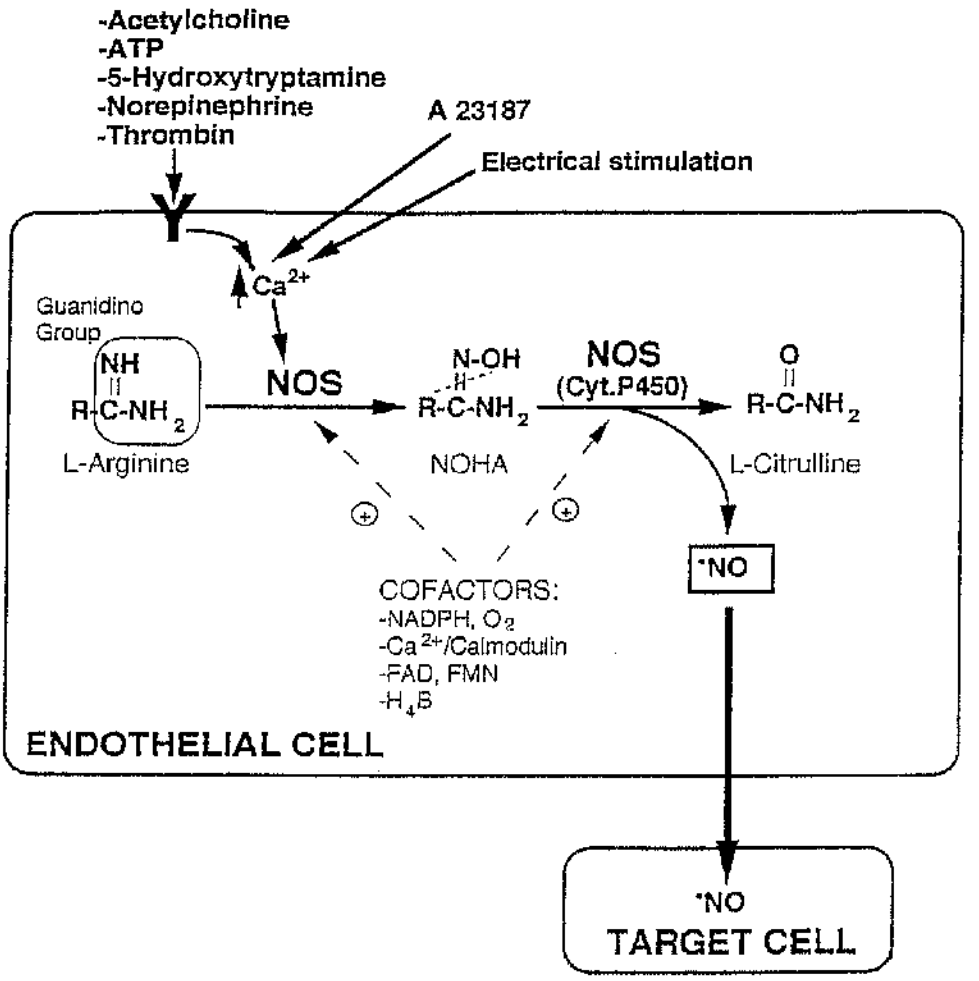


Figure 1.1 Synthesis of nitric oxide (NO). The endothelial cell activation by A23187 (Ca^{2+} ionophore), electrical stimulation or agonists that interact with specific receptors, increasing intracellular Ca^{2+} levels, which leads to activation of NO synthesis. NO formation occurs in two steps catalysed by NO synthase (NOS); the first one consists of an N-oxygenation of L-arginine to N^{G}-hydroxy-L-arginine (NOHA), and the second one, which can also be catalysed by cytochrome P_{450}, consists of an oxidative cleavage of the C = N bond of NOHA leading to L-citrulline and NO formation. Endothelial cell NOS uses as cofactors reduced nicotinamide adenine dinucleotide phosphate (NADPH), O_2, Ca^{2+}/calmodulin, tetrahydrobiopterin (H_4B), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) (modified from Marin & Rodriguez-Martinez, 1995).

Calmodulin binds reversibly to cNOS in response to elevated Ca^{2+} and binding of calmodulin allows NADPH-derived electrons to pass onto the haem group of the enzyme (Abu-Soud & Stuehr, 1993). In contrast, the activity of iNOS is not regulated by Ca^{2+}. Originally it was thought that this isoform was Ca^{2+}/calmodulin independent, but it has recently been shown to bind calmodulin so firmly that dissociation does not occur even in the presence of the Ca^{2+}-chelator, EGTA (Cho *et al.*, 1992). The amino acid sequence is well conserved across species: cNOS has been shown to exhibit over 90 % homology between humans and other species and iNOS exhibits over 80 % homology (Morris & Billiar, 1994).

The first cNOS isoforms purified were from rat and porcine cerebellum (Bredt & Synder, 1990; Nathan, 1992). The isoform in brain is mainly cytosolic and migrates with a molecular mass of 150 to 160 kD on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Bredt & Synder, 1990). This isoform is also present in peripheral non-adrenergic non-cholinergic (NANC) inhibitory neurons in many tissues including the rat anococcygeus and the bovine retractor penis muscle (Martin *et al.*, 1988; Gillespie *et al.*, 1989). The nerves in the bovine retractor penis and rat anococcygeus, however, contain both soluble and particulate NOS (Förstermann *et al.*, 1991). The endothelial form of cNOS has been isolated from native and cultured bovine aortic endothelial cells, shows a molecular mass of 135 kD (Pollock *et al.*, 1991) and is mainly membrane-bound (Sessa *et al.*, 1993).

When cNOS-containing cells are given an appropriate stimulus, e.g. ACh (endothelial cells) or glutamate (cerebellar neurons), receptor activation leads to an increase in cytosolic Ca^{2+}, which activates cNOS to give a burst of NO. This activation is short-lived and the NO produced serves as a diffusible signalling molecule mediating numerous physiological processes including vasodilatation and neurotransmission (Morris & Billiar, 1994).

The inducible NOS can be induced following stimulation with LPS and/or cytokines in marophages (Marletta *et al.*, 1988; Stuehr *et al.*, 1991a) and many other cell types including endothelial cells (Radomski *et al.*, 1990a), vascular smooth muscle cells (Busse & Mülsch, 1990), hepatocytes (Curran *et al.*, 1989), Kupffer cells (Billiar *et al.*, 1989) and neutrophils (Van Dervort *et al.*, 1994). Like nNOS, this isoform is predominantly soluble (Stuehr *et al.*, 1991a; Hevel *et al.*, 1991) and the protein has a molecular mass of 125 to 135 kD (Hevel *et al.*, 1991). Following stimulation, NO synthesis depends on the *de novo* synthesis of iNOS protein and essential cofactors (Beasley *et al.*, 1991; Morris & Billiar, 1994; Fukuto & Chaudhuri, 1995). One of the main characteristics of iNOS is that it releases large amounts of NO continuously since its activity does not depend on Ca^{2+}. The role of NO in activated immune cells is to act as a killer molecule. By releasing massive amounts of NO lethal oxidative injury is inflicted on the target cells (Nussler & Billiar, 1993). In addition to regulation of the activity of iNOS at the transcriptional level, a further level of control appears to be exercised by mRNA stability (Nathan & Xie, 1994; Fukuto & Chaudhuri, 1995). Figure 1.2 shows the structure of endothelial and macrophage NOS and highlights similarities with cytochrome P_{450} reductase.

1.7 Pharmacological regulation of NOS and NO synthesis

Analogues of L-arginine represent the largest and potentially most useful class of NOS inhibitors. The most widely used inhibitors are L-NMMA, N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methyl ester (L-NAME) (Palmer *et al.*, 1988b; Rees *et al.*, 1989; 1990a; Moore *et al.*, 1990; Losonczy *et al.*, 1996). The structure of each of these inhibitors is shown in Figure 1.3. Many analogues of L-arginine act as competitive inhibitors and their inhibitory effects can be prevented or rapidly reversed with L-arginine. Earlier reports indicated

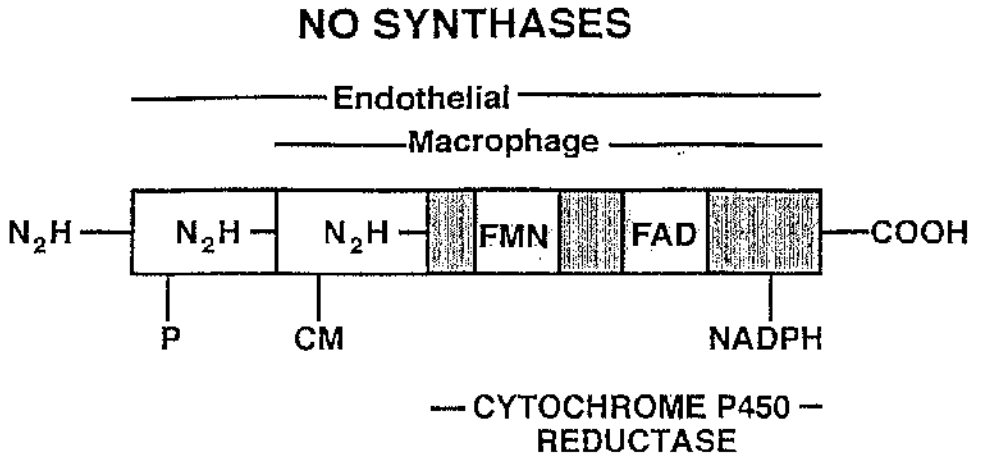
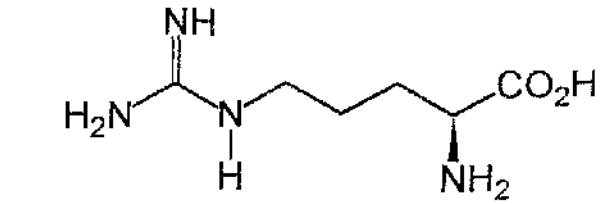
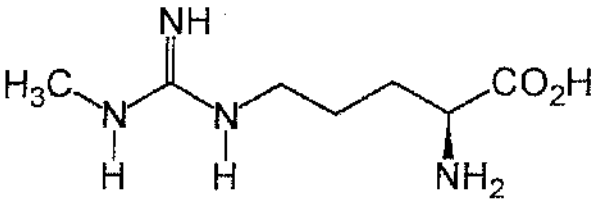


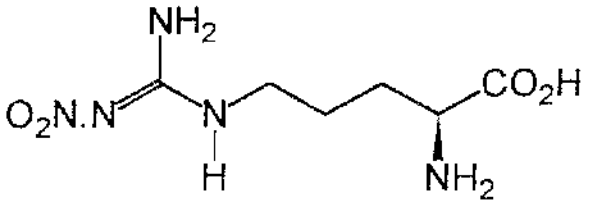
Figure 1.2 Structure of endothelial and macrophage NO synthases and cytochrome P_{450} reductase. The common binding sites for reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) in the carboxyl-terminal domains are shown. The proposed sites for calmodulin (CAM) and protein phosphorylation (P), are also shown (from Marin & Rodriguez-Martinez, 1995).



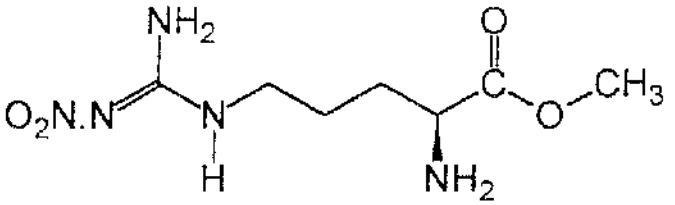
L-ARG



L-NMMA



L-NOARG



L-NAME

Figure 1.3 The chemical structure of some of the substrate analogue inhibitors of NO synthase: N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methyl ester (L-NAME). The structure of the substrate, L-arginine (L-ARG) is also shown.

that L-NMMA was a competitive inhibitor of both cNOS from endothelial cells and iNOS from macrophages (Gross *et al.*, 1990), however, later work showed that it can under certain circumstances irreversibly inhibit iNOS (Olken *et al.*, 1991). In same cells L-NMMA has been shown to undergo both iNOS- and cNOS-catalysed oxidation to generate N-hydroxy-N-methyl-L-arginine, which upon further conversion to L-citrulline, generates an irreversibly binding species (Feldman *et al.*, 1993). An unusual ability of L-NMMA to act as a substrate for production of NO has also been found in porcine brain (Klatt *et al.*, 1994), rat aorta (Frew *et al.*, 1993), bovine retractor penis (Martin *et al.*, 1993) and murine macrophages (Olken & Marletta, 1993). L-NOARG has been shown to essentially inhibit irreversibly cNOS both *in vitro* and *in vivo* (Dwyer *et al.*, 1991), whereas its effects are fully reversible on iNOS (Furfine *et al.*, 1993). Thus, L-NOARG shows some selectivity for cNOS over iNOS. Another N-substituted L-arginine analogue, N^G-amino-L-arginine (L-NAA), is a potent inhibitor of both cNOS and iNOS (Fukuto *et al.*, 1990). The rank order of potency for inhibition of NO synthesis varies in different cell systems (see Fukuto & Chaudhuri, 1995 for review), indicating that some degree of isoform selectively can be accomplished by simple derivatization of L-arginine.

A variety of nitrogen compounds also have the ability to inhibit both iNOS and cNOS, although the exact mechanism by which they do so is yet to be elucidated. 3-Amino-1,2,4-triazole is one such inhibitor (Fukuto & Chaudhuri, 1995). However, we found this compound to have no effect on ACh-induced relaxation in rat aorta (Mian & Martin, 1995b). Another is aminoguanidine, which contains the guanido group of L-arginine linked to hydrazine; this has been shown to be more potent at inhibiting iNOS compared to cNOS (Misko *et al.*, 1993). Further, Moore *et al.* (1993) demonstrated that 7-nitroindazole is a fairly potent and selective inhibitor of nNOS. Most recently, Handy *et al.* (1995) demonstrated that a nitrogen-containing heterocyclic compound, 1-(2-

trifluoromethylphenyl)imidazole, is a potent and relatively selective inhibitor of nNOS both *in vitro* and *in vivo*. Thus, isoform-selective inhibitors of NOS may prove useful for the treatment of a wide variety of disease states.

Since both cNOS and iNOS require NADPH-dependent FAD and FMN, it would be expected that flavoprotein inhibitors would inhibit NOS activity. Indeed, diphenyleneiodonium, an inhibitor of NADPH-flavoprotein oxidation, and several of its analogues, are potent inhibitors of NOS activity (Stuehr *et al.*, 1991b).

Both iNOS and cNOS are iron haem-containing proteins. The haem prosthetic group is required for catalytic activity and haem-binding agents would, therefore, be expected to inhibit the activity of the enzyme. Carbon monoxide is an extremely good ligand for ferrous (Fe^{2+}) haem and is a potent inhibitor of NOS activity (White & Marletta, 1992; Klatt *et al.*, 1992). Other haem ligands such as cyanide (Klatt *et al.*, 1992), and even NO itself (Roger & Ignarro, 1992) have been shown to inhibit NOS activity. H_4B is a further cofactor required for activity of both iNOS and cNOS (Mülsch & Busse, 1991; Schmidt *et al.*, 1992) and its inhibition would therefore be expected to block NOS activity. Indeed, inhibition of the biosynthesis of this cofactor with the drug, 2,4-diamino-6-hydroxypyrimidine which acts by inhibiting the rate-limiting enzyme in the synthesis of H_4B, GTP-cyclohydrolase I, has been shown to inhibit the production of NO from both iNOS and cNOS (Schmidt *et al.*, 1992; Bogdan *et al.*, 1995). Furthermore, cNOS is activated and regulated by Ca^{2+} via the reversible binding of calmodulin, whereas iNOS is not regulated by Ca^{2+} since calmodulin is tightly bound (Cho *et al.*, 1992; Abu-Soud & Stuehr, 1993). It has been shown that calmodulin binders such as calcineurin, trifluoroperazine, calmidazolium, W-7 and fendiline inhibit cNOS activity while having no effect on iNOS (Schini & Vanhoutte, 1992).

Expression of iNOS can be inhibited by the protein inhibitor cycloheximide (Radomski *et al.*, 1990a). Furthermore, steroids such as hydrocortisone and dexamethasone but not progesterone inhibit the expression of iNOS without directly affecting the activity of the enzyme (Radomski *et al.*, 1990a; Rees *et al.*, 1990b). Inhibition of the induction of NOS by glucocorticoids is a receptor-mediated event involving blockade of the synthesis of mRNA for *de novo* synthesis of this enzyme (Radomski *et al.*, 1990a).

The L-arginine/NO pathway can also be interfered with by directly targeting NO. Haemoglobin has been shown to inhibit relaxations induced by endothelium-dependent agonists such as ACh, and by endothelium-independent nitrovasodilators such as glyceryl trinitrate in rabbit aortic rings (Martin *et al.*, 1985a,b). These authors proposed that haemoglobin inhibits relaxations induced by these agents by directly binding NO before it gains access to soluble guanylate cyclase. In addition, as the vasorelaxant effects of NO are mediated through activation of soluble guanylate cyclase and subsequent increase in cGMP (Rapoport & Murad, 1983), this can be targeted as a means of inhibiting the effects of NO. Methylene blue (MeB) has been shown to inhibit the activity of soluble guanylate cyclase (Holzmann, 1982; Gruetter *et al.*, 1979; Martin *et al.*, 1985a). Martin *et al.* (1985a) suggested that MeB may produce inhibition by oxidising the ferrous (Fe^{2+}) haem component of soluble guanylate cyclase. A recent report by Marczin *et al.* (1992) demonstrated that MeB inhibited nitrovasodilator- and EDRF-induced cGMP accumulation in cultured pulmonary arterial smooth muscle cells via an additional action involving generation of superoxide anion. A specific inhibitor of soluble guanylate cyclase, which does not involve generation of superoxide anion, is 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, Cellek *et al.*, 1996). Thus, this compound may be useful in elucidating the role of cGMP in the biological actions of NO.

The activity of NO can be destroyed as a result of interaction with superoxide anion and SOD has been shown to protect it (Gryglewski *et al.*, 1986b; Moncada *et al.*, 1986; Rubanyi & Vanhoutte, 1986a,b; Mian & Martin, 1995a). The mechanism of this interaction and its consequences will be discussed in more detail in the next Chapter.

1.8 Role of NO in mammalian physiology

NO has gained attention as a representative of a newly discovered class of messenger molecule, amongst which is carbon monoxide (Zakhary *et al.*, 1996), serving a variety of functions in the cardiovascular system, central and peripheral nervous systems and immune system.

1.8.1 NO in the cardiovascular system

The vasculature is in a constant state of dilation mediated by NO produced continuously by endothelial cells under physiological conditions (Egléme *et al.*, 1984; Martin *et al.*, 1986; Martin, 1988). Inhibitors of NOS such as L-NAME or L-NMMA, when infused (systemically) into animals (Baylis *et al.*, 1992) or humans (Haynes *et al.*, 1993), contract vascular smooth muscle and increase the blood pressure due to loss of basal dilator tone mediated by NO. Thus, a basal level of NO acts as an endogenous autoregulator of blood flow. The concept of basal release of NO indicates that arterial pressure and vascular resistance are determined as much by active vasodilatation as by active vasoconstriction.

NO dilates blood vessels by directly relaxing vascular smooth muscle cells. Agonists such as ACh, bradykinin, Ca^{2+} ionophore A23187, substance P, thrombin, ADP and 5-HT (Furchgott, 1984; Stork & Cocks, 1994) bind to their

corresponding receptors on endothelial cells causing a transient increase in intracellular Ca^{2+} which then binds to calmodulin and the resulting complex activates endothelial NOS which synthesises NO. NO is a small lipophilic molecule which travels freely through cell membranes and can, therefore, act on neighbouring target cells. For example, NO released from endothelial cells acts on the vascular smooth muscle cell immediately beneath it where it binds to the haem group on the soluble guanylate cyclase. The enzyme is activated to produce cGMP (Murad, 1994) which, through a cascade of protein kinases (see section 1.2.4.3), induces smooth muscle relaxation (Warner *et al.*, 1994).

Shear stress on the vessel wall also activates ion channels in the endothelial cells and leads to increased NO production (Pohl *et al.*, 1986). This effect accounts for the phenomenon of flow-dependent vasodilatation, a form of autoregulation that might control tissue perfusion (Lowenstein *et al.*, 1994).

The release of NO into the vasculature is also controlled by the autonomic nervous system. Parasympathetic nerves containing NOS terminate in the adventitia of certain large vessels such as the cerebral and retinal arteries. NO released from these nerves diffuses into the muscular media from the outside of the vessel, causing vasorelaxation (see Lowenstein *et al.*, 1994 for review).

NO influences other areas of the cardiovascular system besides blood vessels. It has been shown to exert a negative chronotropic effect on the heart and a negative inotropic effect on cardiac muscle cells (Henderson *et al.*, 1992).

NO produced by endothelial cells reduces the blood clotting process by inhibiting platelet aggregation (Radomski *et al.*, 1987a,b; 1990b) and adhesion (Sneddon & Vane, 1988). In addition, platelets also express the L-

arginine/NO system which may have a negative feedback role in the regulation of platelet function (Radomski *et al.*, 1990b). The inhibitory effects of NO on platelets, like on vascular smooth muscle, are mediated by elevation of cGMP levels (Merritt, 1993). In addition, NO synergises with PGI_2 to inhibit platelet aggregation (Radomski *et al.*, 1987b), suggesting that these two mediators play a role in the thrombo-resistant properties of the endothelium (Davies & Hagen, 1993; Curzen *et al.*, 1994). NO has also been shown to inhibit leukocyte adhesion to the endothelium, which is one of the early events in the development of atherosclerosis (Kubes *et al.*, 1991). It additionally exerts an antiproliferative effect on vascular smooth muscle (Lüscher, 1993).

Abnormalities of the L-arginine/NO pathway have been shown to contribute to many cardiovascular pathologies. For example, impaired endothelium-dependent relaxation to ACh has been demonstrated in spontaneously hypertensive rats (Dohi *et al.*, 1990). Similarly, intra-arterial infusion of ACh into the human forearm circulation of hypertensive subjects results in impaired endothelium-dependent vasodilatation (Linder *et al.*, 1990). Also, in patients with essential hypertension, infusion of L-NMMA into the brachial artery causes less vasoconstriction (decreased forearm blood flow) in hypertensives as compared to normotensives (Panza *et al.*, 1993), suggesting a reduced basal formation of NO in hypertension. Hypercholesterolemia and atherosclerosis are also associated with a reduction in vasodilatation mediated by the endothelium (Wennmalm, 1994). The release of biologically active EDRF (NO) from coronary artery from hypercholesterolemic pigs, as assessed by bioassay, has been shown to be significantly reduced (Shimokawa & Vanhoutte, 1989). Moreover, reduced endothelium-dependent vascular relaxation has also been observed in rats with streptozotocin-induced or spontaneous diabetes (Heygate *et al.*, 1995). Calver *et al.* (1992) studied endothelium-dependent vasodilatation in patients with Type 1 (insulin-

dependent) diabetes and in healthy controls and reported a decreased basal formation in the former group. Decreased NO production or activity has also been associated with vasospasm and thrombosis (Quinn *et al.*, 1995).

As mentioned previously, production of NO can be induced in vascular smooth muscle cells and other cells following exposure to endotoxin and/or certain cytokines (Stuehr *et al.*, 1991a), resulting in the massive production of NO for sustained periods. Such an action is believed to underly the pathogenesis of the circulatory failure in endotoxin shock (Thiemermann, 1994).

1.8.2 NO and the central and peripheral nervous system

NO mediates both central and peripheral neurotransmission. Garthwaite *et al.* (1988) showed that a substance similar to EDRF was released through activation of NMDA receptors in the cerebellum. NO may participate in long-term potentiation which may serve as a model for learning and memory. Pathological effects of NO in the central nervous system have been linked to activation of the NMDA receptors. The brain enzyme is implicated in neural cell death following stimulation of the NMDA receptors in stroke, Parkinson's disease, Alzheimer's disease, Huntington's chorea, and AIDS-related dementia (Springall, 1995; Zhang & Synder, 1995).

In the periphery, NO acts as a non-adrenergic non-cholinergic inhibitory transmitter leading to relaxation of smooth muscle in the reproductive, digestive and respiratory systems (see Zhang & Synder, 1995 for review). Neuronal NOS is present in these nerves and blocking NO formation with L-NMMA and L-NOARG or its action with Hb inhibits the effects of nerve stimulation (Bowman *et al.*, 1982; Gillespie *et al.*, 1989). The relaxing effect of NO facilitates propulsion of food through pyloric sphincter, just as NO is

presumably involved in the relaxation phase of peristalsis (Desai *et al.*, 1991). Inhibition of the synthesis or actions of NO may lead to intestinal dysmobility and bronchial asthma (Springall, 1995).

1.8.3 NO and the immune system

iNOS in macrophages and other cells has been proposed to play a primary defence role against intracellular microoarganisms as well as pathogens such as fungi and helminths that are too large to be phagocytosed (Langrehr *et al.*, 1993). NO has also been shown to kill tumour cells (Stuehr & Nathan, 1989) and may therefore act as an endogenous anti-neoplastic agent. The mechanism of the cytostatic effect of macrophage-derived NO on tumour cells, fungi, mycobacteria and parasites is not clear. However, activated macrophages can inhibit a number of key enzymes present in microbial and tumour cells by nitrosylating the non-haem iron moiety (Stuehr & Nathan, 1989). High concentrations of NO released by macrophages inhibit mitochondrial respiratory enzymes, DNA synthesis and aconitase activity in tumour cells (Nathan, 1992; Kam & Govender, 1994). Other immune cells including lymphocytes and neutrophils also synthesise and release NO (Quinn *et al.*, 1995).

Unregulated NO release may be responsible for the vascular leakage (oedema) and tissue damage seen in some inflammatory conditions (Curzen *et al.*, 1994). In addition, NO may damage normal host cells in autoimmune disease in which iNOS is inappropriately induced (Vallance & Collier, 1994).

Chapter 2

2.1 Oxygen free radicals and reactive oxygen species

A free radical is defined as a chemical species possessing an unpaired electron. It can also be considered as fragments of molecules which are generally very reactive. Free radicals can be formed in three ways: (i) by the homolytic cleavage of a covalent bond of a normal molecule, with each fragment retaining one of the paired electrons ($X : Y \rightarrow X^{\bullet} + Y^{\bullet}$); (ii) by the loss of a single electron from a normal molecule; or (iii) by the addition of a single electron to a normal molecule ($A + e^{-} \rightarrow A^{-\bullet}$). The latter, electron transfer, is a common process in biological systems. Free radicals can be positively charged, negatively charged or electrically neutral (Cheeseman & Slater, 1993).

The electrons in one of the most important molecules in free radical biochemistry, oxygen, are distributed in such a way that two of the electrons are "unpaired". Thus, oxygen is sometimes considered a di-radical. The di-radical nature of oxygen enables it to react readily with many other free radicals (Bast *et al.*, 1991) and it reacts relatively slowly with non-radical species (Cheeseman & Slater, 1993).

2.1.1 Superoxide radical

The most important free radicals in biological systems are derivatives of oxygen. A transfer of a single electron to oxygen results in its reduction and subsequent production of superoxide free radical anion (superoxide).

$$O_2 + e^- \rightarrow {}^{\bullet}O_2^-$$ (superoxide anion)

Superoxide anion is formed in all aerobic animal cells (Fridovich, 1983). Under normal circumstances, the major source of superoxide production in cells is electron "leakage" from electron transport chains such as those in mitochondria and endoplasmic reticulum (Cheeseman & Slater, 1993). Liver microsomes, when supplied with NADPH, have been shown to generate superoxide (Rashba-Step & Cederbaum, 1993). The rate of production in mammalian liver has been estimated to be 24 nmole ${}^{\bullet}O_2^-$ min^{-1} gm^{-1} and the intramitochondrial steady state concentration of this radical to be approximately 1 x 10^{-11} M (Chance *et al.*, 1979). Another important source of superoxide production is the respiratory burst of phagocytic cells when they contact foreign particles or immune complexes (Halliwell, 1982). Phagocytic cells known to produce superoxide include neutrophils, monocytes, macrophages and eosinophils (Halliwell & Gutteridge, 1984). In addition, endothelial cells are a potential source of superoxide anion (Rosen & Freeman, 1984). This free radical is also extensively produced by a number of enzymes including xanthine oxidase, aldehyde oxidase and a variety of flavin dehydrogenases (Fridovich, 1983). In addition, autooxidation of certain compounds such as ascorbic acid (vitamin C), catecholamines, haemoglobin and thiols (e.g. glutathione and L-cysteine) (Misra & Fridovich, 1972; Misra, 1974; Freeman & Crapo, 1982; Cheeseman & Slater, 1993) results in the formation of superoxide. Superoxide production has also been shown to be potentiated by hyperoxia (Freeman & Crapo, 1981).

Superoxide has a short half-life (1 x 10^{-6} seconds at 37 °C; Yu, 1994) and spontaneously or enzymatically dismutases to produce more damaging species such as hydrogen peroxide (see later). The dismutation process is also catalysed by superoxide dismutase (SOD) which dramatically increases

the rate of reaction (Salvemini & Botting, 1993). The antioxidant aspects of SOD will be discussed in detail later.

Several roles have been attributed to superoxide anion in the vascular wall. Among these are alterations of endothelial cell integrity, vascular constriction and vasodilatation.

Endothelial cells contain both xanthine dehydrogenase and xanthine oxidase in the ratio of 2:1 which following a contact with activated neutrophils is converted to 1:2 (Ward, 1991). The increase in the xanthine oxidase activity facilitates the generation of superoxide anion. The interaction between endothelial cells and activated neutrophils leads to endothelial cell killing as measured by ^{15}Cr release (Ward, 1991). The role of superoxide anion in endothelial cell killing is supported by experiments demonstrating that preloading of endothelial cells with SOD attenuates injury caused by activated neutrophils (Ward, 1991). Furthermore, superoxide anion generated by xanthine/xanthine oxidase was shown to cause endothelial disruption in rat aortic rings as assessed by scanning electron microscopy (Lawson *et al.*, 1990). Furthermore, studies in our own laboratory (Berman & Martin, 1993) measured albumin transfer across monolayers of bovine aortic endothelial cells as an indicator of endothelial barrier function and demonstrated an increase in albumin transfer across monolayers on addition of hypoxanthine/xanthine oxidase. However, it is likely that superoxide anion may itself be reduced to hydrogen peroxide or it may reduce Fe^{3+} to Fe^{2+} permitting the Fenton reaction and formation of hydroxyl radical (Kvietys *et al.*, 1989; Bast *et al.*, 1991, see later), which may finally be responsible of endothelial cell damage.

Endothelial cells produce superoxide anion in addition to NO (Rosen &

Freeman, 1984). The inactivation of NO by superoxide anion has been well established in a number of studies (Gryglewski *et al.*, 1986b; Moncada *et al.*, 1986; Rubanyi & Vanhoutte, 1986a,b). The interaction between superoxide anion and NO leads to induction of endothelium-dependent vasoconstriction in arterial rings from loss of the dilator actions of basally produced NO (Katusic & Vanhoutte, 1989; Ohlstein & Nichols, 1989). The generation of superoxide anion may be a common mechanism for the inhibitory actions of many other redox compounds, such as hydroquinone and pyrogallol, on NO (Hutchinson *et al.*, 1987; Halliwell & Gutteridge, 1989).

The ability of superoxide anion to inactivate NO has been mentioned above. Of a special note is the role of NO in the decrease of superoxide anion levels by direct removal of this radical. Rubanyi *et al.* (1991) demonstrated that exogenously added NO inactivated superoxide anion produced by human leukocytes. The antioxidant role of NO may be important under pathological conditions.

Superoxide anion has also been shown to attenuate the contractions induced by catecholamines (Wolin & Belloni, 1985). This action was suggested to be due to oxidation of catecholamines by superoxide anion since it was prevented by SOD (Wolin & Belloni, 1985).

In contrast to the above, vasodilatory effects resulting from superoxide anion are suggested to be mediated via formation of peroxynitrite (Liu *et al.*, 1994). This will be discussed in detail later.

2.1.2 Hydrogen peroxide

Hydrogen peroxide is a normal cellular metabolite (Chance *et al.*, 1979) formed in all subcellular compartments (Boveris *et al.*, 1972) capable of undergoing oxidation-reduction reactions. Mitochondria are potential source of hydrogen peroxide in cells (Boveris & Chance, 1973; Dionisi *et al.*, 1975) where it is predominantly formed from superoxide anion by the dismutation reaction:

$$^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide (H_2O_2) has no unpaired electrons (i.e. is not a free radical). In addition to mitochondrial electron transport, hydrogen peroxide is generated in tissues by various mechanisms: enzymatic activities e.g. glucose oxidase, xanthine oxidase and cytochrome P_{450} (Sevanian & Hochstein, 1985); autooxidation of normal physiological reductants such as glutathione and ascorbate (Chance, 1950; Misra, 1974); and autooxidation of small molecules such as catecholamines and flavins (Freeman & Crapo, 1982; Cheeseman & Slater, 1993). Furthermore, hydrogen peroxide is generated by phagocytic granulocytes during inflammation processes (Setty *et al.*, 1984).

Unlike superoxide anion, hydrogen peroxide is fairly stable and is enzymatically broken down to water and oxygen by catalase (Halliwell & Gutteridge, 1989; Cheeseman & Slater, 1993, see later). Additionally, glutathione peroxidase catalyses the breakdown of hydrogen peroxide (Little & O'Brien, 1968; Christophersen, 1969; Smith *et al.*, 1989).

Hydrogen peroxide is potentially more toxic than superoxide anion since it diffuses through cell membranes with relative ease and directly attacks certain

target components within the cell (Yu, 1994). Hydrogen peroxide has the ability to generate highly reactive radicals through its interaction with redox-active transitional metals.

Hydrogen peroxide performs two main effects in the vascular wall. The first of these effects is its ability to produce cytotoxicity of cultured endothelial cells. Oxidation injury is thought to be an important mechanism in vascular damage. Hydrogen peroxide provokes a variety of adverse biological effects on the endothelial cells such as morphological alterations (Weiss *et al.*, 1981; Krautschick *et al.*, 1995), modulation of prostacyclin production (Harlan & Callahan, 1984; Whorton *et al.*, 1985), enhancement of neutrophil adhesion (Lewis *et al.*, 1988), and increases in permeability of endothelial monolayers (Johnson *et al.*, 1989; Berman & Martin, 1993). Exposure of endothelial cells to hydrogen peroxide produced a concentration-dependent reduction of glutathione and ATP levels (Spragg *et al.*, 1985; Varani *et al.*, 1990). In addition, hydrogen peroxide produces numerous effects on the vascular smooth muscle: inhibition of contractions induced by various vasoactive agents (Sheehan *et al.*, 1993; Iesaki *et al.*, 1994), stimulation of membrane associated protein kinase C activity and phospholipase A activity, and arachidonic acid release (Stäuble *et al.*, 1994; Rao *et al.*, 1995). Hydrogen peroxide-induced cytotoxicity has been reported to be mediated, at least in part, by an enhanced release of intracellular Ca^{2+} in target cells (Hirosumi *et al.*, 1988; Sato *et al.*, 1989; Krippeit-Drews *et al.*, 1995).

In addition to its actions as an oxidant, hydrogen peroxide can also induce vascular relaxation (Burke & Wolin, 1987; Furchgott, 1991; Zembowicz *et al.*, 1993). This occurs by both endothelium-dependent and endothelium-independent mechanisms. The endothelium-dependent component is thought to be mediated via enhanced endothelial synthesis of NO, whilst the

endothelium-independent component is due to a direct stimulatory effect on smooth muscle soluble guanylate cyclase (Burke & Wolin, 1987, Furchgott, 1991; Zembowicz *et al.*, 1993). The mechanism by which hydrogen peroxide stimulates soluble guanylate cyclase is not fully elucidated but it has been proposed that the presence of catalase is vital for this to occur and that the active species which stimulates the enzyme is compound I, i.e. the catalase-hydrogen peroxide complex formed as an intermediate (Burke & Wolin, 1987; Wolin & Burke, 1987).

2.1.3 Hydroxyl radical

The hydroxyl radical is considered to be potentially the most potent oxidant encountered in biological systems. Addition of an electron to hydrogen peroxide generates hydroxyl radical ($^{\bullet}OH$). This reaction is, however, slow and a metal catalyst is needed to accelerate the reaction. The presence of traces of transition metal ions (Fe^{2+} and Cu^{+}) leads to formation of hydroxyl radical by the Fenton reaction:

$$Fe^{3+} + {}^{\bullet}O_2^{-} \rightarrow Fe^{2+} + O_2$$

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

Although hydroxyl radical is highly reactive, its *in vivo* formation is contingent upon the availability of physiological iron (Cross *et al.*, 1987). Two thirds of the 4 g of iron present in an adult is stored in haemoglobin. Ten percent is found in myoglobin with a small portion in iron-containing enzymes and in the transport protein, transferrin (Bast *et al.*, 1991). The majority of intracellular iron is stored in ferritin as Fe^{3+} (Halliwell & Gutteridge, 1984). Iron, as Fe^{3+}, does not participate in the Fenton reaction, so it must be reduced to Fe^{2+} and dissociated from ferritin. Such processes are achieved either in a reductive

environment caused by superoxide anion or in an acid medium as occurs in hypoxic and ischemic tissues (Bast *et al.*, 1991). Ferrous iron (Fe^{2+}), as shown above, then reacts with hydrogen peroxide and promotes generation of hydroxyl radical. Hydroxyl radical can also be generated by an iron-independent pathway, via decomposition of peroxynitrite (Beckman *et al.*, 1990).

The interaction of hydroxyl radical with a number of cellular targets initiates events that lead eventually to cell death. The most important of such targets include: the phospholipids of cellular membranes with the initiation and propagation of lipid peroxidation; the mitochondrial inner membrane with the loss of energization and cellular stores of ATP; and DNA with the appearance of single-strand breakage (Halliwell & Gutteridge, 1984; Farber *et al.*, 1990).

Being highly reactive hydroxyl radical has a very short half-life of about 1×10^{-9} seconds at 37 °C (Yu, 1994) and therefore does not diffuse far from its site of production (Cheeseman & Slater, 1993). Generation of this radical also plays an essential role in the tissue damage caused by high energy radiation (Cross *et al.*, 1987), and could explain part or all of the cytotoxicity attributed to excess of superoxide anion and hydrogen peroxide, since both species can lead to formation of hydroxyl radical.

To protect against hydroxyl radical damage, scavengers such as dimethylsulfoxide, mannitol, N,N-dimethyl thiourea, uric acid and compounds with thiol or sulphydryl groups (RSH) have been used under experimental conditions (Rubanyi, 1988; Bast *et al.*, 1991). Besides that, chelators of divalent metal ions e.g. deferoxamine, which inhibits the formation of hydroxyl radical, have also been utilised (Cross *et al.*, 1987; Rubanyi, 1988).

Hydroxyl radical has been suggested to facilitate ACh-induced relaxation in canine coronary arteries with endothelium (Rubanyi & Vanhoutte, 1986b). This conclusion was prompted by the inhibitory effects on ACh-induced relaxation of mannitol, which scavenges hydroxyl radicals (Cheeseman & Slater, 1993). Similarly to hydrogen peroxide, hydroxyl radical may trigger the synthesis/release of EDRF (Rubanyi, 1988). In smooth muscle cells, this radical may mediate vasodilatation by activating the soluble form of guanylate cyclase (see Rubanyi, 1988 for review).

2.1.4 Peroxynitrite

Peroxynitrite is formed as a result of the interaction between NO and superoxide anion by the following reaction:

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

In alkaline solutions, peroxynitrite ($ONOO^{-}$) is stable. However, at physiological pH peroxynitrite has a half-life of 1.9 seconds and has been shown to be the source of a strong oxidising agent with properties identical to those of the hydroxyl radical (Beckman *et al.*, 1990). It has been proposed that peroxynitrite when protonated can lead to formation of nitrogen dioxide radical ($^{\bullet}NO_2$) and hydroxyl radical ($^{\bullet}OH$) as follows:

$$ONOO^{-} + H^{+} \rightarrow ONOOH \text{ (peroxynitrous acid)}$$

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

$$\downarrow$$

$$NO_3^{-}$$

Furthermore, peroxynitrite can be generated by cells such as macrophages,

neutrophils and vascular endothelium by the simultaneous generation of both superoxide anion and NO (Hogg *et al.*, 1992).

Peroxynitrite is a powerful oxidant. In addition to producing oxidising hydroxyl radicals (shown above), it directly attacks sulphydryl groups of small molecules and proteins and initiates lipid peroxidation (Beckman *et al.*, 1990; Radi *et al.*, 1991; Hogg *et al.*, 1992), and may also be involved in endothelial damage (Kooy & Royall, 1994). Transition metals including copper in the active site of SOD catalyse the formation of NO_2^+-like species, which results in the nitration of aromatics substances (Ischiropoulos *et al.*, 1992). Moreover, due to its ability to modify proteins by nitration of tyrosine residues, peroxynitrite has been shown to inactivate some important proteins including manganese-containing SOD (Ischiropoulos & Al-Mehdi, 1995), thus compromising an important component of the intrinsic antioxidant defence mechanism of the cell.

In addition to its actions as an oxidant, peroxynitrite is a powerful vasorelaxant. Wu *et al.* (1994) demonstrated relaxation of bovine pulmonary arteries by peroxynitrite which appeared to be due to nitrosylating tissue thiol groups that subsequently release NO. More recently, Mayer *et al.* (1995) demonstrated peroxynitrite-induced accumulation of cyclic GMP in endothelial cells and stimulation of purified soluble guanylate cyclase.

2.2 Defences against reactive oxygen species

All forms of aerobic organisms are constantly subjected to oxidant pressure from molecular oxygen (O_2) and from reactive oxygen metabolites produced both during the biochemical utilisation of O_2 and by prooxidant stimulation of O_2 metabolism (Cotgreave *et al.*, 1988). Substances that neutralise the

potential ill effects of free radicals are generally grouped in the so-called antioxidant defence system. The antioxidant systems responsible for cellular protection against oxidative stress are as diverse as the free radicals themselves. A variety of substances capable of scavenging many different species of free radicals are compartmentalised in the subcellular organelles within the cells to provide maximum protection. There are two main strategies of antioxidant defence. The first strategy is to prevent the generation of free radicals e.g. allopurinol by inhibiting xanthine oxidase activity prevents the production of superoxide anion, and transition metal-binding proteins, such as transferrin, by tightly binding the metal prevents its accessibility to act as a catalyst in the production of free radicals. The second strategy is to scavenge the radicals once they have been formed with antioxidants such as vitamin E, ascorbic acid (vitamin C), β-carotene (a metabolic precursor to vitamin A), or with specific enzymes such as superoxide dismutase, catalase and glutathione peroxidase (Cross *et al.*, 1987; Cotgreave *et al.*, 1988; Cheeseman & Slater, 1993).

2.2.1 Superoxide dismutase

Superoxide dismutases (SODs) are a family of metalloenzymes that catalyse the dismutation reaction, involving oxidation of one molecule of superoxide anion ($^{\bullet}O_2^-$) to oxygen (O_2) and reduction of another $^{\bullet}O_2^-$ to hydrogen peroxide (H_2O_2) (Halliwell, 1991):

$$\xrightarrow{\text{SOD}}$$

$$^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The levels of superoxide anion within the cells are maintained at a low level (at 10^{-12} -10^{-11} M) by SOD (Chance *et al.*, 1979). The superoxide dismutases are very efficient catalysts and operate over a broad range of pH values (Fridovich, 1985). It is proposed that the clustering of positive charges around the active site of SOD, coupled with the broad distribution of negative charges elsewhere on the protein, attracts the negatively charged superoxide anion to the active site (Getzoff *et al.*, 1983).

Superoxide dismutases are classified into four distinct classes. In mammalian systems three isoenzymes: Cu-Zn SOD, Mn-SOD and extracellular SOD (EC-SOD, also Cu-Zn-containing) are known (Marklund, 1984). Table 2.1 indicates the general properties of the mammalian isoenzymes. The fourth enzyme, Fe-SOD is structurally homologous to Mn-SOD and is only found in bacteria. All types catalyse the same reaction (shown above) with comparable efficiency.

Cu-Zn SOD is widely distributed mainly in the cytosol of eukaryotes (Hassan, 1988) but also exists in the mitochondrial intermembrane space and in lysosomes (Taniguchi, 1992). Cu-Zn SOD is also present in some bacteria and chloroplasts (James, 1994). Cu-Zn SOD is composed of two identical subunits (i.e. a dimer) of molecular weight 16,000. Each subunit contains one Cu and one Zn molecule, noncovalently linked (Fridovich, 1975). Cu-Zn SOD is encoded by chromosome 21 in humans. It contains a glycated site and may undergo a non-enzymatic glycosylation under hyperglycemic conditions. Increased glycation is thought to be involved in the structural and functional changes in body proteins during normal aging and at accelerated rates in diabetes. Indeed, a high percentage of glycated Cu-Zn SOD has been reported in diabetic patients (Taniguchi, 1992).

Property	Cu-Zn SOD	Mn-SOD	EC-SOD
Distribution	Cytosol	Mitochondrial matrix	Extracellular
Molecular weight	32,000	88,000	135,000
Subunits (molecular weight)	Dimer (16,000)	Tetramer (22,000)	Tetramer (35,000)
Metal/monomer	1 Cu, 1 Zn	1 Mn	1 Cu, 1 Zn
Inhibition by cyanide	+	—	++
Inhibition by diethyldithiocarbamate	+	—	++
Inhibition by chloroform/ethanol	—	+	—
Rate constant for reaction with $^{\bullet}O_2^-$ (M^{-1} s^{-1})	0.62×10^9	1.2×10^9	$0.72\text{-}1.0 \times 10^9$

Table 2.1 Some properties of human superoxide dismutase (SOD) isoenzymes (modified from Taniguchi, 1992)

Cyanide is a reversible inhibitor of Cu-Zn SOD (Fridovich, 1978). Both intracellular and extracellular forms of the enzyme are also inhibited by diethyldithiocarbamate (DETCA, Heikkila *et al.*, 1976; Cocco *et al.*, 1981; Kelner *et al.*, 1989). This irreversibly binds Cu at the active site and removes the metal from the enzyme. The copper ion appears to function in the enzymatic reaction, whereas the zinc stabilises the enzyme (Taniguchi, 1992). Furthermore, hydrogen peroxide has the additional action of inhibiting Cu-Zn SOD activity (Bray *et al.*, 1974; Taniguchi, 1992).

Some bacteria contain either Mn-SOD or Fe-SOD while some contain both types (Fridovich, 1985; James, 1994). In eukaryotes, Mn-SOD is encoded by a nuclear gene and its activity is mainly restricted to the mitochondria (Hassan, 1988). In humans, the Mn-SOD gene is located on chromosome 6 (Taniguchi, 1992). Bacterial Mn-SOD can be either homodimeric or tetrameric, whereas eukaryotic Mn-SOD is tetrameric (James, 1994). The monomeric size of Mn-SOD and Fe-SOD is about 20-24 kDa and both types contain one metal ion per monomer. The metal ion is specific and replacement results in the loss of activity (James, 1994). In contrast to Cu-Zn SOD, Mn-SOD is not inhibited by cyanide or DETCA but is destroyed by chloroform and ethanol (Fridovich, 1978). Involvement of Mn-SOD in aging and disease has also been implicated. High levels of Mn-SOD as determined by the ELISA are present in the serum of patients with certain malignant conditions including gastric cancer, primary hepatoma, epithelial ovarian cancer, and acute myeloid leukemia (see Taniguchi, 1992 for review).

Extracellular SOD (EC-SOD) is a tetrameric high molecular weight (about 135,000) enzyme and is found in numerous extracellular fluids including plasma, lymph, and synovical fluid (Marklund, 1990). EC-SOD shows a sensitivity to heparin suggesting that it is bound to the cell surface (James, 1994).

Control of biosynthesis of SODs by their substrates, or by products derived from them, provide cells with important regulatory mechanisms. Bacteria possesses a series of elaborate and interacting genes that can sense changes in intracellular levels of superoxide anion and hydrogen peroxide and accordingly regulate the synthesis of the enzymes. In higher organisms, metal ion cofactors and cytokines such as interleukin-1 and tumour necrosis factor (TNF-α) impose pre- and post-translational control over the genetic expression of the enzymes (see Fridovich, 1983; 1985; Harris, 1992 for review).

The biological importance of SODs is demonstrated in simple organisms such as bacteria and yeast mutants devoid of the enzyme. Such mutants are more susceptible to oxidative stress and develop greater resistance to oxidative challenge following reintroduction of the SOD gene (Harris, 1992; Warner, 1994). In humans, altered expression of SOD has been linked to two genetic diseases: Downs syndrome in which an extra copy of chromosome 21 is present and a 50 % elevation in Cu-Zn SOD levels has been observed (Garber *et al.*, 1979), and familial amyotrophic lateral sclerosis (ALS) which is an age-dependent degenerative disorder of motor neurons in the cortex, brain stem and spinal cord arising from the mutation in the gene encoding the cytosolic Cu-Zn SOD (Rosen *et al.*, 1993). Taken together, SOD plays an important role in the survival of an organism. The potential role of SOD in protecting endothelial NO from inactivation by superoxide anion remains to be determined.

2.2.2 Catalase

With the exception of certain micro-organisms such as the bacterium *Bacillus popilliae*, catalase activity is present in nearly all forms of life (Halliwell & Gutteridge, 1989). As with SOD, human catalase levels show great tissue

heterogeneity; they are highest in erythrocytes, liver and kidney and low in brain, skeletal muscle, pancreas, and lung tissue (Halliwell & Gutteridge, 1989; Yu, 1994). Catalase consists of 4 protein subunits (i.e. is tetrameric), each of which contains a haem (Fe^{3+}-protoporphyin) group bound to its active site and has a molecular weight of about 60,000 (Brill, 1962). Catalase is mainly localised in subcellular organelles such as peroxisomes (microbodies) or in much smaller aggregates such as the microperoxisomes of the cells (Schonbaum & Chance, 1976; Chance *et al.*, 1979).

Catalase is a major primary antioxidant defence component that primarily works to catalyse the decomposition of hydrogen peroxide to water, sharing this function with glutathione peroxidase (Halliwell & Gutteridge, 1989; Cheeseman & Slater, 1993). Both enzymes detoxify oxygen reactive radicals by catalysing the removal of hydrogen peroxide derived from superoxide. In addition to the difference in their substrate specificities (Chance *et al.*, 1979; Yu, 1994), 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 (Cotgreave *et al.*, 1988) whereas, glutathione peroxidase and other peroxidases have a low capacity but a high affinity. Consequently, it is generally believed that low levels of hydrogen peroxide are preferentially removed by peroxidases. However, higher concentrations of hydrogen peroxide are metabolised by catalase (Cohen & Hochstein, 1963; Nicholls, 1972; Jones *et al.*, 1981; Dobrina & Patriarca, 1986; Verkerk & Jondkind, 1992).

The mechanism by which catalase acts is rather complex. It appears to be the only antioxidant enzyme that exhibits dual activities: (1) decomposition of hydrogen peroxide (H_2O_2) to give water and oxygen (catalytic activity, Chance *et al.*, 1952); and (2) oxidation of hydrogen donor substances (AH_2)

such as ascorbate, ferrocyanide, phenols, short chain alcohols, sodium azide and hydroxylamine in the presence of low concentrations of hydrogen peroxide (peroxidatic activity, Theorell & Ehrenberg, 1952; Keilin & Hartree, 1954; Keilin & Nicholls, 1958; Nicholls & Schonbaum, 1963; Nicholls, 1964; Schonbaum & Chance, 1976). The three key steps shown below provide a complete description of the kinetics of catalase (CAT) in its catalytic (Eqn 1 and 2) and peroxidatic (Eqn 1 and 3) functions:

$$\text{CAT-Fe}^{3+} + H_2O_2 \rightarrow \text{compound I} \quad (1)$$

$$\text{compound I} + H_2O_2 \rightarrow \text{CAT-Fe}^{3+} + 2H_2O + O_2 \quad (2)$$

$$\text{compound I} + AH_2 \rightarrow \text{CAT-Fe}^{3+} + 2H_2O + A \quad (3)$$

The predominant reaction depends on the concentration of the hydrogen donor and the steady-state concentration or rate of production of H_2O_2 in the system. The catalytic reaction proceeds exceedingly rapidly whereas peroxidatic reactions proceed relatively slowly (Schonbaum & Chance, 1976). Both reactions involve the formation of the intermediate, compound I. The green compound I (Fe^{5+}) observed in cells and tissues involves the transfer of two-electrons from the ferric (Fe^{3+}) iron of the haem in the resting state of catalase to the peroxide, and thus peroxide is reduced (Chance *et al.*, 1979; Du & Loew, 1995). Catalase in the native form (Fe^{3+}) shows characteristic optical spectra with α, β and Soret bands around 600, 500, and 400 nm, respectively (Schonbaum & Chance, 1976). The decrease in absorbance at 405 nm in the Soret band and the red shift of the α-band to 660 nm are characteristics of compound I (Schonbaum & Chance, 1976).

Two well characterised hydrogen donors which act as substrates for peroxidation are sodium azide and hydroxylamine. These undergo oxidation to nitrous oxide, nitrogen, and nitric oxide (Theorell & Ehrenberg, 1952; Keilin &

Hartree, 1954; Nicholls, 1964). The nitric oxide thus produced is believed to underly ability of sodium azide and hydroxylamine to activate soluble guanylate cyclase and promote vascular relaxation (Katsuki *et al.*, 1977; Waldman & Murad, 1987; Murad, 1994). Although biochemical evidence supporting the role of catalase in the activation of soluble guanylate cyclase by sodium azide and hydroxylamine is strong (Miki *et al.*, 1976; Katsuki *et al.*, 1977; Craven *et al.*, 1979), direct proof of the involvement of this enzyme in vascular relaxation is lacking.

Catalase activity can be inhibited by sodium azide and hydroxylamine in the presence of hydrogen peroxide. This phenomenon depends on the reduction of catalase compound I to a ferrous derivative; the derivative with bands at 587 and 559 nm is probably nitric oxide ferrocatalase (Keilin & Nicholls, 1958). Cyanide by accelerating the reduction of compound I to compound II (inactive form) inhibits the activity of catalase (Keilin & Nicholls, 1958). Furthermore, superoxide anion has been shown to inhibit the activity of catalase by reducing compound I to compound II (Shimizu *et al.*, 1984). This inhibition is rapidly reversed by SOD and may provide the basis for a synergism between SOD and catalase (Kono & Fridovich, 1982). A commonly used inhibitor is 3-amino-1,2,4-triazole (AT) which inhibits endogenous catalase activity when fed to animals or plant tissues. Heim *et al.* (1956) demonstrated that AT irreversibly inhibits the catalytic function in liver. The reaction was originally found to occur only *in vivo* and not with the isolated enzyme. This behaviour was explained by Margoliash and Novogrodsky (1958), who noted that the inhibition depends upon the presence of peroxides or compounds susceptible to autooxidation, and proposed a reaction between catalase compound I and AT:

$$\text{catalase} + H_2O_2 \rightarrow \text{compound I}$$

$$\text{compound I} + \text{AT} \rightarrow \text{inhibited enzyme}$$

Thus, AT binds to the intermediate compound I, and not to the native enzyme.

As for SOD, catalase activity can also be up-regulated in some conditions by exposure of cells or organisms to oxidative stress (Cotgreave *et al.*, 1988). It has been demonstrated that the development of resistance to high H_2O_2 concentrations in Chinese hamster fibroblasts was concomitant with 20-fold increase in catalase activity. These cells were also more resistant to hyperoxia (Spitz *et al.*, 1992). Furthermore, an increase in the levels of catalase has been demonstrated in diabetic rats (Langenstroer & Pieper, 1992). These studies suggest an important role for catalase in the overall antioxidant capacity of the cell. The potential role of catalase in protecting vascular function from the oxidant, hydrogen peroxide, remains to be determined.

2.2.3 Glutathione peroxidase

Glutathione peroxidase (Gpx) is present in most species and is located intracellularly in the cytosol and mitrochondrial matrix (Chance *et al.*, 1979; Meister & Anderson, 1983). It catalyses the reduction of hydrogen peroxide (H_2O_2) and organic hydroperoxides (ROOH), while oxidising two molecules of glutathione (GSH) to form oxidised glutathione disulphide (GSSG) and water (Little & O'Brien, 1968; Christophersen, 1969; Smith *et al.*, 1989). The reactions are as follows:

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

or

$$2GSH + ROOH \rightarrow GSSG + H_2O + ROH$$

Gpx needs reduced GSH to detoxify peroxides. A high concentration of reduced GSH is maintained within the cell. Glutathione reductase is a dimeric

enzyme containing FAD in the active sites and utilises NADPH to reduce the oxidised GSSG back to GSH (Meister & Anderson, 1983). Gpx enzyme exists in two broad families; selenium-dependent (Se-Gpx) and selenium-independent (non Se-Gpx). Both types have been shown to catalyse the above reactions and thus protect against oxidant damage by reducing peroxides. Se-Gpx is tetrameric with a molecular weight of about 76,000 and non Se-Gpx is dimer with a molecular weight of about 46,000 (Chance *et al.*, 1979). The non Se-Gpx utilises organic hydroperoxides as preferred substrates over H_2O_2 (Yu, 1994). Gpx being a major pathway for the metabolism of lipidic peroxides and H_2O_2 in many cells is important for the protection of biological membranes against lipid peroxidation (McCay *et al.*, 1976).

The activity of Gpx is regulated by the levels of NADPH and reduced glutathione (GSH) in the cells (Meister & Anderson, 1983; Michiels *et al.*, 1994). In addition, the levels of the enzyme may be up-regulated in oxidative stress. Prolonged exposure to hyperoxia has been shown to raise the levels of Gpx and glutathione reductase in rat lungs (see Cotgreave *et al.*, 1988; Harris, 1992 for review).

A key protective role for glutathione peroxidase has been suggested in a variety of tissues including the heart (Doroshow *et al.*, 1980; Simmons & Jamall, 1989), liver (Simmons & Jamall, 1988), endothelial cells (Harlan *et al.*, 1984; Tsan *et al.*, 1985; Suttorp *et al.*, 1986), tumour cells (Nathan *et al.*, 1980; Arrick *et al.*, 1982), red blood cells (Lal *et al.*, 1980), cultured gastric mucous cells (Hiraishi *et al.*, 1991), and human fibroblasts (Michiels & Remacle, 1988).

2.3 Reactive oxygen species in pathological processes

Enzymatic and non-enzymatic reactions continuously generate reactive oxygen metabolites and free radicals in cells and tissues. The organism possesses a delicate equilibrium between free radical production and antioxidant systems which may be altered in many pathological processes, such as ischemia-reperfusion injury, hypertension, hypercholesterolemia/atherosclerosis and diabetes (Cheeseman & Slater, 1993).

Ischemia is the deprivation of oxygen to tissues. The extent of the damage caused to the affected tissue is dependent upon the duration of the ischemic period and the level of oxygen deprivation (Halliwell & Gutteridge, 1989). Tissues respond to ischemia in number of ways, including a fall in ATP levels and degradation of AMP resulting in the accumulation of hypoxanthine (Kloner *et al.*, 1989). In addition, cytosolic Ca^{2+} concentrations increase owing to an anoxia-dependent cation gradient alteration which leads to the activation of proteases that convert xanthine dehydrogenase to xanthine oxidase (McCord, 1985). When the ischemic tissue is reperfused, hypoxanthine reacts with molecular oxygen in the presence of xanthine oxidase to form superoxide and xanthine; xanthine is in turn converted to superoxide and uric acid by xanthine oxidase (Parks & Granger, 1986; Rubanyi, 1988). Activated neutrophils are another potential source of oxygen free radicals (Werns & Lucchesi, 1990; Ward, 1991). Evidence supporting the role of free radicals in ischemia-reperfusion damage has been provided by studies in the intestine (Granger, 1988), myocardium (Downey, 1990; Flaherty, 1991), kidney (Greene & Paller, 1994) and brain (Tsung *et al.*, 1989; Liu *et al.*, 1989). Furthermore, the protective effects of exogenous SOD and catalase alone or when combined are well demonstrated in myocardial and brain ischemia-reperfusion injury

(Jolly *et al.*, 1984; Gross *et al.*, 1986; Aoki *et al.*, 1988; Tamura *et al.*, 1988; Liu *et al.*, 1989; Tsung *et al.*, 1989; Moore & Traystman, 1994).

In acute hypertension, the generation of superoxide and other radicals (Kontos *et al.*, 1981) and impairment of endothelium-dependent vasodilatation (Wei *et al.*, 1985) has been demonstrated. Vega *et al.* (1995) demonstrated a lower EDRF/NO availability in vessels of hypertensive rats and suggested that this was due to enhanced production of superoxide anion. Furthermore, reductions in the SOD activity are reported to occur in the myocardium (Ito *et al.*, 1995) and aorta of the spontaneously hypertensive rat (SHR) (Vega *et al.*, 1995), and the erythrocytes of patients with pregnancy-induced hypertension (Chen *et al.*, 1994). Moreover, the infusion of a membrane-permeant form of SOD has been shown to lower blood pressure in SHR rats but not in their normotensive Wistar Kyoto controls (Nakazono *et al.*, 1991). Angiotensin II-mediated hypertension in rats has also been shown to be associated with increased vascular superoxide production and, again, membrane-permeant SOD improves endothelial NO activity in hypertensive vessels (Rajagopalan *et al.*, 1996).

Another major cardiovascular disease where the role of superoxide anion has been implicated is hypercholesterolemia/atherosclerosis. In this case, the enhanced production of superoxide anion has been suggested to arise from the oxidation of low density lipoproteins (LDL, Kimura *et al.*, 1995; Ohara *et al.*, 1995). Oxidised LDL has been demonstrated to inhibit endothelium-dependent relaxations in various preparations (Jacobs *et al.*, 1990; Taner *et al.*, 1991; Plane *et al.*, 1992; Jiang *et al.*, 1995). Furthermore, a massive breakdown of NO has been reported to occur in atherosclerotic rabbits (Minor *et al.*, 1990). This observation may suggest an increased formation of superoxide anion and/or decreased activity of SOD in the vascular wall leading

to enhanced destruction of NO. Furthermore, administration of membrane-permeant SOD, polyethylene-glycolated (PEG-SOD) partially restores the impaired endothelium-dependent relaxation in vessels from hypercholesterolemic rabbits (Mügge *et al.*, 1991a).

There is considerable evidence to show that the generation of free radicals is enhanced in diabetes (Langenstroer & Pieper, 1992; Chang *et al.*, 1993) and that these reactive species mediate impairment of endothelium-dependent relaxations (Pieper & Gross, 1988; Diederich *et al.*, 1994; Tesfamariam, 1994). Furthermore, administration of SOD has been shown to restore the impaired endothelium-dependent relaxation in diabetic rat aorta (Hattori *et al.*, 1991).

The role of free radicals has also been implicated in inflammation (Halliwell, 1982; Cross *et al.*, 1987) and cytotoxic brain injury (Lipton *et al.*, 1993).

SOD clearly, therefore, offers enormous therapeutic potential in a large number of vascular diseases. Liposome encapsulated SOD or CAT may provide a useful means of delivering these enzymes to the tissues (Freeman *et al.*, 1985).

2.4 Aims of the study

The aims of the study were:

1. To determine if basal and ACh-stimulated activity of NO were equally sensitive to destruction by superoxide anion. The effects of superoxide anion were assessed by generating the free radical either using the hypoxanthine/xanthine oxidase (HX/XO) system or the drug pyrogallol.

2. To determine the extent to which endogenous Cu-Zn SOD protects basal and ACh-stimulated activity of NO against destruction by superoxide anion. The inhibition of endogenous Cu-Zn SOD was achieved with the copper chelator, diethyldithiocarbamate (DETCA).

3. To test the hypothesis that metabolism by catalase is necessary in order to express the relaxant activities of hydrogen peroxide and the nitrovasodilators, sodium azide and hydroxylamine.

4. To assess the impairment induced by hydrogen peroxide of vascular reactivity.

5. To determine the protective role of endogenous catalase against hydrogen peroxide-induced damage. The inhibition of endogenous catalase was achieved with 3-amino-1,2,4-triazole (AT).

METHODS

Chapter 3

Rat Aortic Rings

3.1 Preparation of tissues, tension recording and experimental conditions

3.1.1 Preparation of rat aortic rings

Male Wistar rats (200-300 g) were killed by stunning and exsanguination. The thoracic aorta was carefully dissected and removed into Krebs' solution in a Petri dish. The aorta was cleared of adhering fat and connective tissue and cut into 2.5 mm long transverse rings using a razor blade slicing device. In some experiments, the endothelium was removed to eliminate the contribution of constitutively produced nitric oxide (NO). This was achieved by locating the aortic ring between two stainless steel hooks, placing a 2 g weight on the bottom hook and gently rubbing the intimal surface with a moist matchstick for 10-20 s. Removal of the endothelium was deemed successful if no relaxation took place in response to the endothelium-dependent relaxant, acetylcholine (ACh; 1 μM) following development of phenylephrine (PE; 10-30 nM)-induced tone.

3.1.2 Tension recording

The aortic rings were mounted on stainless-steel hooks under 1 g resting tension in 20 ml organ baths and bathed at 37 °C in Krebs' solution aerated with 95 % O_2 and 5 % CO_2. Great care was taken during the whole procedure to avoid unnecessary stretch of the tissue. Tension was recorded isometrically by means of Grass FTO3C transducers and responses were displayed on a

Grass polygraph, model 7. The tissues were allowed to equilibrate for 60-90 min before experiments were carried out, during which time the resting tension was re-adjusted to 1 g, as required. The Grass polygraph and isometric transducers were calibrated at the start of each day.

3.1.3 Krebs' Solution

The composition (mM) of the Krebs' solution was based on that described by Krebs and Henseleit (1932):

NaCl 118.0	KCl 4.8	$CaCl_2$ 2.5
$MgSO_4$ 1.2	KH_2PO_4 1.2	$NaHCO_3$ 24.0
glucose 11.0		

All components, except glucose and $NaHCO_3$, were prepared as a 10-times concentrated stock solution with distilled water. The stock solution was diluted by 1:10 with distilled water. Glucose and $NaHCO_3$ were added last and the final Krebs' solution was bubbled with 5 % CO_2 and 95 % O_2 for 15-20 min before use.

3.1.4 Temperature

The Krebs' solution in organ baths and in the tubes delivering Krebs to the baths was maintained at 37 °C by a water jacket heated by a thermostatically controlled pump (Techne Tempette Junior TE-8J).

3.1.5 Induction of tone

The alpha$_1$-adrenoreceptor agonist, phenylephrine (PE, 10-100 nM), was used to induce contractile tone in the rings. Once the contraction to PE had stabilised, the relaxant stimuli were added. Responses to relaxant stimuli were expressed as a percentage (%) relaxation of the PE-induced tone.

3.2 Experimental protocol for assessing the effects of superoxide anions on basal and agonist-stimulated activity of nitric oxide (NO) and role of superoxide dismutase

3.2.1 Basal activity of NO

Basal activity of NO was assessed indirectly by measuring the endothelium-dependent depression of phenylephrine (PE)-induced vasoconstriction (Martin *et al.*, 1986). The rationale for these experiments is that basal production of NO in endothelium-containing rings of rat aorta exerts a tonic vasodilator action opposing the effects of vasoconstrictor agents (Eglème *et al.*, 1984; Martin *et al.*, 1986; Martin, 1988). Consequently, this endothelium-dependent depression of vasoconstriction would expected to be blocked or enhanced by agents which inhibit or enhance the activity of NO, respectively.

3.2.1.1 Effects of superoxide anion generators and superoxide dismutase on basal activity of NO

We utilised two well known superoxide anion generators; hypoxanthine (HX)/xanthine oxidase (XO) and pyrogallol to produce superoxide anion in the solution surrounding the tissue.

$$\begin{array}{c} XO \\ \downarrow \\ HX \Leftrightarrow \text{xanthine} + {}^{\bullet}O_2^- \\ \downarrow XO \\ \text{uric acid} + {}^{\bullet}O_2^- \end{array}$$

HX/XO is a simple enzymic system where xanthine oxidase catalyses the conversion of hypoxanthine to xanthine and superoxide anion. Xanthine is further converted to uric acid by xanthine oxidase with concomitant release of another superoxide anion (Halliwell & Gutteridge, 1989).

$$\text{pyrogallol} \rightarrow \text{semiquinone} + O_2 \Leftrightarrow \text{quinone} + {}^{\bullet}O_2^-$$

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

In these experiments, endothelium-containing rings were contracted with PE (10-30 nM) and when the contraction had stabilised, the effects of hypoxanthine (HX, 0.1 mM)/xanthine oxidase (16 mu ml^{-1}) and pyrogallol (0.1 mM) were examined on tone.

The effects of superoxide dismutase (SOD, 1-300 u ml^{-1}) were also studied on PE (10-30 nM)-contracted endothelium-containing rings. SOD scavenges superoxide anion by converting it to hydrogen peroxide (H_2O_2).

$$\begin{array}{c} SOD \\ \downarrow \\ {}^{\bullet}O_2^- + 2H_2O \rightarrow 2H_2O_2 \end{array}$$

This dismutation can also occur spontaneously, but the presence of SOD dramatically accelerates the reaction (Salvemini & Botting, 1993). SOD acts as an important antioxidant defence mechanism against the damage resulting from the overproduction of superoxide anion in the cells.

In the early experiments with HX/XO, it was clear that a delayed relaxant effect was produced resulting from the generation of hydrogen peroxide (H_2O_2). Consequently, where indicated in the Results, experiments were performed in the presence of catalase (3000 u ml^{-1}) to prevent the accumulation of hydrogen peroxide.

$$\text{CAT} \downarrow \quad 2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase is present in many cell types and acts as a natural defence against the accumulation of hydrogen peroxide (Halliwell, 1982; Cheeseman & Slater, 1993).

3.2.1.2 Effects of diethyldithiocarbamate on basal activity of NO

In certain experiments, we wished to use diethyldithiocarbamate (DETCA), an inhibitor of Cu-Zn SOD (Heikkila *et al.,* 1976; Cocco *et al.*, 1981, Halliwell & Gutteridge, 1989), to inhibit the activity of endogenous SOD. 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 (Hassan, 1988). The copper chelator, DETCA, inhibits Cu-Zn SOD both intracellularly and extracellularly (Kelner *et al.*, 1989),

but has no effect on Mn-SOD. The effects of inhibiting endogenous Cu-Zn SOD were investigated on the endothelium-dependent depression of PE-induced tone. In these experiments, endothelium-containing rings were incubated with DETCA (0.1 mM) for 1h, followed by extensive washing, before the contractile actions of PE (20 nM) were examined. Since DETCA irreversibly inhibits the SOD by chelating copper in the enzyme, once it has produced its effect it no longer needs to be present. Adopting this procedure also meant that any non-specific effects of the drug would have been removed following washing. Moreover, washing the DETCA out permitted the option of putting back exogenous SOD (250 u ml^{-1}) to try to reverse the inhibitory effects of the drug.

3.2.1.3 Effects of NO synthase inhibitors on basal activity of NO

Inhibitors of the NO synthesis are known to block the endothelium-dependent depression of vasoconstriction induced by basal NO (Frew *et al.*, 1993) and so can be used to determine the magnitude of basal NO activity in a tissue. In these experiments, endothelium-containing rings were contracted with PE (20 nM) and when the tone had stabilised, the effects of inhibiting the NO synthesis with analogues of L-arginine: N^G-monomethyl-L-arginine (L-NMMA, 30 μM; Rees *et al.*, 1989; 1990a) or N^G-nitro-L-arginine (L-NOARG, 30 μM; Moore *et al.*, 1990) alone or in combination with HX (0.1 mM)/XO (16 mu ml^{-1}) were examined on PE (20 nM)-induced tone.

Each of the above experiments (sections 3.2.1.1; 3.2.1.2 and 3.2.1.3) was also conducted in endothelium-denuded rings but the concentration of PE used was lowered to 1-3 nM so as to attain a similar degree of tone to that obtained in endothelium-containing rings.

3.2.2 Agonist-stimulated activity of NO

The production of NO is stimulated by a large number of biological mediators such as acetylcholine (ACh, Furchgott & Zawadzki, 1980a) and by the physical shearing force of the flowing blood (Pohl *et al.*, 1986).

Agonist-stimulated activity of NO was determined by assessing ACh-induced relaxation. Cumulative concentration-response curves to ACh (10 nM-3 μM) were constructed in endothelium-containing rings following induction of sub-maximal PE (30-100 nM)-induced tone. The baths were washed out, and the tissues allowed to re-equilibrate.

3.2.2.1 Effects of superoxide anion generators on ACh-stimulated activity of NO

In experiments in which the effects of HX (0.1 or 1 mM)/XO (16 mu ml^{-1}) and pyrogallol (0.1 or 0.3 mM) were to be studied on ACh (10 nM-3 μM)-induced relaxation, we ensured that the level of tone prior to inducing relaxation was similar to that of untreated preparations. In order to achieve this, tissues were initially precontracted with lower concentrations of PE (10-30 nM). Subsequent addition of the free radical generators produced a further elevation of tone in endothelium-containing tissues by destroying basal NO activity, and when the contraction had stabilised, a further cumulative concentration-response curve to ACh was obtained.

In some experiments, the ability of exogenously added SOD (250 u ml^{-1}, 20 min) to protect against the inhibitory effects of HX/XO and pyrogallol was also studied. All experiments were conducted in the presence of catalase (3000 u ml^{-1}) to prevent the relaxant effects of HX/XO and pyrogallol resulting

from the accumulation of hydrogen peroxide.

3.2.2.2 Effects of superoxide dismutase on ACh-stimulated activity of NO

In these experiments, endothelium-containing rings were initially contracted with PE (10-30 nM). Subsequent addition of superoxide dismutase (SOD, 50 u ml^{-1}) produced a relaxation of PE-induced tone. We ensured that the level of tone prior to inducing relaxation to ACh (10 nM-3 μM) was similar to that of untreated preparations. In order to achieve this, the concentration of PE was increased to 100 nM.

3.2.2.3 Effects of diethyldithiocarbamate on ACh-stimulated activity of NO

In studies involving inhibition of endogenous Cu-Zn SOD, the rings were incubated with DETCA for 1h followed by extensive washing. Various concentrations of DETCA were tested, and 0.1 mM was chosen for most experiments since it produced a sub-maximal (25-30 %) inhibition of ACh-induced relaxation by itself. Cumulative concentration-response curves to ACh (10 nM-3 μM) in the presence of HX (0.1 mM)/XO (16 mu ml^{-1}) and pyrogallol (0.1 mM) were also constructed in DETCA-treated rings. All experiments involving HX/XO, pyrogallol or DETCA were conducted in the presence of catalase (3000 u ml^{-1}), added as 5 min pretreatment to prevent the accumulation of hydrogen peroxide.

In some experiments, the ability of exogenously added SOD (250 u ml^{-1}) to protect against the inhibitory effects of HX/XO, pyrogallol or DETCA was studied and, in these, it was added as a 20 min pretreatment.

3.3 Experimental protocol for assessing the role of catalase in relaxations induced by hydrogen peroxide and nitrovasodilators

3.3.1 Hydrogen peroxide-induced relaxations

The effects of hydrogen peroxide (H_2O_2, 100 nM-1 mM) were examined on both endothelium-containing and endothelium-denuded rings, precontracted with PE 100 nM and 10 nM, respectively.

In a separate series of experiments, the effects of methylene blue, an inhibitor of soluble guanylate cyclase (MeB, 30 μM, Gruetter *et al.*, 1979; Holzmann, 1982; Martin *et al.*, 1985a) and N^G-nitro-L-arginine methyl ester (L-NAME, 30 μM; Rees *et al.*, 1990a) alone or in combination with L-arginine (L-arg, 2 mM) were studied on hydrogen peroxide-induced relaxations. In these experiments, rings were incubated with MeB or L-NAME for 20 min. We ensured that the level of tone prior to inducing relaxation to hydrogen peroxide was similar to that of untreated preparations. In order to achieve this, tissues were initially precontracted with a lower concentration of PE (3-30 nM) before addition of the inhibitors. This was necessary since both L-NAME and MeB potentiated tone by blocking the synthesis and actions of basal NO, respectively.

The effects of superoxide dismutase (SOD, 50 u ml^{-1}) and catalase (CAT, 1000 or 3000 u ml^{-1}) were also studied on hydrogen peroxide-induced relaxation precontracted with PE (10-30 nM). In these experiments rings were incubated with SOD or CAT for 20 min and cumulative concentration-response curves to hydrogen peroxide were constructed.

3.3.2 Vasodilator actions of sodium azide, hydroxylamine and glyceryl trinitrate

In a separate series of experiments, the vasodilator actions of three nitrovasodilators: sodium azide (NaN_3, 1-300 nM), hydroxylamine (NH_2OH, 1-300 nM) and glyceryl trinitrate (GTN, 1-100 nM) were investigated in endothelium-denuded rings, precontracted with PE (10-30 nM). The effects of the inhibitor of soluble guanylate cyclase, MeB (30 μM), were also studied on relaxations induced by these nitrovasodilators.

3.3.3 Use of 3-amino-1,2,4-triazole

In certain experiments, we wished to use 3-amino-1,2,4-triazole (AT), a selective inhibitor of catalase (Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958), to inhibit the endogenous catalase activity of rat aortic rings.

In order to establish the conditions necessary to produce optimum inhibition, we first conducted a series of experiments using exogenous catalase (bovine liver). In these experiments, endothelium-denuded rings were incubated either with catalase (1000 u ml^{-1}) or with catalase together with AT (50 mM) for 30 and 90 min. As shown in the Results (Figure 5.8), incubation of catalase with AT for 90 min was required to maximally inhibit the ability of the enzyme to block hydrogen peroxide-induced relaxation. Consequently, in all subsequent experiments involving AT, tissues were incubated with the drug for 90 min.

Pretreatment with AT (50 mM, 90 min) depressed PE (10 nM)-induced tone by 31.8 ± 2.2 % (n=15), but we ensured that the level of tone prior to inducing relaxation was similar to that of untreated preparations by increasing the concentration of PE (to 30-100 nM). AT (50 mM), when added to the tissue

baths, remained in solution and had no effect on the pH of the Krebs' solution. The addition of AT clearly resulted in an increase of the osmotic strength of Krebs' solution. This, however, played no role in the actions of AT since addition of the inert substance, mannitol, at the same concentration (50 mM, 90 min) had no effect on relaxations induced by sodium azide (1-300 nM), hydroxylamine (1-300 nM), glyceryl trinitrate (1-100 nM) or hydrogen peroxide (10 μM-1 mM) (data not shown).

In order to determine if the depression of PE-induced tone by AT was due to activation of soluble guanylate cyclase, the effects of the inhibitor of guanylate cyclase, MeB, were studied on AT-induced relaxation. In these experiments, endothelium-denuded rings were precontracted with PE (30-100 nM) and cumulative concentration-response curves to AT (1-100 mM) were constructed in the presence of MeB (30 μM, 20 min).

3.3.4 Effects of 3-amino-1,2,4-triazole on relaxations induced by sodium azide, hydroxylamine, glyceryl trinitrate and hydrogen peroxide

Catalase has been proposed to play an important role in mediating relaxations induced by certain nitrovasodilators and hydrogen peroxide. This hypothesis was tested by inhibiting endogenous catalase with AT (1-50 mM, 90 min). Consequently, the effects of this inhibition were studied on relaxations of endothelium-denuded rings induced by sodium azide (NaN_3, 1-300 nM), hydroxylamine (NH_2OH, 1-300 nM), glyceryl trinitrate (GTN, 1-100 nM) and hydrogen peroxide (H_2O_2, 10 μM-1 mM), following precontraction with PE (30-100 nM).

3.4 Measurement of nitrite production

It has been proposed that conversion of azide and hydroxylamine to NO by catalase requires the presence of low concentrations of hydrogen peroxide and thus occurs as a consequence of the peroxidase activity of the enzyme (Theorell & Ehrenberg, 1952; Keilin & Hartree, 1954; Nicholls, 1964; Klebanoff & Nathan, 1993; Figure 3.1). Using bovine liver catalase we measured the production of nitrite, a major stable product of NO, from certain nitrovasodilators.

3.4.1 Nitrite assay

NO has a short half life of around 5 seconds and decays to form the stable end products of nitrite and nitrate.

$$NO \rightarrow NO_2^- \text{ (nitrite)} + NO_3^- \text{ (nitrate)}$$

The majority of NO decays to nitrite *in vitro* (Stuehr & Marletta, 1987a,b). *In vivo*, however, NO is mainly converted to nitrate in whole blood, but in plasma, a ratio of 5:1 nitrite: nitrate is formed (Wennmalm *et al.*, 1992). The presence of haemoglobin in blood is thought to be responsible for the conversion to nitrate. However, Ignarro *et al.* (1993) demonstrated that in aqueous solutions in the absence of any haem containing proteins, nitrite is the major oxidation product of NO.

3.4.2 Greiss reaction

The accumulation of nitrite was measured by the formation of a diazo product using a variant of the method of Green *et al.* (1982), i.e. the samples (80

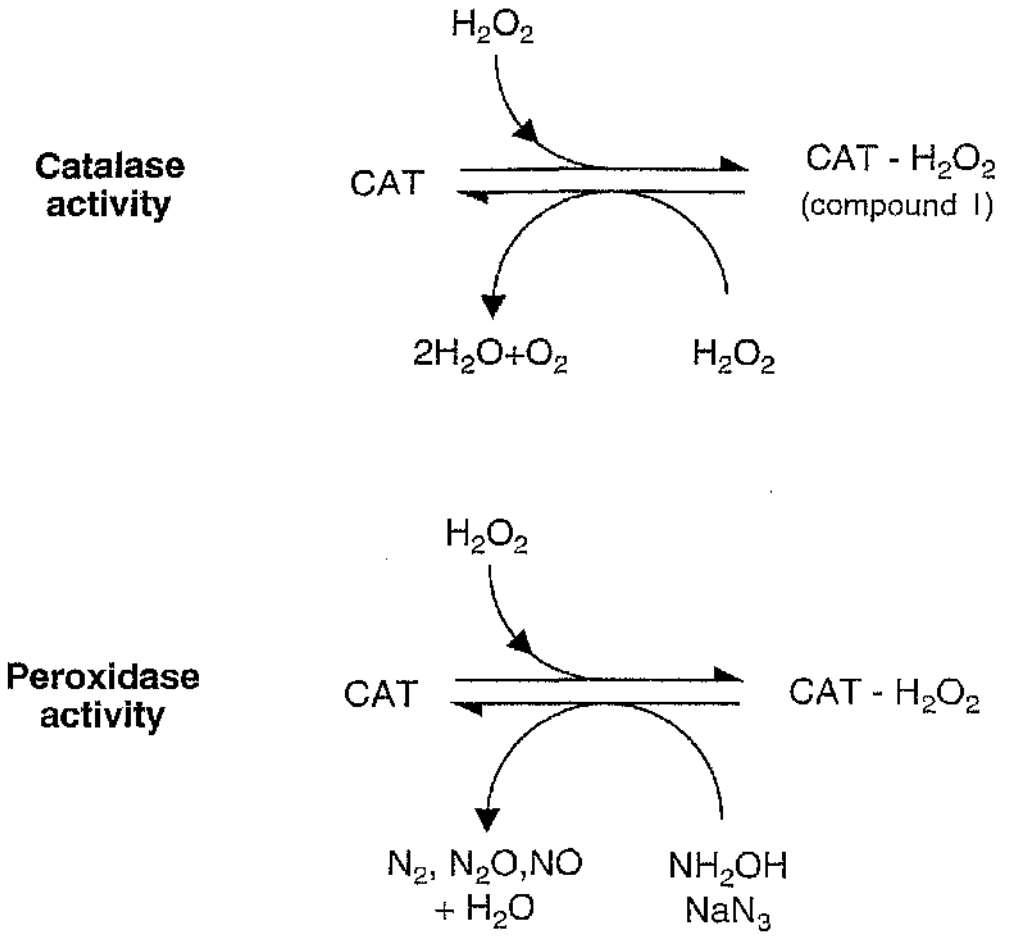


Figure 3.1 Proposed dual function of catalase: breakdown of hydrogen peroxide (H_2O_2) to water and oxygen (catalase activity) and oxidation of hydroxylamine (NH_2OH) and sodium azide (NaN_3) in the presence of H_2O_2 (peroxidase activity) (Keilin & Hartree, 1945; Chance *et al.*, 1952; Theorell & Ehrenberg, 1952; Nicholls, 1964).

µl) were mixed with 80 µl of 1% sulphanilic acid in 2M HCl. After 5 min, 80 µl of 1% (w/v) aqueous N-(1-napthyl)-ethylenediamine dihydrochloride was added, and the absorbance of the pink complex was determined at 550 nm using a Dynatech Microplate Reader model no MR 5000/7000. A standard curve was prepared with solutions of sodium nitrite (1-30 µM, Figure 3.2). The nitrite concentration of unknowns was determined automatically by the computer-controlled plate reader by interpolation from the standard curve.

3.4.3 Catalase-dependent formation of nitrite

We measured nitrite, the major breakdown product of NO, formed from nitrovasodilators by the peroxidase activity of catalase. Briefly, the reaction mixtures containing phosphate buffer (pH 5.6, 0.08 M), bovine liver catalase (300 or 1000 u ml^{-1}), hydrogen peroxide (1 mM) or the glucose (5 mM)/glucose oxidase (300 mu ml^{-1}) system, and a nitrovasodilator, i.e. sodium azide (10 µM-3 mM), hydroxylamine (10 µM-30 mM) or glyceryl trinitrate (10 µM-1 mM) were incubated in a final volume of 80 µl at 37 °C in an incubator for the times indicated in the Results. At the end of these times, nitrite content was determined as above.

We also measured nitrite formation from nitrovasodilators by the thiol, L-cysteine. In these experiments the reaction mixtures containing HEPES buffer (pH 7.4, 5 mM), L-cysteine (3 mM), and a nitrovasodilator, i.e. sodium azide (1 mM), hydroxylamine (1 mM) or glyceryl trinitrate (1 mM) were incubated for 1h at 37 °C. At the end of 1h incubation period, nitrite content was determined as above.

Phosphate buffer contained two components: potassium dihydrogen orthophosphate (A) and disodium phosphate (B). Solutions of A and B

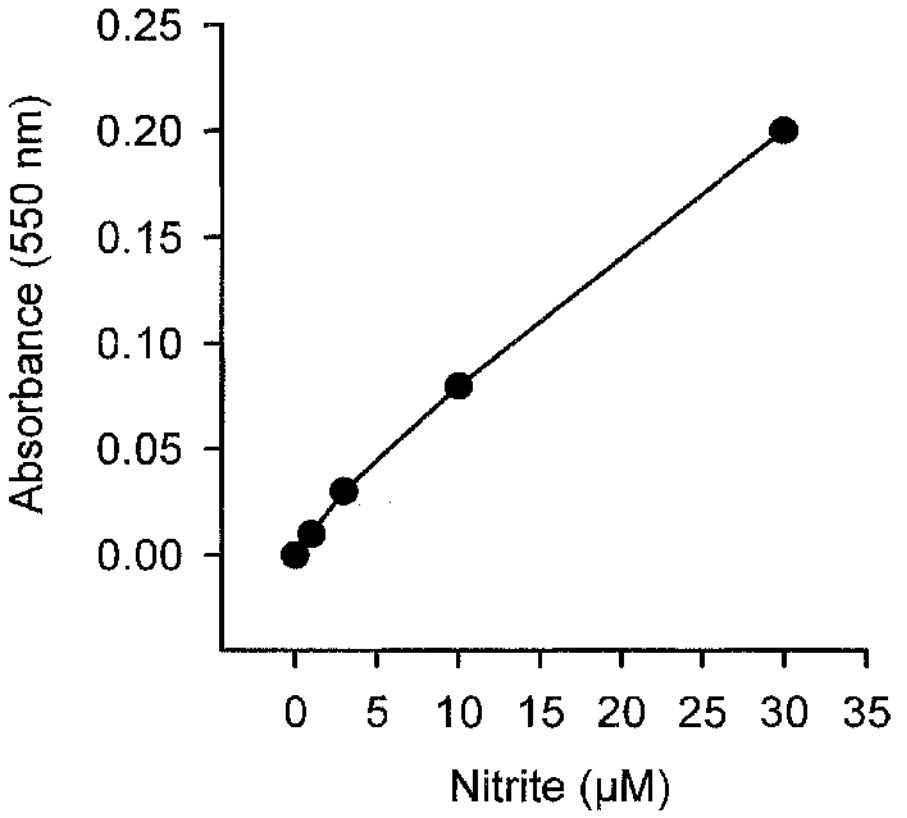


Figure 3.2 Standard curve for measurement of nitrite formation in experimental samples using the Dynatech Microplate Reader model no MR 5000/7000. Standards consisted of sodium nitrite in phosphate buffer (pH, 5.6, 0.08 M). The concentration of nitrite (1-30 μM) is plotted against absorbance at 550 nm. The instrument was zeroed using blanks consisting of phosphate buffer (pH 6.5, 0.08 M).

were prepared with saline. The composition of phosphate buffer was as follows:

x ml A + (100 - x) ml B (for pH 5.6, x= 95.5 ml)

Finally, 4.5 ml of solution B was added slowly to 95.5 ml of solution A and pH was measured constantly.

HEPES-buffered Krebs was prepared from 10-times concentrated stock Krebs' solution containing NaCl, KCl, $CaCl_2$, $MgSO_4$ and KH_2PO_4. The concentrated stock solution of Krebs was diluted with distilled water, and glucose, HEPES and $NaHCO_3$ were added. The final concentration of reagents was: NaCl 118.0 mM, KCl 4.8 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, glucose 11.0 mM, $NaHCO_3$ 24.0 mM and HEPES 5 mM. The final solution was taken to pH 7.4 with 1 M NaOH.

3.4.4 Effects of 3-amino-1,2,4-triazole on catalase-dependent formation of nitrite

In some experiments, we studied the effects of catalase inhibitor, 3-amino-1,2,4-triazole (AT) on catalase-dependent formation of nitrite from sodium azide. The reaction mixtures containing phosphate buffer (pH 5.6, 0.08 M), AT (0.1-100 mM), bovine liver catalase (300 or 1000 u ml^{-1}), hydrogen peroxide (1 mM) and sodium azide (0.3 mM) were incubated in a final volume of 80 µl at 37 °C in an incubator for the times indicated in the Results.

3.5 Experimental protocol for assessing hydrogen peroxide-induced impairment of vascular reactivity and its potentiation by 3-amino-1,2,4-triazole

3.5.1 Hydrogen peroxide-induced impairment of vascular reactivity

The effects of incubation with hydrogen peroxide (0.1 or 1 mM) for 15, 30, or 60 min followed by washout were examined on PE (10 nM)-induced contraction in endothelium-containing rings of rat aorta.

The effects of incubation with hydrogen peroxide were also examined on ACh-induced relaxation. In these experiments the endothelium-containing rings, precontracted with PE (10-30 nM), were incubated with hydrogen peroxide 0.1 or 1 mM for 15, 30 or 60 min. The tissues were then washed out and cumulative concentration-response curves to ACh (10 nM-3 μM) were constructed to determine if any impairment of endothelium-dependent relaxation had taken place.

The ability of exogenous catalase (bovine liver, 1000 u ml^{-1}) to protect against hydrogen peroxide-induced impairment was also studied. In these experiments catalase was added to the baths 15 min before the addition of hydrogen peroxide. Again the tissues were washed out and cumulative concentration-response curves to ACh were constructed to assess the level of relaxation obtained.

In early experiments (see Results, Chapter 7), incubation with hydrogen peroxide (1 mM) for 30 min produced a sub-maximal (20-30 %) inhibition of ACh-induced relaxation by itself. In all subsequent experiments involving

hydrogen peroxide at 0.1 or 1 mM, tissues were incubated with the drug for 30 min. Hydrogen peroxide was washed out at the end of 30 min incubation period.

The effects of incubation with hydrogen peroxide were also examined on endothelium-independent relaxations induced by glyceryl trinitrate and isoprenaline. In these experiments the endothelium-denuded rings, precontracted with PE (3-10 nM), were incubated with hydrogen peroxide (0.1 or 1 mM) for 30 min. The tissues were then washed out and cumulative concentration-response curves to glyceryl trinitrate (1-100 nM) and isoprenaline (10 nM-3 μM) were constructed to determine if any impairment of endothelium-independent relaxation had taken place.

3.5.2 Use of 3-amino-1,2,4-triazole

In certain experiments, we wished to use 3-amino-1,2,4-triazole (AT), a selective inhibitor of catalase (Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958), to inhibit the activity of endogenous catalase. This was done to determine if endogenous catalase exerted a protective action against exogenous hydrogen peroxide. In all experiments involving AT, tissues were incubated with the drug (50 mM) for 90 min. AT was washed out at the end of 90 min incubation period.

3.5.3 Effects of 3-amino-1,2,4-triazole

To assess the selectivity of action of AT (50 mM, 90 min), its effects were studied on relaxations of endothelium-containing rings induced by ACh (10 nM-3 μM) and endothelium-denuded rings induced by glyceryl trinitrate (1-100 nM) and isoprenaline (10 nM-3 μM) following precontraction with PE (10-30 nM).

3.5.4 Effects of 3-amino-1,2,4-triazole on hydrogen peroxide-induced impairment of vascular reactivity

We examined the ability of AT (50 mM) to enhance hydrogen peroxide (0.1 or 1 mM)-induced impairment by assessing relaxation induced by all 3 vasodilators, i.e. ACh, glyceryl trinitrate and isoprenaline. In these experiments, following incubation with AT for 60 min, the tissues were contracted with PE (10-30 nM) and then hydrogen peroxide at 0.1 or 1 mM was added and left for 30 min. All the drugs were then washed out and cumulative concentration-response curves to ACh, glyceryl trinitrate and isoprenaline were constructed.

The effects of incubation with AT (50 mM, 90 min, followed by washout) alone or together with hydrogen peroxide (0.1 or 1 mM, present during the final 30 min of 90 min incubation with AT, followed by washout) were also examined on PE (10 nM)-induced contraction.

3.6 Drugs

Acetylcholine chloride, 3-amino-1,2,4-triazole, L-arginine hydrochloride, catalase (bovine liver), diethyldithiocarbamate, glucose oxidase, hydrogen peroxide (30 %), hydroxylamine chloride, hypoxanthine, isoprenaline hydrochloride, N^G-nitro-L-arginine, N^G-nitro-L-arginine methyl ester, N-(1-napthyl)-ethylenediamine dihydrochloride, phenylephrine hydrochloride, methylene blue, sodium azide, sodium nitrite, sulphanilic acid, superoxide dismutase (bovine erythrocyte, Cu-Zn-containing) and xanthine oxidase (buttermilk) were obtained from Sigma (Poole, Dorset).

Glucose and pyrogallol were obtained from BDH (Poole, Dorset).

N^G-monomethyl-L-arginine was a gift from Wellcome Laboratories (Beckenham, Kent).

Glyceryl trinitrate (GTN) was obtained from NAPP Laboratories (Cambridge, UK).

All drugs were dissolved and dilutions made in saline (0.9 %), except for hypoxanthine which was dissolved in 0.1 % sodium hydroxide. N^G-nitro-L-arginine at 10^{-2} was soluble only after sonication. Concentrations of drugs are expressed as the final molar concentration in the organ bath.

3.7 Statistical analysis

Results are expressed throughout as the mean ± the standard error of the mean for n separate experiments. Statistical significance was determined by one-way analysis of variance followed by Fisher's test. A probability of 0.05 or less was considered significant.

RESULTS

Chapter 4

Assessment of the effects of superoxide anion on basal and agonist-stimulated activity of nitric oxide

In vascular endothelium, the production of nitric oxide (NO) is subject to complex control; it is stimulated by a large number of biological mediators such as acetylcholine (ACh, Furchgott & Zawadzki, 1980a) and by the physical shearing force of the flowing blood (Pohl *et al.*, 1986). Agonist-stimulated activity of NO was determined by assessing ACh-induced relaxation.

In addition, there is a basal production of NO which exerts a tonic vasodilator action on endothelium-containing arterial rings (Eglème *et al.*, 1984; Martin *et al.*, 1986; Martin, 1988). Basal activity of NO was assessed indirectly by measuring the endothelium-dependent depression of phenylephrine (PE)-induced vasoconstriction.

In this part of the study, we determined if basal and ACh-stimulated activity of NO in rat aorta were equally sensitive to destruction by superoxide anion. The effects of superoxide anion were assessed either by generating the free radical using the hypoxanthine/xanthine oxidase (HX/XO) system or the drug, pyrogallol (Halliwell & Gutteridge, 1989), or by increasing the background level of the free radical by inhibiting the endogenous Cu-Zn form of SOD with diethyldithiocarbamate (DETCA, Heikkila *et al.*, 1976; Cocco *et al.*, 1981; Halliwell & Gutteridge, 1989).

4.1 Effects of superoxide dismutase (SOD) on basal and ACh-stimulated activity of NO

4.1.1 Effects of SOD on basal activity of NO

Superoxide dismutase (SOD) scavenges superoxide anions (Halliwell & Gutteridge, 1989; Cheeseman & Slater, 1993). Following induction of phenylephrine (PE, 10-30 nM)-induced tone (1.6 ± 0.1 g, n=8) in endothelium-containing rings of rat aorta, SOD (1-300 u ml^{-1}) produced a powerful concentration-dependent relaxation (maximum relaxation obtained was 79.9 ± 2.0 %, Figure 4.1, 4.2, 4.5 and 4.6). In endothelium-denuded rings, lower concentrations of PE (3-10 nM) were required to induce a similar degree of tone (1.5 ± 0.2 g, n=6) as in endothelium-containing rings, but in these, SOD produced no relaxation (Figure 4.1, 4.2 and 4.7).

We reasoned that if the relaxation of aortic rings produced by SOD was mediated by NO, then this action would be expected to be blocked by inhibitors of NO synthase. Indeed, pretreatment with N^G-nitro-L-arginine (L-NOARG, 30 μM, Moore *et al.*, 1990) for 10 min completely blocked SOD-induced relaxation in endothelium-containing rings (Figure 4.2).

The relaxant effects of SOD are therefore likely to have arisen from removal of superoxide anions generated either within the tissue or in the oxygenated Krebs' solution, which were destroying the basally produced NO.

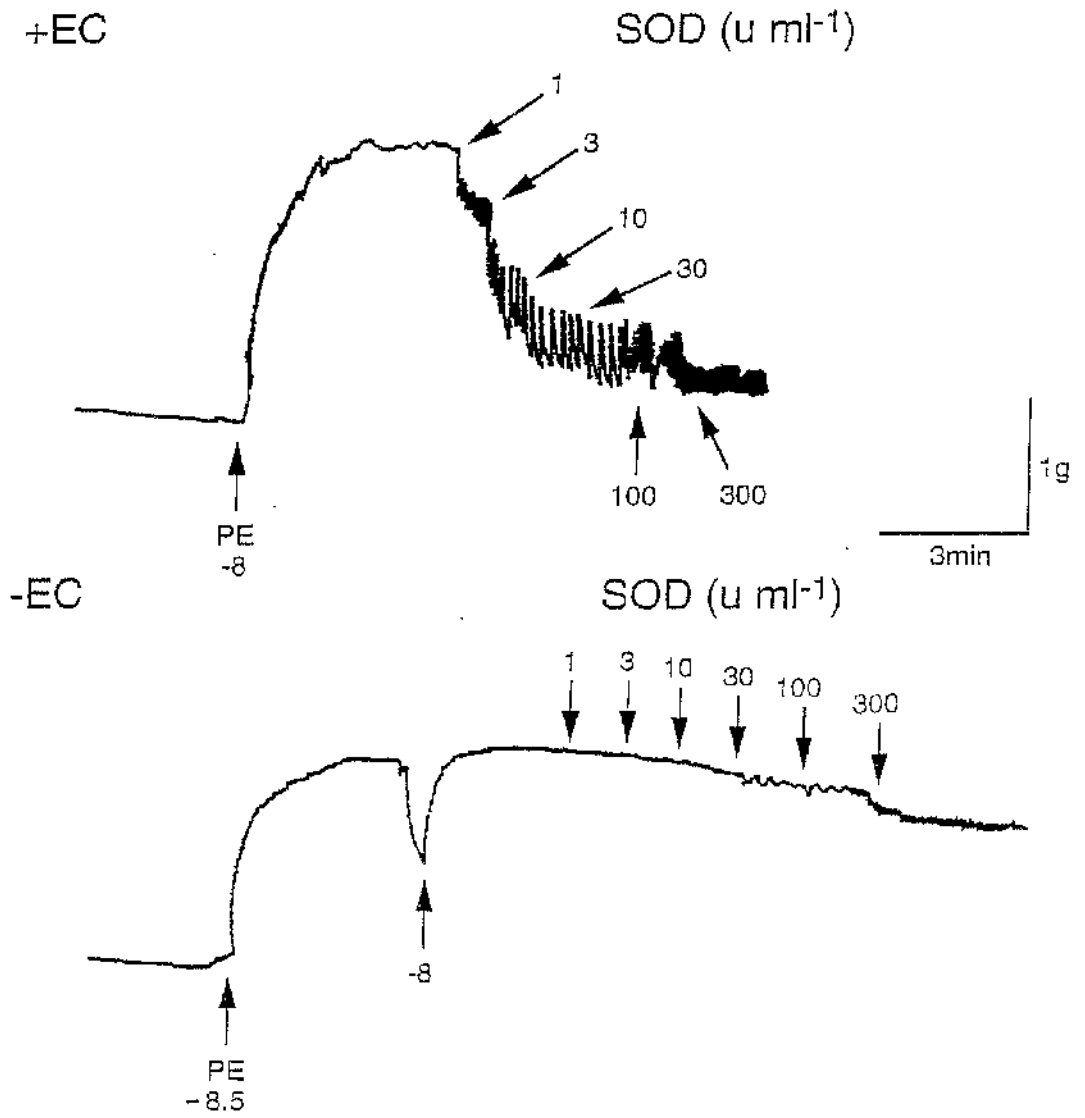


Figure 4.1 Individual experimental tracings showing the ability of superoxide dismutase (SOD) to relax phenylephrine (PE)-contracted endothelium-containing (+EC) but not endothelium-denuded (-EC) rings of rat aorta. Drug concentrations are expressed in log molar units and enzyme concentrations are expressed in units ml^{-1}.

4.1.1.1 Effects of diethyldithiocarbamate (DETCA) on SOD-induced relaxation

Mammalian cells contain two major forms of SOD: Cu-Zn SOD which is located both intracellularly and extracellularly and Mn-SOD which is mainly present in the mitochondria (Hassan, 1988). The Cu-Zn SOD, but not Mn-SOD, is inactivated by the copper chelator DETCA (Heikkila *et al.*, 1976; Cocco *et al.*, 1981; Halliwell & Gutteridge, 1989). DETCA inhibits the Cu-Zn-containing form both intracellularly and extracellularly (Kelner *et al.*, 1989). Consequently, its effects were examined on SOD-induced relaxation in endothelium-containing rings. The rings were pretreated with DETCA (0.1 mM) for 1h and this was followed by extensive washing to permit the effects of exogenous SOD to be studied. Pretreatment with DETCA led to a 30.0 ± 1.4 % (n=6) reduction in the maximal relaxation induced by SOD (300 u ml^{-1}) (Figure 4.2 and 4.11), indicating that loss of basal activity of NO had occurred as a consequence of destruction by the greater steady-state levels of superoxide anion.

4.1.2 Effects of SOD on ACh-stimulated activity of NO

Having established the effects of exogenous SOD on basal activity of NO, we sought to determine the effects of this enzyme on ACh-stimulated activity of NO. In endothelium-containing rings pretreated with SOD (50 u ml^{-1}), higher concentrations of PE (100 nM) were required to induce a similar degree of tone (1.1 ± 0.1 g, n=6) to untreated rings (1.1 ± 0.1 g, n=6) contracted with PE (10-30 nM). In contrast to potentiation of basal activity NO, SOD produced absolutely no effect on ACh (10 nM-3 μM)-induced relaxation (Figure 4.3).

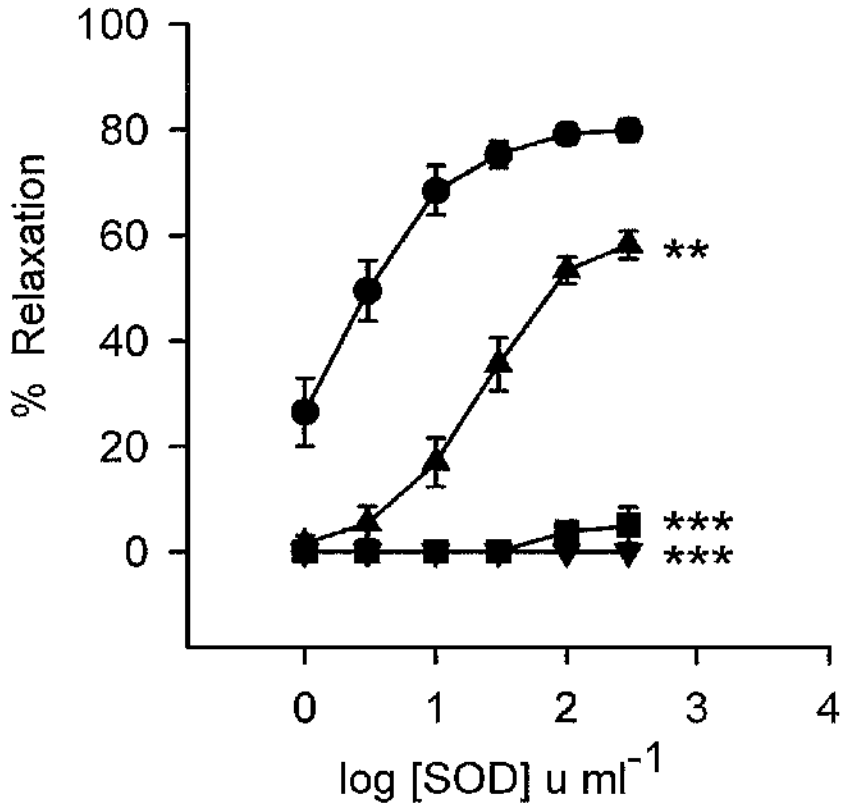


Figure 4.2 Concentration-response curves showing relaxation to superoxide dismutase (SOD) on phenylephrine-contracted endothelium-containing (●) and endothelium-denuded (■) rings of rat aorta and blockade of this relaxation following inhibition of nitric oxide synthesis with N^G-nitro-L-arginine (L-NOARG, 30 μM, ▼) and the inhibition of endogenous Cu-Zn SOD with diethyldithiocarbamate (DETCA, 0.1 mM, 1h, wash, ▲). Each point is the mean ± s.e. mean of 6 observations. **P<0.005 and ***P<0.001 indicate a significant difference from maximal relaxation in untreated endothelium-containing rings, respectively.

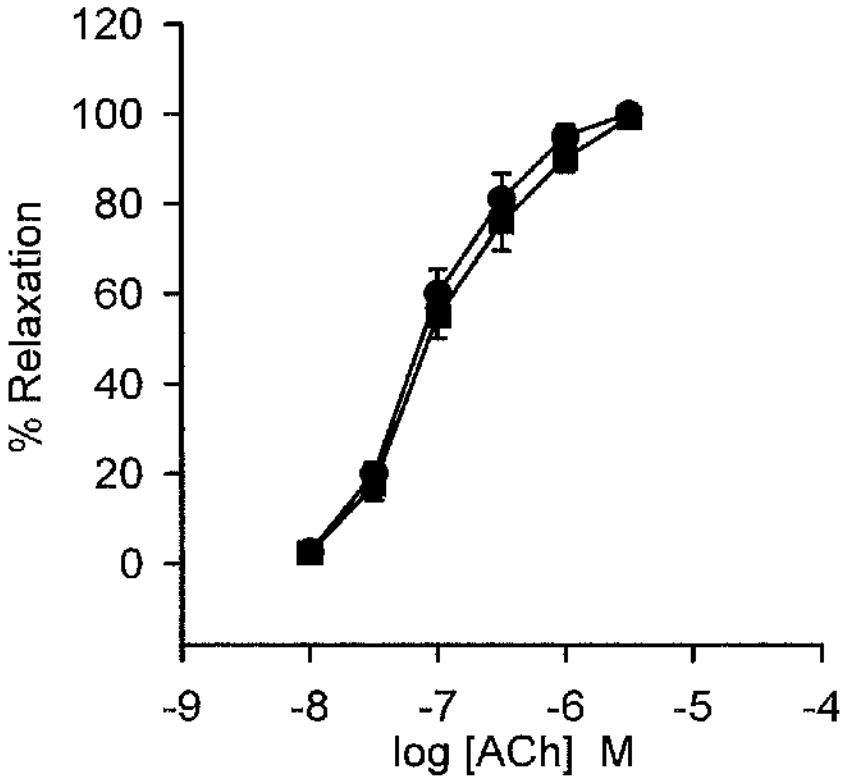


Figure 4.3 Concentration-response curves showing relaxation to acetylcholine (ACh, ●) on phenylephrine-contracted endothelium-containing rings of rat aorta and the inability of superoxide dismutase (SOD, 50 u ml^{-1}, ■) to affect this relaxation. Each point is the mean ± s.e. mean of 6 observations.

These findings suggested a selective potentiation of basal but not ACh-stimulated activity of NO by SOD. In order to gain an insight into the mechanisms that may be responsible for this difference we adopted different approach by examining the effects of direct generation of superoxide anion.

4.2 Effects of hypoxanthine/xanthine oxidase (HX/XO) on basal and ACh-stimulated activity of NO

HX/XO system is commonly used to generate superoxide anion (Fridovich, 1970; Halliwell & Gutteridge, 1989; Berman & Martin, 1993). We utilised this system to study the effects of superoxide anion on basal and agonist-stimulated activity of NO.

4.2.1 Effects of HX/XO on basal activity of NO

Following induction of tone with PE (3-30 nM) subsequent application with HX (0.1 mM)/XO (16 mu ml^{-1}) produced two effects on rings of rat aorta. The first of these effects was an immediate and profound rise in PE-induced tone (from 0.8 ± 0.1 g to 1.5 ± 0.1 g, n=6) in endothelium-containing (Figure 4.4, 4.5 and 4.6) but not endothelium-denuded rings (Figure 4.4 and 4.7).

In order to determine whether this rise in PE-induced tone had resulted from destruction of basally produced NO by superoxide anion, it was necessary to assess the actions of SOD on this enhancement of tone. In these experiments, addition of SOD (35 u ml^{-1}) produced an immediate reversal of potentiation of PE-induced tone by HX/XO (Figure 4.4). Furthermore, in endothelium-containing rings pretreated with SOD, subsequent application with HX/XO failed to enhance the tone (Figure 4.5 and 4.6).

The second of the effects of HX/XO was a delayed (about 5 min) fall in tone which was seen in both endothelium-containing and endothelium-denuded rings (Figure 4.4). It was considered likely that this relaxation was due to production of hydrogen peroxide from spontaneous dismutation of superoxide anion. A test of this hypothesis was carried out using catalase which eliminates hydrogen peroxide by converting it to water and oxygen (Halliwell, 1982; Halliwell & Gutteridge, 1989; Cheeseman & Slater, 1993). Addition of catalase (30-3000 u ml^{-1}) was seen to produce an immediate reversal of this delayed relaxant action of HX/XO in both endothelium-containing and endothelium-denuded rings (Figure 4.4). Moreover, in endothelium-containing rings pretreated with catalase (3000 u ml^{-1}), subsequent application with HX/XO produced only one effect; an immediate and sustained rise in PE-induced tone (Figure 4.4 and 4.5).

In our study we wished to examine specifically the interaction between NO and superoxide anion and, consequently, catalase at a concentration of 3000 u ml^{-1} was included in all subsequent experiments in order to prevent the accumulation of hydrogen peroxide.

4.2.1.1 Effects of L-NMMA and L-NOARG alone or in combination with HX/XO on basal activity of NO

The effects of inhibiting the basal synthesis of NO were also examined on PE-induced tone. Following induction of tone with PE (1-20 nM, 0.8 ± 0.1 g, n=6) subsequent treatment with the NO synthase inhibitors, L-NMMA (30 μM; Rees *et al.*, 1989; 1990a) or L-NOARG (30 μM; Moore *et al.*, 1990) significantly potentiated tone in endothelium-containing (maximum potentiation 2.1 ± 0.1 g and 2.2 ± 0.1 g, respectively, n=6) (Figure 4.5 and 4.6) but not in

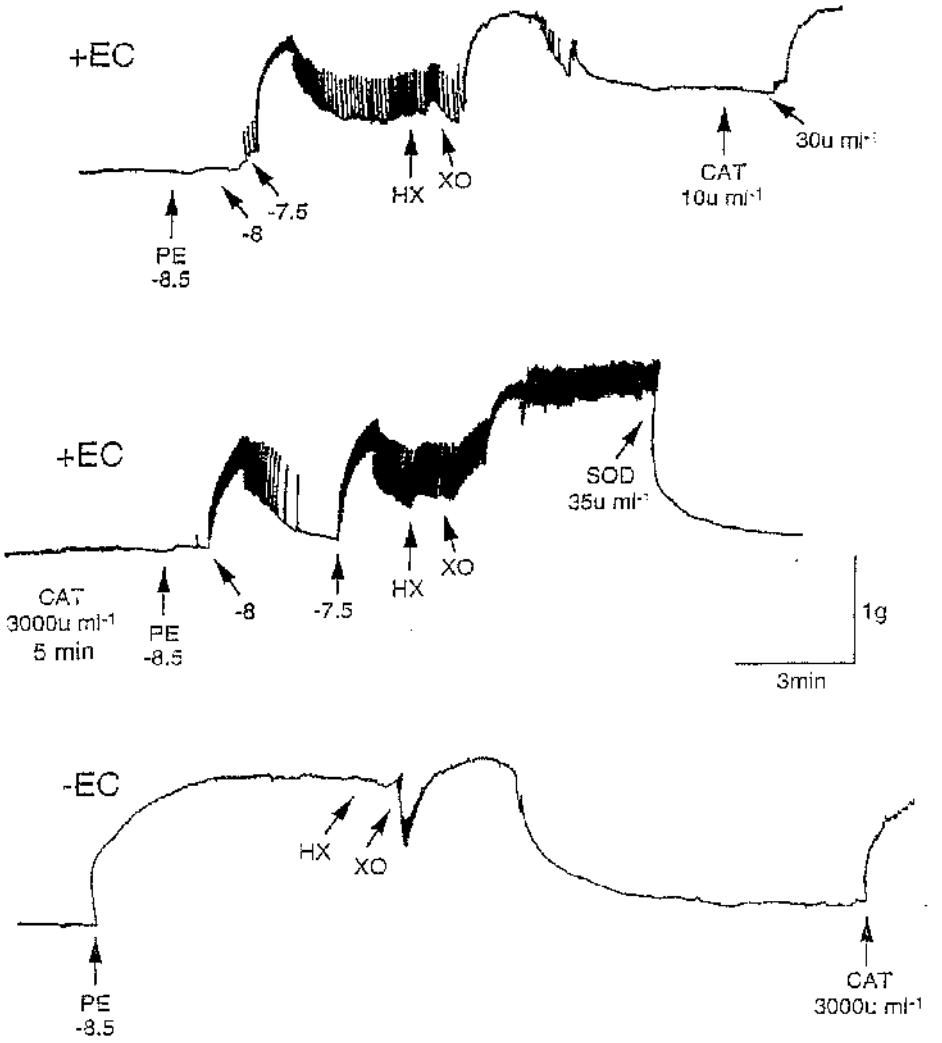


Figure 4.4 Individual experimental tracings showing the two separate effects of hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}) on rings of rat aorta; an immediate rise in phenylephrine (PE)-induced tone in endothelium-containing (+EC) but not endothelium-denuded (-EC) rings which was reversed by superoxide dismutase (SOD, 35 u ml^{-1}), and a delayed fall in tone in both endothelium-containing and endothelium-denuded rings which was inhibited by catalase (CAT, 30-3000 u ml^{-1}). Drug concentrations are expressed in log molar units and enzyme concentrations are in units ml^{-1}

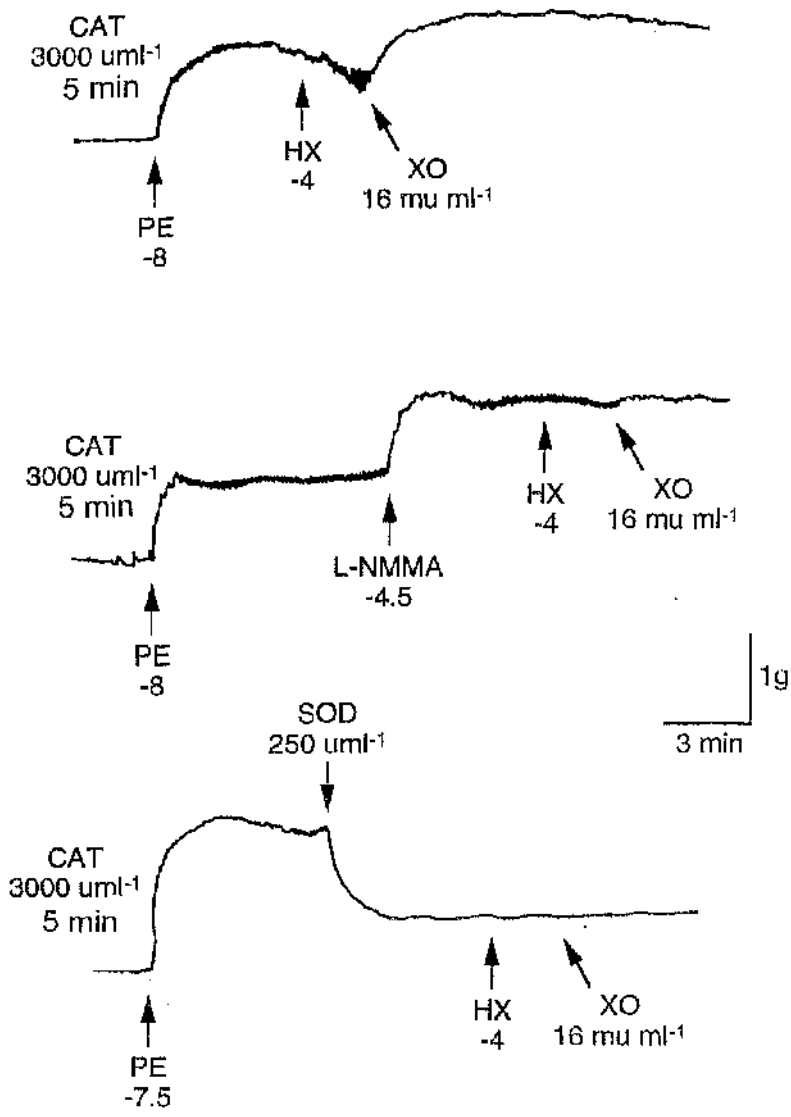


Figure 4.5 Individual experimental tracings showing the potentiation of phenylephrine (PE)-induced tone by hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}) in endothelium-containing rings of rat aorta. N^G-mono-methyl-L-arginine (L-NMMA, 30 μM) also potentiated PE-induced tone and, moreover, prevented the ability of HX/XO to enhance tone. Superoxide dismutase (SOD, 250 u ml^{-1}) relaxed PE-induced tone and prevented the potentiation induced by HX/XO. All experiments were conducted in the presence of catalase (CAT, 3000 u ml^{-1}) to prevent the relaxant effects of hydrogen peroxide. Drug concentrations are expressed in log molar units and enzyme concentrations are expressed in units ml^{-1}.

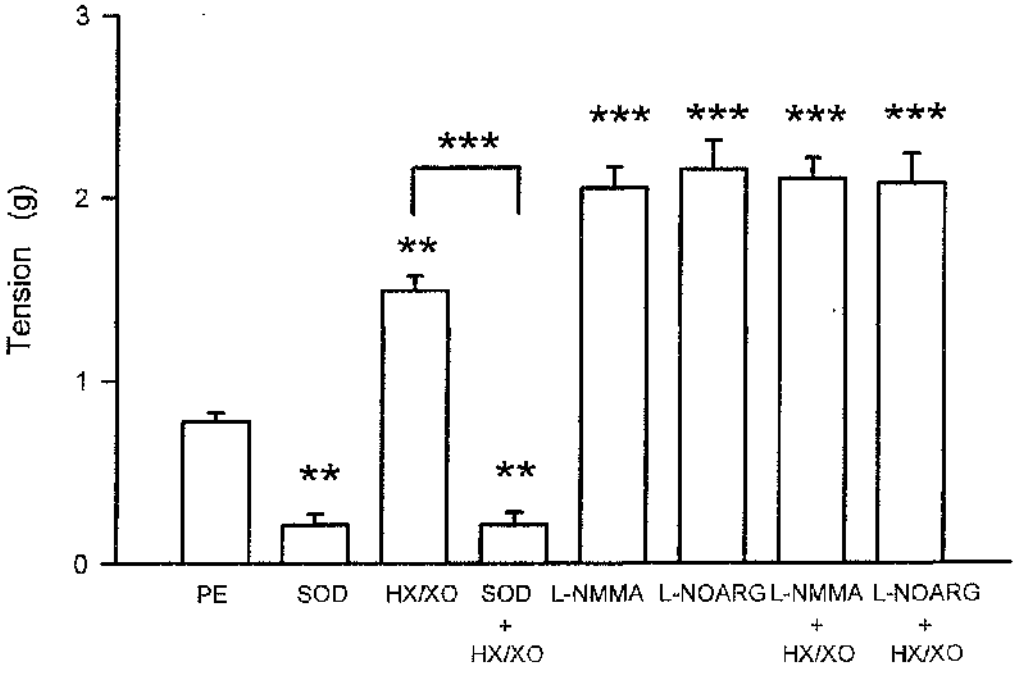


Figure 4.6 Hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}), N^G-monomethyl-L-arginine (L-NMMA, 30 μM) and N^G-nitro-L-arginine (L-NOARG, 30 μM) all potentiated phenylephrine (PE, 20 nM)-induced tone in endothelium-containing rings of rat aorta. Superoxide dismutase (SOD, 250 u ml^{-1}) relaxed PE-induced tone and prevented the potentiation induced by HX/XO. Furthermore, following treatment with L-NMMA or L-NOARG, subsequent treatment with HX/XO failed to enhance tone further. Each column is the mean ± s.e. mean of 6 observations. **P<0.005 and ***P<0.001 indicate a significant difference from rings receiving PE only, respectively, or between groups joined by a bracket.

endothelium-denuded rings (Figure 4.7). Furthermore, HX/XO failed to enhance PE-induced tone in L-NMMA- or L-NOARG-treated endothelium-containing rings (Figure 4.5 and 4.6), thus confirming that HX/XO augments tone through destruction of basal NO by superoxide anion.

4.2.2 Effects of HX/XO on ACh-stimulated activity of NO

Having established the ability of HX/XO to powerfully destroy basal activity of NO, through production of superoxide anion, its actions were investigated on ACh-stimulated activity of NO.

In the presence of HX (0.1 mM)/XO (16 mu ml^{-1}), lower concentrations of PE (10-30 nM) were required to induce a similar degree of tone (1.3 ± 0.1 g, n=11) to that of control rings (1.4 ± 0.1 g, n=11) contracted with PE (30-100 nM). HX/XO despite substantially blocking basal activity of NO, had no significant effect on relaxations produced by ACh at any concentration in endothelium-containing rings (Figure 4.8).

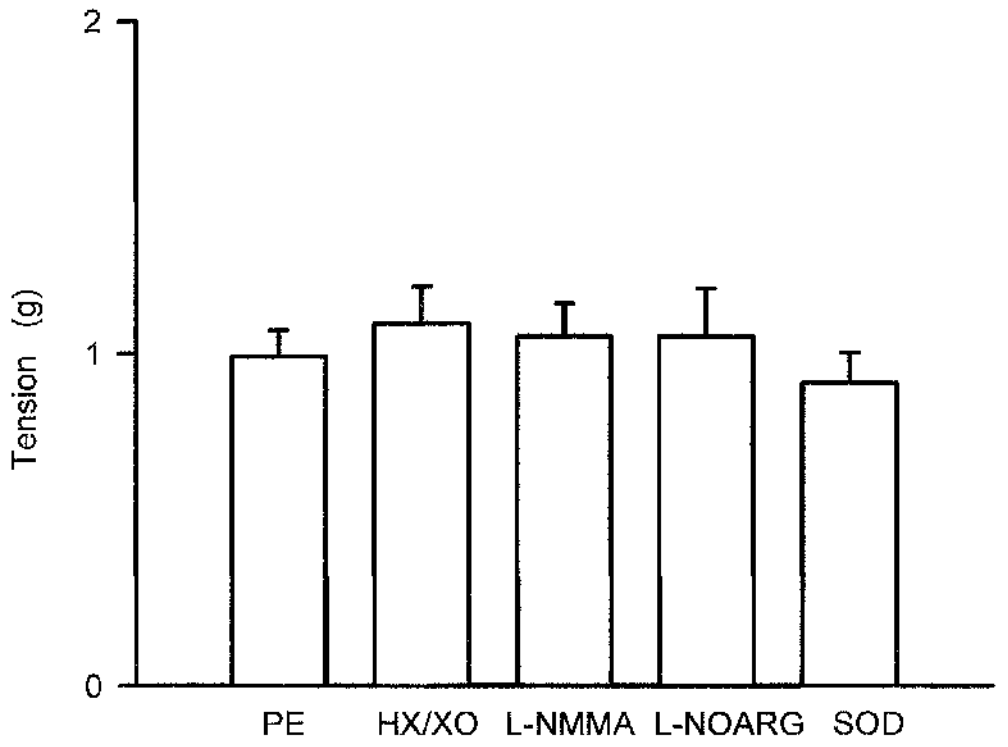


Figure 4.7 Graph showing the lack of effect of hypoxanthine (HX, 0.1 mM)/xanthine oxidase (16 mu ml^{-1}), N^G-monomethyl-L-arginine (L-NMMA, 30 µM), N^G-nitro-L-arginine (L-NOARG, 30 µM) and superoxide dismutase (SOD, 250 u ml^{-1}) on phenylephrine (PE, 1 nM)-induced tone in endothelium-denuded rings of rat aorta. Each column is the mean ± s.e. mean of 6 observations.

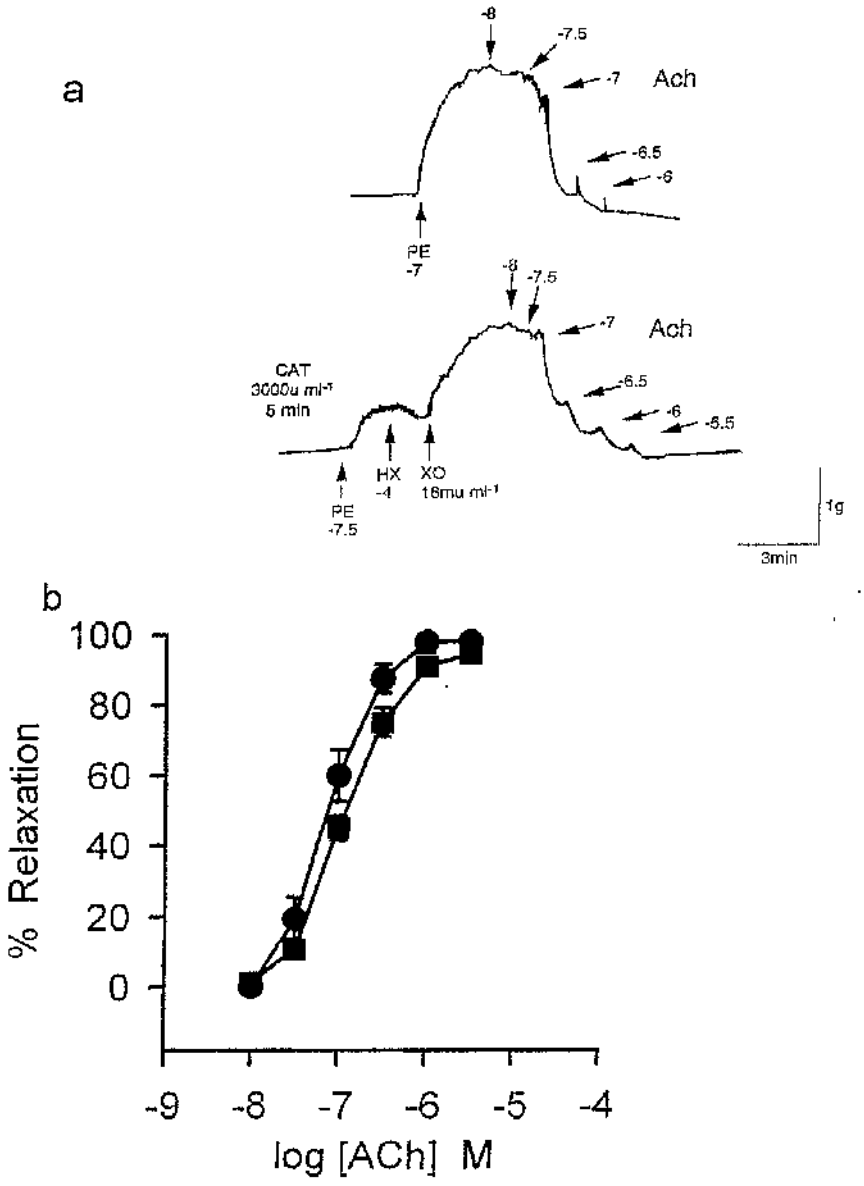


Figure 4.8 Individual experimental traces (a) and concentration-response curves (b) showing relaxation to acetylcholine (ACh, ●) on phenylephrine (PE)-contracted endothelium-containing rings of rat aorta and the inability of hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}, ■) to block this relaxation. All experiments were conducted in the presence of catalase (CAT, 3000 u ml^{-1}) to prevent the relaxant effects of hydrogen peroxide. Each point is the mean ± s.e. mean of 11 observations. Drug concentrations are expressed in log molar units and enzyme concentrations are in units ml^{-1}.

4.2.2.1 Effects of higher concentrations of HX/XO on ACh-stimulated activity of NO

The data presented so far suggest a greater sensitivity of basal than ACh-stimulated activity of NO to destruction by superoxide anion. In order to determine whether this difference was absolute, the levels of superoxide anion were increased by employing higher concentrations of HX/XO.

Increasing the concentration of HX to 1 mM which meeting XO (16 mu ml^{-1}) did, however, lead to a profound blockade of ACh-induced relaxation in PE (30-100 nM)-contracted endothelium-containing rings. The maximal relaxation induced by ACh at 3 μM was inhibited by 40.0 ± 3.5 % (n= 5-12, Figure 4.9 and 4.10). HX (1 mM)/XO (16 mu ml^{-1}) also reduced the duration of relaxation induced by ACh at 3 μM (Figure 4.9).

It was important to establish if the blockade by HX/XO occurred as a consequence of destruction of ACh-stimulated activity of NO by superoxide anion. The tissues were, therefore, pretreated with exogenous SOD (250 u ml^{-1}) for 20 min and its ability to protect against the inhibitory effects of HX (1 mM)/ XO (16 mu ml^{-1}) was assessed. SOD offered complete protection against this blockade (Figure 4.10).

These results suggested that higher concentrations of superoxide anion were required to destroy ACh-stimulated activity of NO than basal NO.

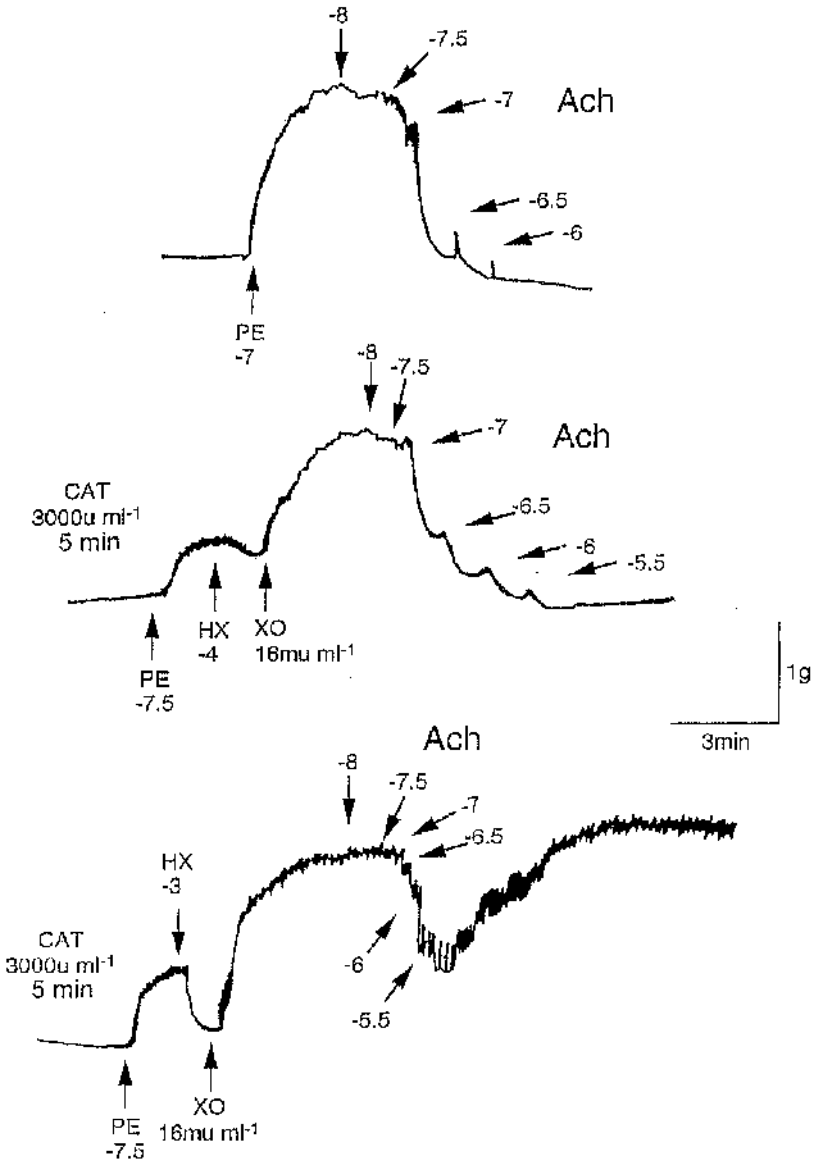


Figure 4.9 Individual experimental tracings showing the ability of hypoxanthine (HX, 0.1 mM and 1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}) to inhibit acetylcholine (ACh)-induced relaxation in phenylephrine (PE)-contracted endothelium-containing rings of rat aorta. All experiments were conducted in the presence of catalase (CAT, 3000 u ml^{-1}) to prevent the relaxant effects of hydrogen peroxide generated by HX/XO. Drug concentrations are expressed in log molar units and enzyme concentrations are in units ml^{-1}.

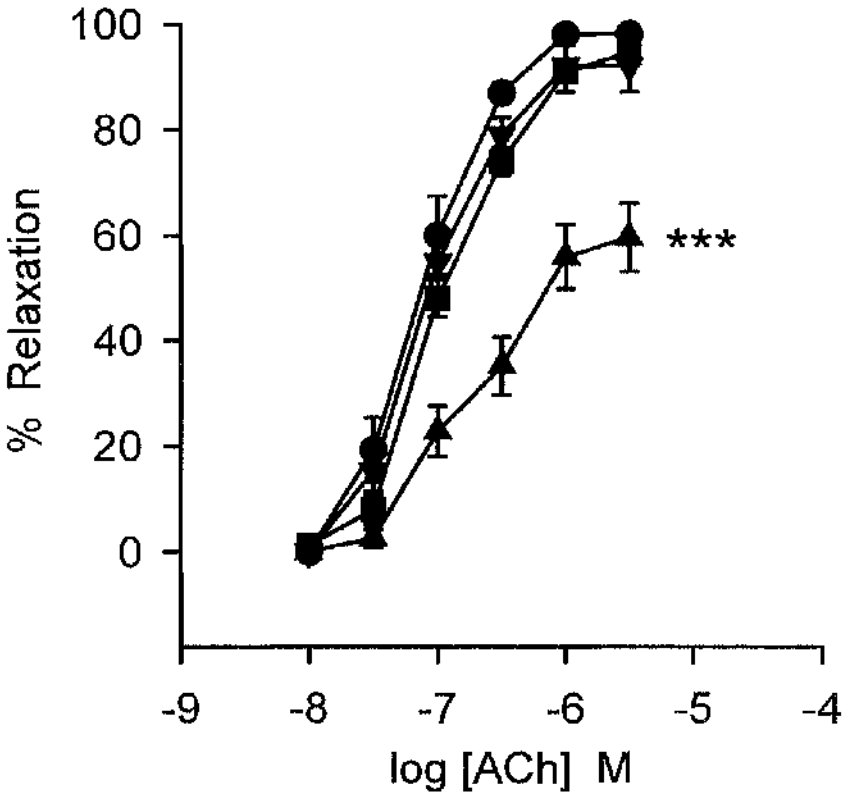


Figure 4.10 Concentration-response curves showing relaxation to acetylcholine (ACh, ●) on phenylephrine-contracted endothelium-containing rings of rat aorta, blockade of relaxation by hypoxanthine (HX, 1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}, ▲) but not by HX (0.1 mM)/XO (16 mu ml^{-1}, ■), and protection against blockade by the former by superoxide dismutase (SOD, 250 u ml^{-1}, ▼). Each point is the mean ± s.e. mean of 5-12 observations. ***P<0.001 indicates a significant difference from maximal relaxation in untreated rings.

4.3 Effects of DETCA on basal and ACh-stimulated activity of NO

It was thought that the greater sensitivity of basal than ACh-stimulated activity of NO to destruction by superoxide anion might reflect differential protection by endogenous SOD. Consequently, the effects of inhibiting the endogenous Cu-Zn SOD with copper chelator, DETCA (Heikkila *et al.*, 1976; Cocco *et al.*, 1981; Halliwell & Gutteridge, 1989), were assessed.

4.3.1 Effects of DETCA on basal activity of NO

Treatment for 1h with DETCA (0.1 mM) to inhibit endogenous Cu-Zn SOD followed by washout enhanced PE (20 nM)-induced tone (from 0.6 ± 0.1 g to 0.9 ± 0.1 g, n=6) in endothelium-containing rings (Figure 4.11 and 4.12). Furthermore, HX (0.1 mM)/XO (16 mu ml^{-1}), L-NMMA (30 μM) or the combined presence of L-NMMA and HX/XO, which would ordinarily augment PE-induced tone in endothelium-containing rings through loss of basal activity of NO, failed to do so in DETCA-treated rings (Figure 4.11). These findings suggested that DETCA had led to destruction of all basal activity of NO.

The potential ability of exogenous SOD (250 u ml^{-1}) to reverse the inhibition by DETCA of basal activity of NO was also studied. Pretreatment with SOD for 20 min depressed PE (20 nM)-induced tone by 30.0 ± 1.4 % (n=6, Figure 4.11) in DETCA-treated endothelium-containing rings. The restoration of basal activity of NO following addition of SOD to DETCA-treated tissues, albeit reduced in comparison to control tissues, suggested that the loss had occurred as a consequence of destruction by the greater steady-state levels of superoxide anion.

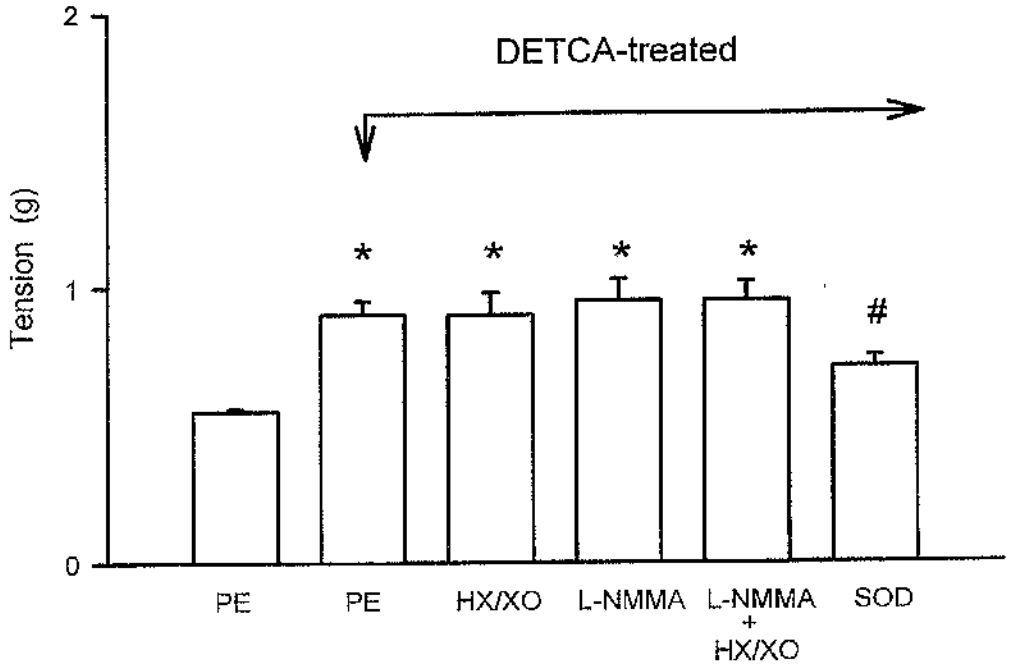


Figure 4.11 Diethyldithiocarbamate (DETCA, 0.1 mM, 1h, wash) enhanced phenylephrine (PE, 20 nM)-induced tone in endothelium-containing rings of rat aorta and prevented the ability of hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}) and N^{G}-monomethyl-L-arginine (L-NMMA, 30 μM) to potentiate tone. Superoxide dismutase (SOD, 250 u ml^{-1}) produced a partial relaxation of PE-induced tone in DETCA-treated endothelium-containing rings. Each column is the mean ± s.e. mean of 6 observations. *$P<0.05$ indicates a significant difference from rings receiving PE only. #$P<0.05$ indicates a significant difference from DETCA-treated, endothelium-containing rings receiving PE only.

The effects of pretreatment with DETCA (0.1 mM, 1h) followed by washout, were also studied on endothelium-denuded rings. In these experiments, the concentration of PE was reduced from 20 nM to 1 nM to attain the same level of tone as in experiments with endothelium-containing rings. DETCA had no effect on the sensitivity of endothelium-denuded rings to PE (Figure 4.12). These findings confirm the ability of DETCA to augment tone of endothelium-containing rings by destruction of basal activity of NO.

The effects of DETCA suggest that Cu-Zn SOD plays a vital role in protecting basal activity of NO from destruction by superoxide anion.

4.3.2 Effects of DETCA on ACh-stimulated activity of NO

The effects of inhibition of endogenous Cu-Zn SOD with DETCA (0.1-3 mM, 1h) were studied on ACh-induced relaxation. In these experiments, pretreatment with DETCA (0.1-3 mM) followed by washout led to a concentration-dependent inhibition of ACh-induced relaxation (Figure 4.13). The maximal relaxation induced by ACh at 3 μM was reduced by 23.1 ± 1.4 %, 55.4 ± 3.5 % and 70.9 ± 6.1 % by DETCA at concentrations of 0.1, 1 and 3 mM, respectively (n=6-12).

The ability of exogenous SOD to reverse the inhibitory effects of the lowest (0.1 mM) and the highest (3 mM) concentrations of DETCA on ACh-induced relaxation was also investigated. In these experiments, treatment with SOD (250 u ml^{-1}) for 20 min had no significant effect on the ability of DETCA (0.1 or 3 mM) to inhibit ACh-induced relaxation (Figure 4.13).

These findings suggest that DETCA destroys the activity of NO mainly intracellularly in endothelial cells where exogenous SOD cannot penetrate.

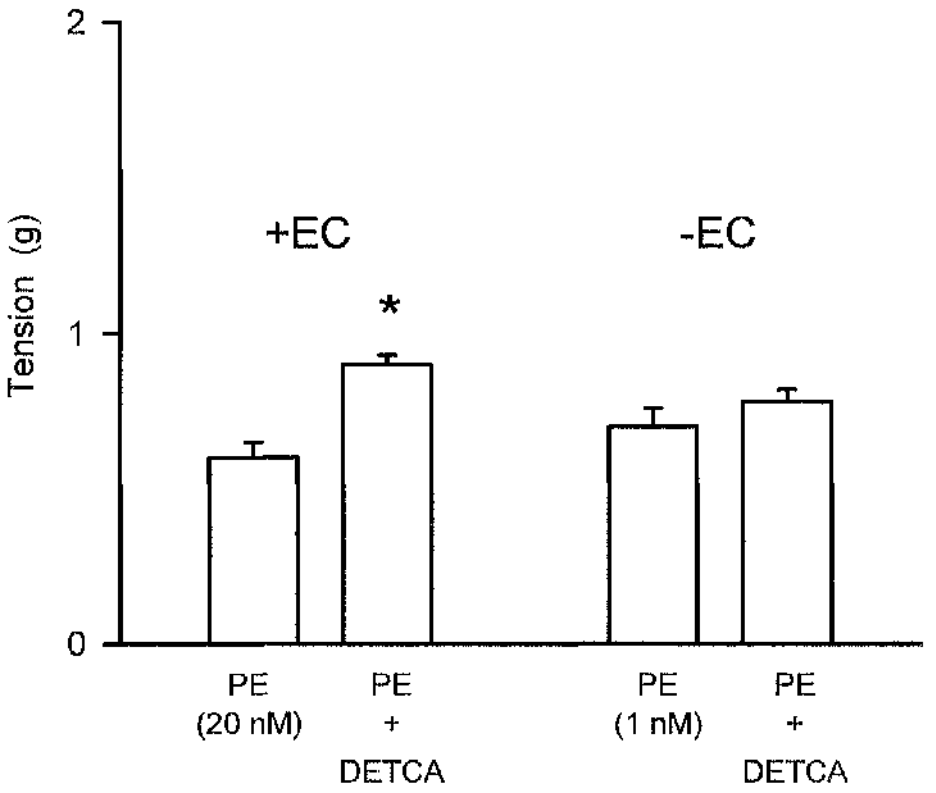


Figure 4.12 Inhibition of endogenous Cu-Zn superoxide dismutase with diethyldithiocarbamate (DETCA, 0.1 mM, 1h, wash) enhanced phenylephrine (PE, 20 nM)-induced tone in endothelium-containing (+EC) rings of rat aorta. The endothelium-denuded (-EC) rings were contracted with a lower concentration of PE (1 nM) to attain the same level of tone as in +EC, but in these, pretreatment with DETCA had no effect on tone. Each column is the mean ± s.e. mean of 6 observations. *P<0.05 indicates a significant difference from rings receiving PE only.

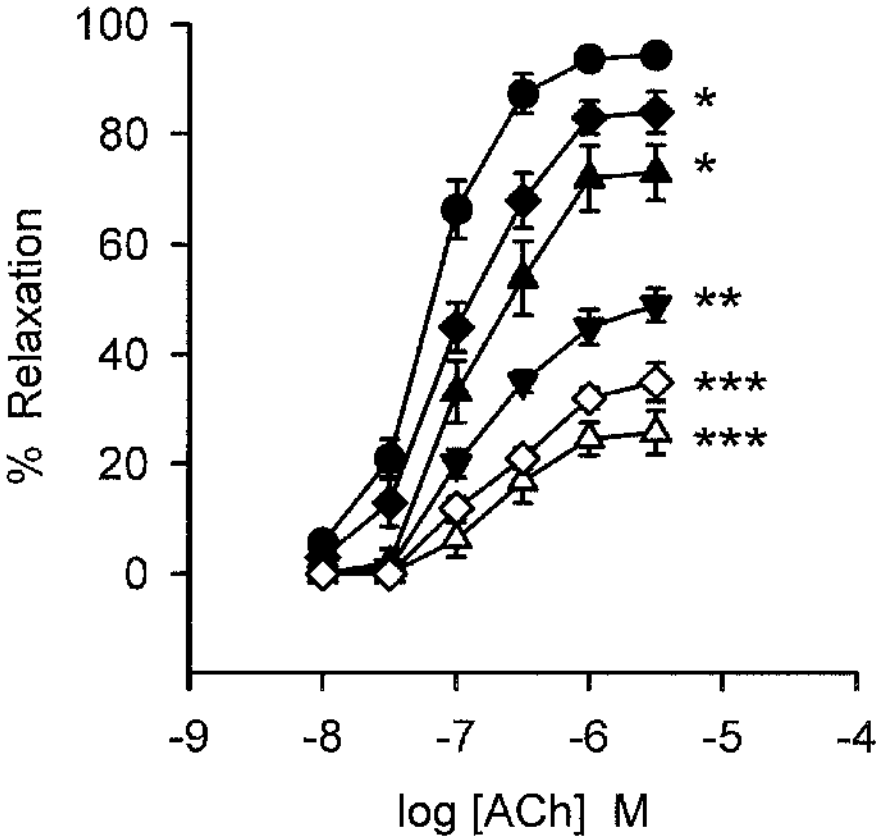


Figure 4.13 Concentration-response curves showing relaxation to acetylcholine (ACh, ●) on phenylephrine-contracted endothelium-containing rings of rat aorta. Inhibition of endogenous Cu-Zn superoxide dismutase (Cu-Zn SOD) with diethyldithiocarbamate (DETCA, 1h, wash) at 0.1 mM (▲), 1 mM (▼) and 3 mM (Δ) blocked ACh-induced relaxation in a concentration-dependent manner. Furthermore, exogenous SOD (250 u ml^{-1}, 20 min) had no significant effect on the blockade by DETCA at 0.1 mM (◆) and 3 mM (◊) of ACh-induced relaxation. Each point is the mean ± s.e. mean of 6-12 observations. *P<0.05, **P<0.005 and ***P<0.001 indicate a significant difference from maximal relaxation in untreated rings, respectively.

4.3.3 Effects of the combination of DETCA and HX/XO on ACh-stimulated activity of NO

If Cu-Zn SOD is important in protecting NO then its inactivation with DETCA will be expected to potentiate the inhibitory effects of HX (0.1 mM)/XO (16 mu ml^{-1}). Consequently, experiments were carried out to investigate the effects of DETCA on the ability of HX/XO to inhibit ACh-induced relaxation. In these experiments, HX (0.1 mM)/XO (16 mu ml^{-1}) that had no effect on ACh-induced relaxation by itself in control tissues led to a profound blockade of relaxation in DETCA (0.1 mM, 1h, wash)-treated tissues (Figure 4.14 and 4.15). The maximal relaxation induced by ACh at 3 μM was inhibited by 58.4 ± 3.5 % (n=8, Figure 4.15).

It should be noted in Figure 4.14 that in DETCA-treated tissues no rise PE-induced tone was obtained when HX/XO was added due to prior destruction of all basal activity of NO.

It was necessary to establish if the inhibition of ACh-induced relaxation seen with HX/XO in DETCA-treated tissues occurred as a consequence of elevation of superoxide anion. The effects of exogenous SOD were, therefore, examined on this inhibition. SOD (250 u ml^{-1}) given as a 20 min pretreatment almost completely protected against the blockade of ACh-induced relaxation by HX (0.1 mM)/XO (16 mu ml^{-1}) in DETCA-treated tissues (n=8, Figure 4.15).

The effects of DETCA suggest that endogenous Cu-Zn SOD plays a vital role in protecting ACh-stimulated activity of NO from destruction by superoxide anion.

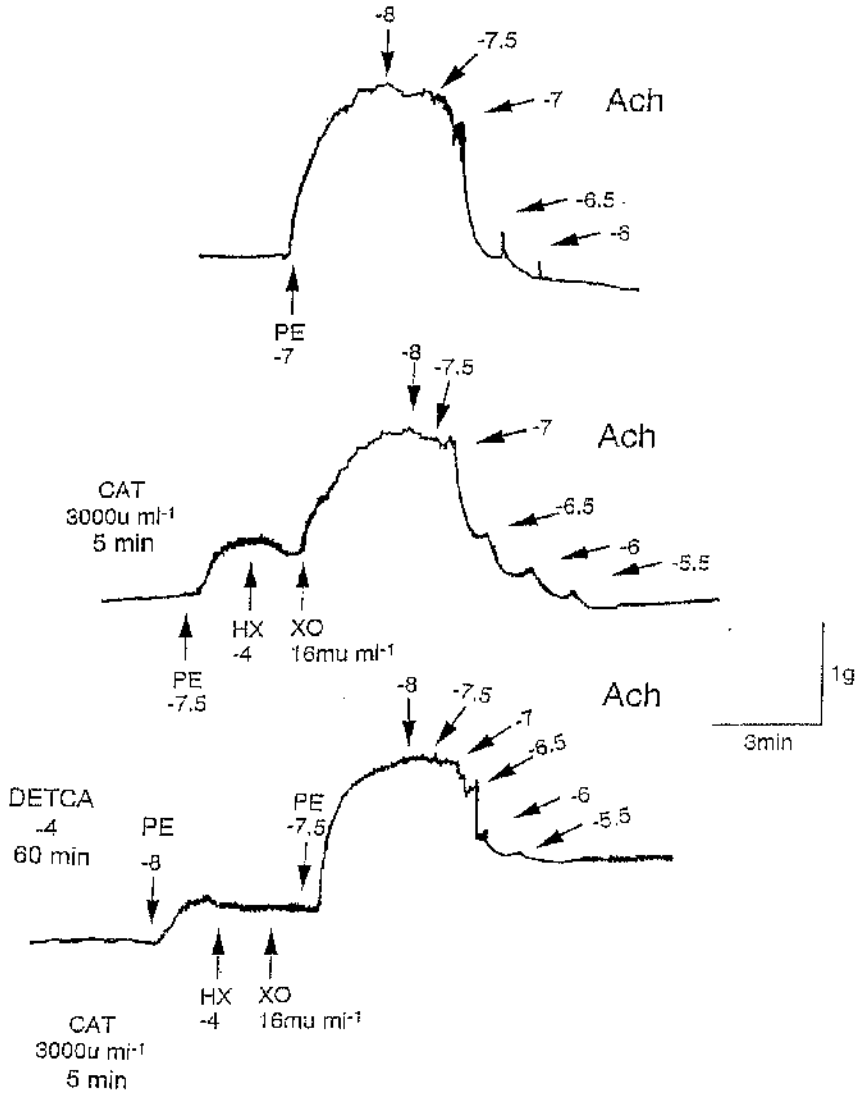


Figure 4.14 Individual experimental tracings showing the inability of hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}) to inhibit acetylcholine (ACh)-induced relaxation in phenylephrine (PE)-contracted endothelium-containing rings of rat aorta. Following treatment with diethyldithiocarbamate (DETCA, 0.1 mM, 1h, wash), subsequent treatment with HX/XO now produced a profound blockade. All experiments were conducted in the presence of catalase (CAT, 3000 u ml^{-1}) to prevent the relaxant effects of hydrogen peroxide generated by HX/XO. Drug concentrations are expressed in log molar units and enzyme concentrations are expressed in units ml^{-1}.

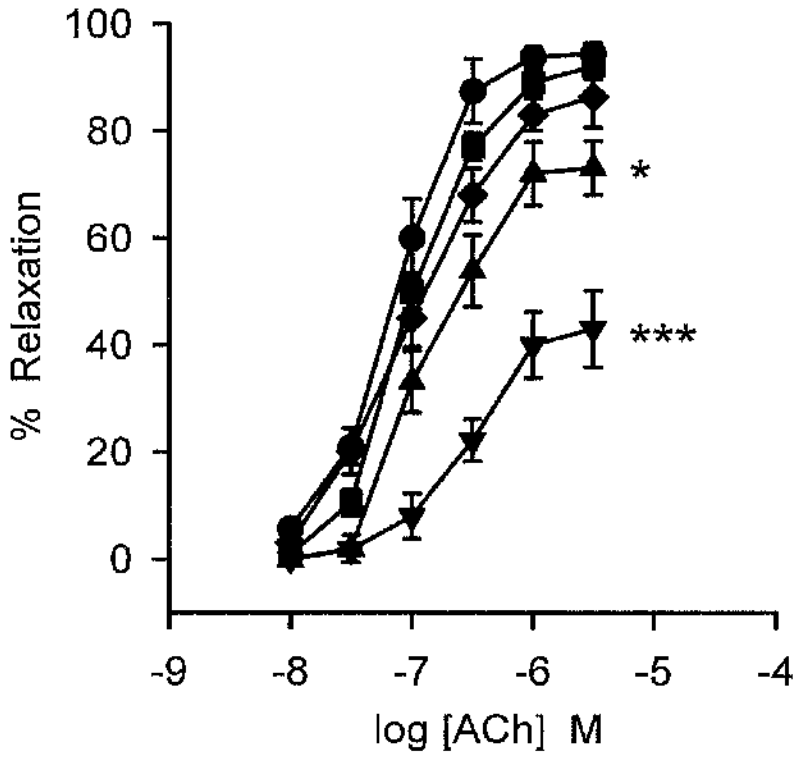


Figure 4.15 Concentration-response curves showing relaxation to acetylcholine (ACh, ●) on phenylephrine-contracted endothelium-containing rings of rat aorta, and the inability of hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}, ■) to block this relaxation. Following inhibition of endogenous Cu-Zn superoxide dismutase (Cu-Zn SOD) with diethyldithiocarbamate (DETCA, 0.1 mM, 1h, wash, ▲) ACh-induced relaxation was partially inhibited and subsequent treatment with HX/XO (▼) now produced a profound blockade. Furthermore, this blockade was prevented by pretreatment for 20 min with exogenous SOD (250 u ml^{-1}, ◆). Each point is the mean ± s.e. mean of 6-12 observations. *P<0.05 and ***P<0.001 indicate a significant difference from maximal relaxation in untreated rings, respectively.

4.4 Effects of pyrogallol on basal and ACh-stimulated activity of NO

Pyrogallol is known to generate superoxide anions by autooxidation (Halliwell & Gutteridge, 1989, see Methods). We utilised pyrogallol, as another source of superoxide anion generator, to determine if it affected basal and ACh-stimulated activity of NO in a similar way to HX/XO.

4.4.1 Effects of pyrogallol on basal activity of NO

Subsequently, the effects of pyrogallol (0.1 mM) were studied on basal activity of NO. Like HX (0.1 mM)/XO (16 mu ml^{-1}), pyrogallol (0.1 mM) produced an immediate and profound rise in PE (20 nM)-induced tone in endothelium-containing rings (Figure 4.16 and 4.17). The tone increased from 0.3 ± 0.1 g to 1.7 ± 0.1 g (n=9, Figure 4.17). However, unlike with HX/XO exogenous SOD (250 u ml^{-1}) only partially reversed (by 26.1 ± 1.1 %, n=9, Figure 4.17) the augmentation of tone induced by pyrogallol, suggesting that only part of this rise is mediated through generation of superoxide anion.

Also unlike HX/XO which augmented PE-induced tone in endothelium-containing rings only, pyrogallol (0.1 mM) augmented tone in endothelium-denuded rings. The rise in PE (1 nM)-induced tone (from 0.3 ± 0.1 to 0.9 ± 0.1 g, n=6), however, was less than that obtained in endothelium-containing rings (Figure 4.16 and 4.17). Note that the concentration of PE was lowered to 1 nM to attain the same level of tone as in endothelium-containing rings. Furthermore, the endothelium-independent component of the rise in tone induced by pyrogallol was not affected by pretreatment with exogenous SOD (250 u ml^{-1}, Figure 4.17).

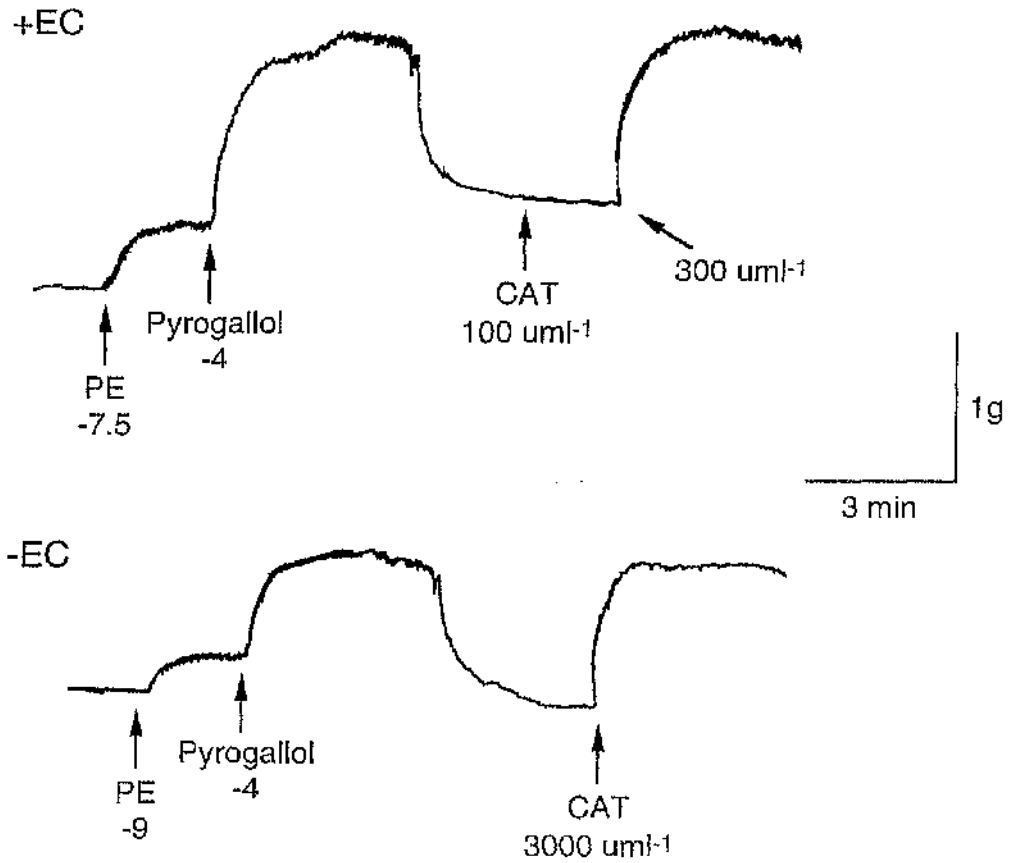


Figure 4.16 Individual experimental tracings showing the two separate effects of pyrogallol (0.1 mM) on rings of rat aorta; an immediate rise in phenylephrine (PE)-induced tone in both endothelium-containing (+EC) and endothelium-denuded (-EC) rings, although lesser than that obtained in endothelium-containing rings, and a delayed fall in tone in both endothelium-containing and endothelium-denuded rings which was inhibited by catalase (CAT, 100-3000 u ml^{-1}). Drug concentrations are expressed in log molar units and enzyme concentrations are in units ml^{-1}.

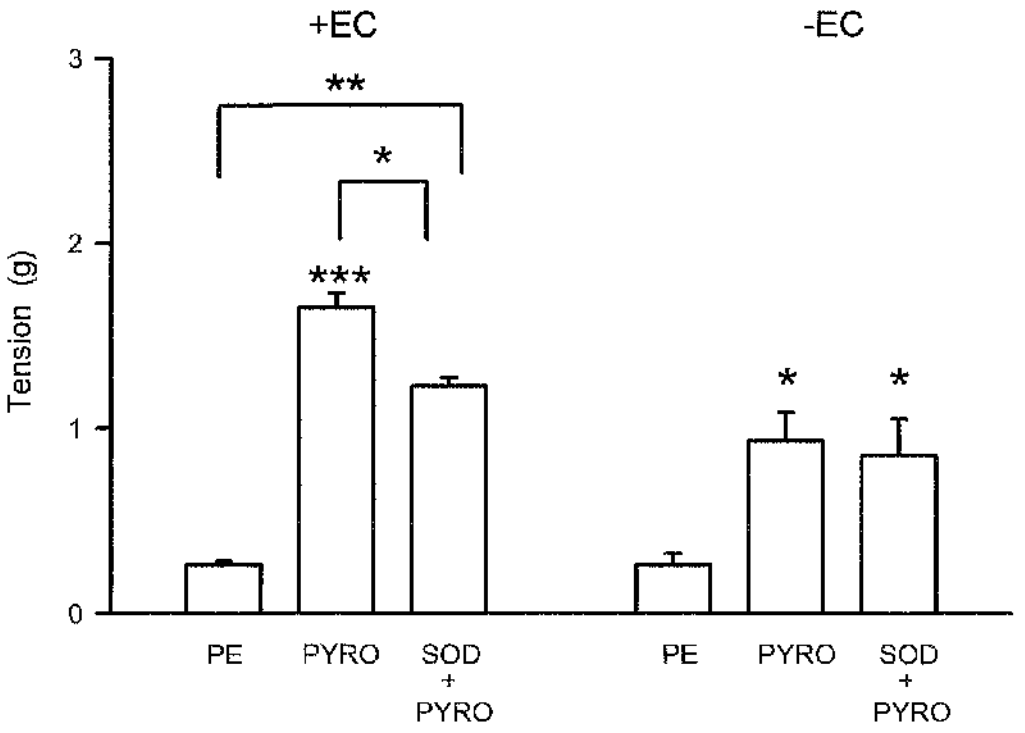


Figure 4.17 Augmentation of phenylephrine (PE)-induced tone by pyrogallol (0.1 mM) in endothelium-containing (+EC) and endothelium-denuded (-EC) rings of rat aorta and blockade of the endothelium-dependent but not endothelium-independent component by superoxide dismutase (SOD, 250 u ml^{-1}). Each column is the mean ± s.e. mean of 6-9 observations. *P<0.05, **P<0.005 and ***P<0.001 respectively indicate a significant difference from rings receiving PE only or between groups joined by brackets.

Pyrogallol, like HX/XO, also produced a delayed (about 5 min) fall in tone in both endothelium-containing and endothelium-denuded rings which was blocked by catalase (100-3000 u ml^{-1}, Figure 4.16) and was therefore likely to be due to the build up of hydrogen peroxide. Consequently, all experiments with pyrogallol were conducted in the presence of catalase (3000 u ml^{-1}).

4.4.2 Effects of pyrogallol on ACh-stimulated activity of NO

Having established the effects of pyrogallol on basal activity of NO, its actions were assessed on ACh-stimulated activity of NO. In the presence of pyrogallol (0.1 mM), lower concentrations of PE (10-30 nM) were required to induce a similar degree of tone (1.4 ± 0.1 g, n=6) to that of control rings (1.5 ± 0.1 g, n=6) contracted with PE (30-100 nM).

In common with HX (0.1 mM)/XO (16 mu ml^{-1}), pyrogallol (0.1 mM) had no significant effect on relaxations produced by ACh at any concentration in endothelium-containing rings (Figure 4.18, 4.19 and 4.20).

4.4.2.1 Effects of higher concentrations of pyrogallol on ACh-stimulated activity of NO

The effects of higher concentrations of pyrogallol on ACh-induced relaxation were also studied. In these experiments, increasing the concentration of pyrogallol to 0.3 mM did, however, lead to a profound blockade of ACh-induced relaxation in PE (30-100 nM)-contracted endothelium-containing rings. The maximal relaxation induced by ACh at 3 μM was inhibited by 38.1 ± 2.8 % (n=7, Figure 4.18 and 4.19) in the presence of pyrogallol (0.3 mM) and this concentration of pyrogallol also reduced the duration of relaxation induced by ACh at 3 μM (Figure 4.18).

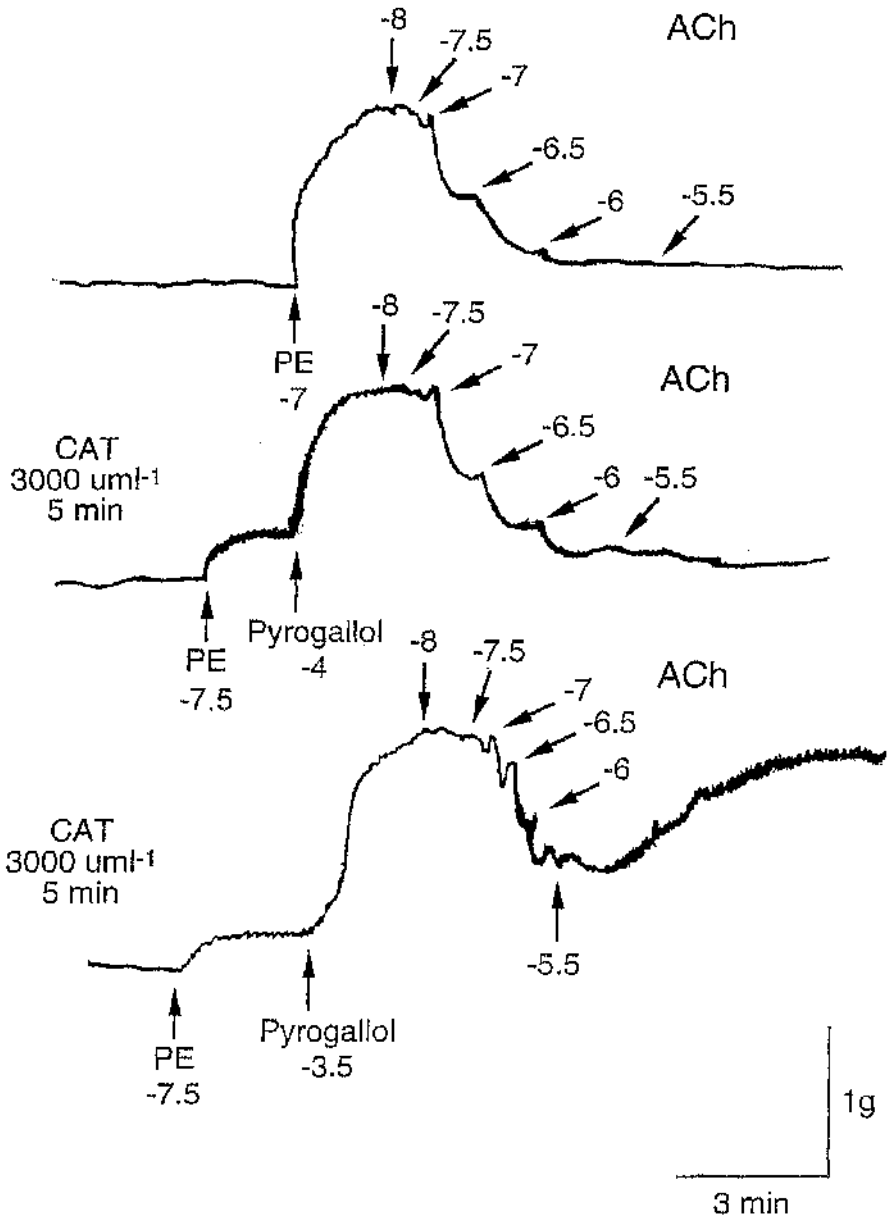


Figure 4.18 Individual experimental tracings showing the ability of pyrogallol (0.1 mM and 0.3 mM) to inhibit acetylcholine (ACh)-induced relaxation in phenylephrine (PE)-contracted endothelium-containing rings of rat aorta. All experiments were conducted in the presence of catalase (CAT, 3000 u ml^{-1}) to prevent the relaxant effects of hydrogen peroxide generated by pyrogallol. Drug concentrations are expressed in log molar units and enzyme concentrations are in units ml^{-1}.

It was important to establish if the blockade by pyrogallol occurred as a consequence of destruction of ACh-stimulated activity of NO by superoxide anion. The tissues were, therefore, pretreated with exogenous SOD (250 u ml^{-1}) for 20 min and its ability to protect against the inhibitory effects of pyrogallol (0.3 mM) was assessed. SOD offered almost complete protection against this inhibition (Figure 4.19). These results, like those obtained with HX/XO, suggested that higher concentrations of superoxide anion were required to destroy ACh-stimulated activity of NO than basal NO.

4.4.3 Effects of the combination of DETCA and pyrogallol on ACh-stimulated activity of NO

If Cu-Zn SOD is important in protecting NO then its inactivation with DETCA will be expected to potentiate the inhibitory effects of pyrogallol (0.1 mM). Consequently, experiments were carried out to investigate the effects of DETCA on the ability of pyrogallol to inhibit ACh-induced relaxation. In these experiments, pretreatment with DETCA (0.1 mM, 1h) followed by washout led to a partial inhibition of ACh-induced relaxation by itself. The maximal relaxation induced by ACh at 3 μM was reduced by 22.6 ± 1.3 % (n=6, Figure 4.20). Furthermore, the concentration of pyrogallol (0.1 mM) that had no effect on ACh-induced relaxation by itself in control tissues led to a profound blockade of this relaxation in DETCA-treated tissues (Figure 4.20). The maximal relaxation induced by ACh at 3 μM was inhibited by 75.8 ± 5.0 % (n=6-12, Figure 4.20).

It was necessary to establish if the inhibition seen with pyrogallol in DETCA-treated tissues occurred as a consequence of the action of superoxide anion. The effects of exogenous SOD were, therefore, assessed on this inhibition. SOD (250 u ml^{-1}) given as 20 min pretreatment almost completely protected

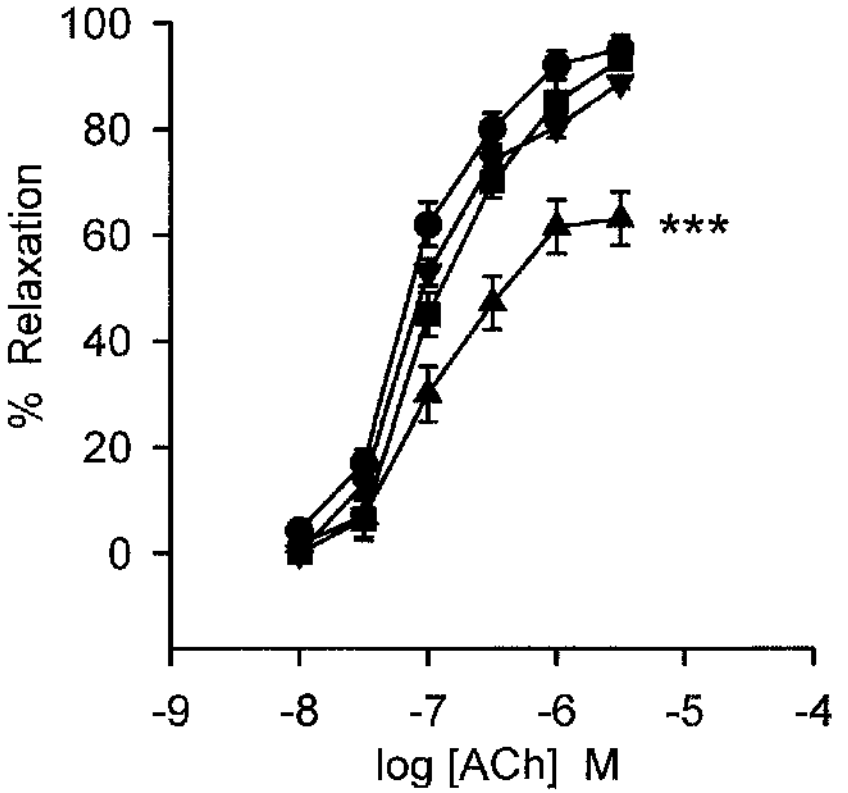


Figure 4.19 Concentration-response curves showing relaxation to acetylcholine (ACh, ●) on phenylephrine-contracted endothelium-containing rings of rat aorta, blockade of relaxation by pyrogallol at 0.3 mM (▲) but not at 0.1 mM (■), and protection against blockade by the former by superoxide dismutase (250 u ml^{-1}, ▼). Each point is the mean ± s.e. mean of 6-12 observations. ***P<0.001 indicates a significant difference from maximal relaxation in untreated rings.

against the blockade of ACh-induced relaxation by pyrogallol (0.3 mM) in DETCA-treated tissues (Figure 4.20). Thus, these results taken together with those obtained with HX/XO in DETCA-treated tissues demonstrate the importance of endogenous Cu-Zn SOD in protecting ACh-stimulated activity of NO from destruction by superoxide anion.

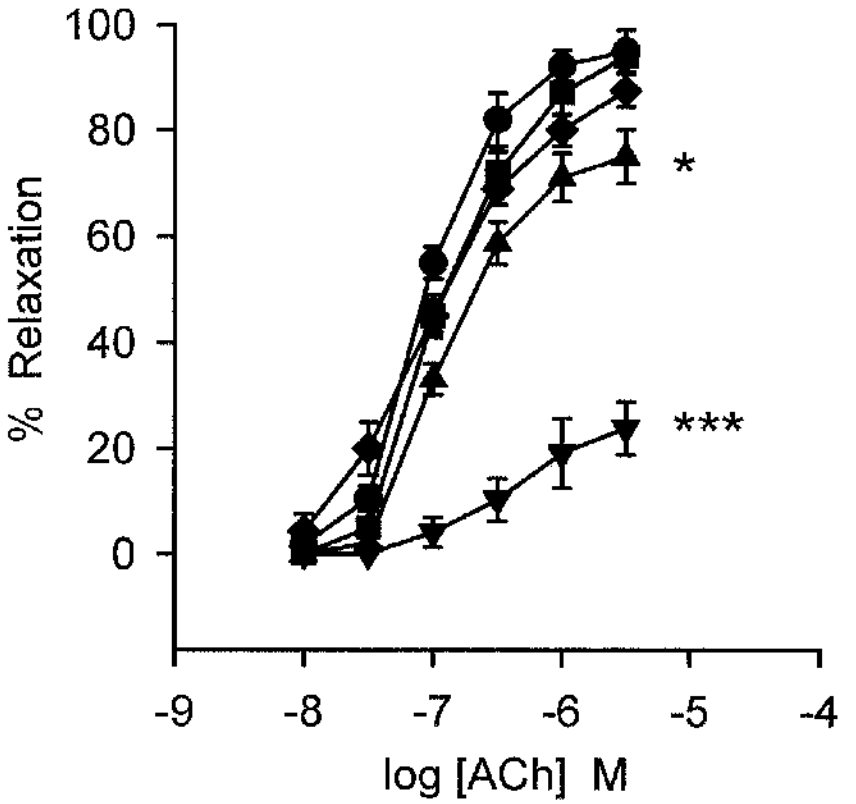


Figure 4.20 Concentration-response curves showing relaxation to acetylcholine (ACh, ●) on phenylephrine-contracted endothelium-containing rings of rat aorta, and the inability of pyrogallol (0.1 mM, ■) to block this relaxation. Following inhibition of endogenous Cu-Zn superoxide dismutase (Cu-Zn SOD) with diethyldithiocarbamate (DETCA, 0.1 mM, 1h, wash, ▲) ACh-induced relaxation was partially inhibited and subsequent treatment with pyrogallol (▼) now produced a profound blockade. Furthermore, this blockade was prevented by pretreatment for 20 min with exogenous SOD (250 u ml^{-1}, ♦). Each point is the mean ± s.e. mean of 6-12 observations. *P<0.05 and ***P<0.001 indicate a significant difference from maximal relaxation in untreated rings, respectively.

Chapter 5

Assessment of the role of catalase in relaxations induced by hydrogen peroxide and nitrovasodilators

In the previous chapter (Chapter 4), the delayed relaxant effect of superoxide anion generators in rat aorta was discussed and it was established that this effect was mediated by hydrogen peroxide since it was blocked by exogenously added catalase.

From the literature it is clear that hydrogen peroxide-induced relaxation has two components; low concentrations produced relaxation which is endothelium-dependent and higher concentrations act in an endothelium-independent manner (Burke & Wolin, 1987; Furchgott, 1991; Zembowicz *et al.*, 1993). The endothelium-dependent component is thought to be mediated via enhanced synthesis of NO, whilst the endothelium-independent component is due to a direct stimulatory effect on smooth muscle soluble guanylate cyclase. It has been proposed that the presence of catalase is vital for the latter effect to occur and that the active species which stimulates soluble guanylate cyclase is compound I, i.e. the catalase-hydrogen peroxide complex formed as an intermediate (Burke & Wolin, 1987; Wolin & Burke, 1987).

Certain nitrovasodilators, i.e. hydroxylamine and sodium azide are believed to be metabolised by catalase to NO (Theorell & Ehrenberg, 1952; Keilin & Hartree, 1954; Nicholls, 1964) and thereby activate soluble guanylate cyclase (Miki *et al.*, 1976; Katsuki *et al.*, 1977; Craven *et al.*, 1979) in cell free systems. This metabolism by catalase is therefore believed to account for the

relaxant actions of these two agents (Katsuki *et al.*, 1977; Waldman & Murad, 1987; Murad, 1994), but this has not been tested directly in an intact blood vessel. Other nitrovasodilators such as glyceryl trinitrate are also converted to NO but in this case conversion is achieved by glutathione-S-transferase (Armstrong *et al.*, 1980), and catalase is not involved.

In this study we made a use of an inhibitor of catalase, 3-amino-1,2,4-triazole (AT, Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958), in rat aorta to test the hypothesis that metabolism by catalase is necessary to express the relaxant activity of hydrogen peroxide, sodium azide and hydroxylamine.

5.1 Hydrogen peroxide-induced relaxations in endothelium-containing and endothelium-denuded rings

It was necessary to establish the nature of hydrogen peroxide-induced relaxation prior to addressing the more complex topic of the role of catalase.

5.1.1 Hydrogen peroxide-induced relaxation in endothelium-containing rings

Following induction of PE (100 nM)-induced tone (1.6 ± 0.1 g, n=7) in endothelium-containing rings of rat aorta, hydrogen peroxide (100 nM-1 mM) produced powerful concentration-dependent relaxation. The maximum relaxation induced by hydrogen peroxide at 1 mM was 96.2 ± 1.0 % (n=7, Figure 5.1 and 5.2). Addition of hydrogen peroxide elicited a biphasic response: a brief contraction which was more pronounced for concentrations ≥ 0.1 mM followed by a powerful relaxation (Figure 5.1).

5.1.2 Hydrogen peroxide-induced relaxation in endothelium-denuded rings

Hydrogen peroxide also relaxed endothelium-denuded rings. In endothelium-denuded rings, a lower concentration of PE (10 nM) was required to induce a similar degree of tone (1.5 ± 0.1 g, n=7) as in endothelium-containing rings (1.6 ± 0.1 g, n=7), but in these higher concentrations of hydrogen peroxide (30 μM-1 mM) were required to produce relaxation. The maximal relaxation induced by hydrogen peroxide in endothelium-denuded rings at 1 mM was 96.0 ± 1.6 % (n=7, Figure 5.1 and 5.2). As seen in endothelium-containing rings, addition of hydrogen peroxide at concentrations ≥ 0.1 mM produced a biphasic response i.e. a brief contraction followed by a powerful relaxation (Figure 5.1)

5.2 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) on hydrogen peroxide-induced relaxation

5.2.1 Effects of L-NAME on hydrogen peroxide-induced relaxation in endothelium-containing rings

The effects of the NO synthase inhibitor, L-NAME (Rees *et al.*, 1990a), were investigated in order to determine if NO, derived from L-arginine/NO pathway, mediated the endothelium-dependent component of hydrogen peroxide-induced relaxation. Endothelium-containing rings were pretreated with L-NAME (30 μM) for 20 min. Under these conditions, lower concentrations of PE (10-30 nM) were required to induce a similar degree of tone (1.5 ± 0.1 g, n=7) to that of control rings (1.6 ± 0.1 g, n=7) contracted with PE (100 nM). This was necessary since L-NAME potentiated PE-induced tone by blocking the

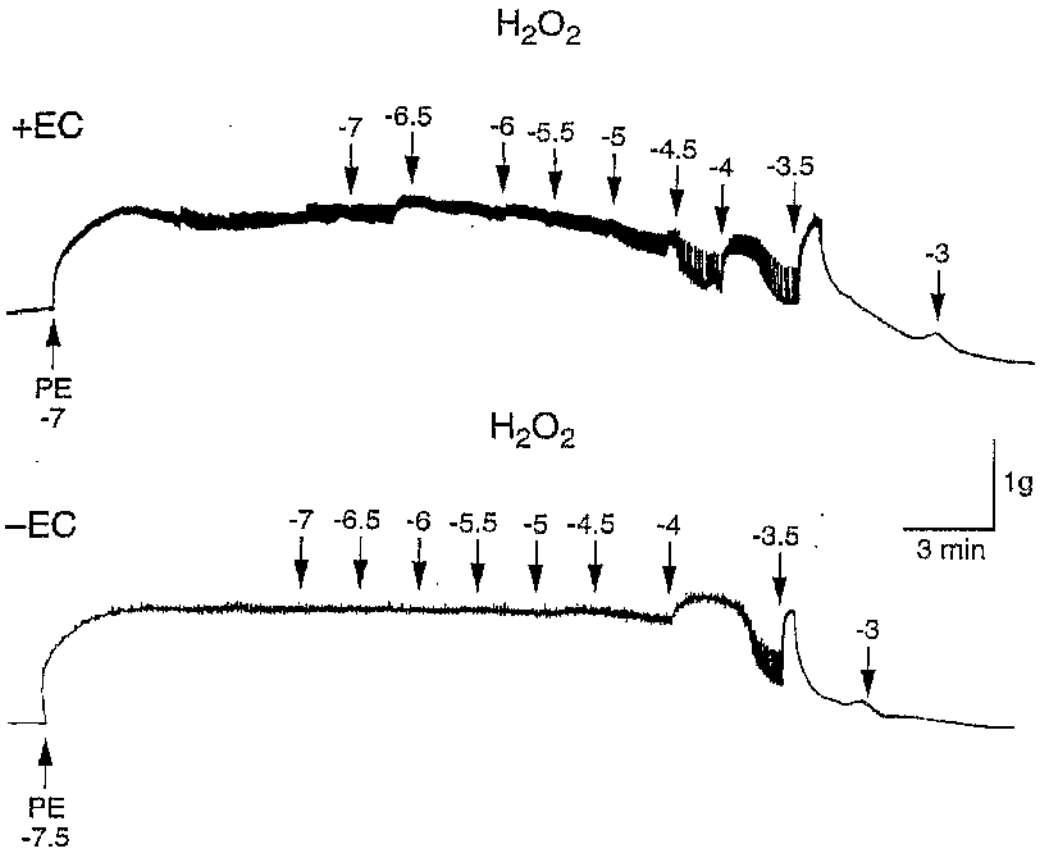


Figure 5.1 Individual experimental tracings showing the ability of hydrogen peroxide (H_2O_2) to produce a concentration-dependent relaxation of phenylephrine (PE)-contracted endothelium-containing (+EC) and endothelium-denuded (-EC) rings of rat aorta. Drug concentrations are expressed in log molar units.

synthesis of basal NO. In the presence of L-NAME hydrogen peroxide-induced relaxation at concentrations of 100 nM-30 μM was abolished, but no effect was seen on relaxation induced at higher concentrations (≥ 0.1 mM) (Figure 5.2 and 5.3). In addition, L-NAME had no effect on the contractile response observed at concentrations ≥ 0.1 mM of hydrogen peroxide (Figure 5.3). These findings suggested that both the contractile and the relaxant actions of hydrogen peroxide at concentrations ≥ 0.1 mM were not mediated by NO.

Furthermore, the inhibitory action of L-NAME on relaxation induced by hydrogen peroxide at lower concentrations (100 nM-30 μM) was prevented in the presence of the substrate for NO synthesis, L-arginine (2 mM, Figure 5.3). These results supported the conclusion of earlier studies that endothelium-dependent component is mediated via enhanced production of NO (Furchgott, 1991; Zembowicz *et al.*, 1993).

5.2.2 Effects of L-NAME on hydrogen peroxide-induced relaxation in endothelium-denuded rings

In contrast to the effects in endothelium-containing rings, L-NAME (30 μM) had no effect on hydrogen peroxide (0.1-1 mM)-induced relaxation in endothelium-denuded rings (Figure 5.2). In endothelium-denuded rings the concentration of PE was lowered to 10 nM to induce a similar degree of tone (1.5 ± 0.1 g, n=7) to endothelium-containing rings (1.6 ± 0.1 g, n=7).

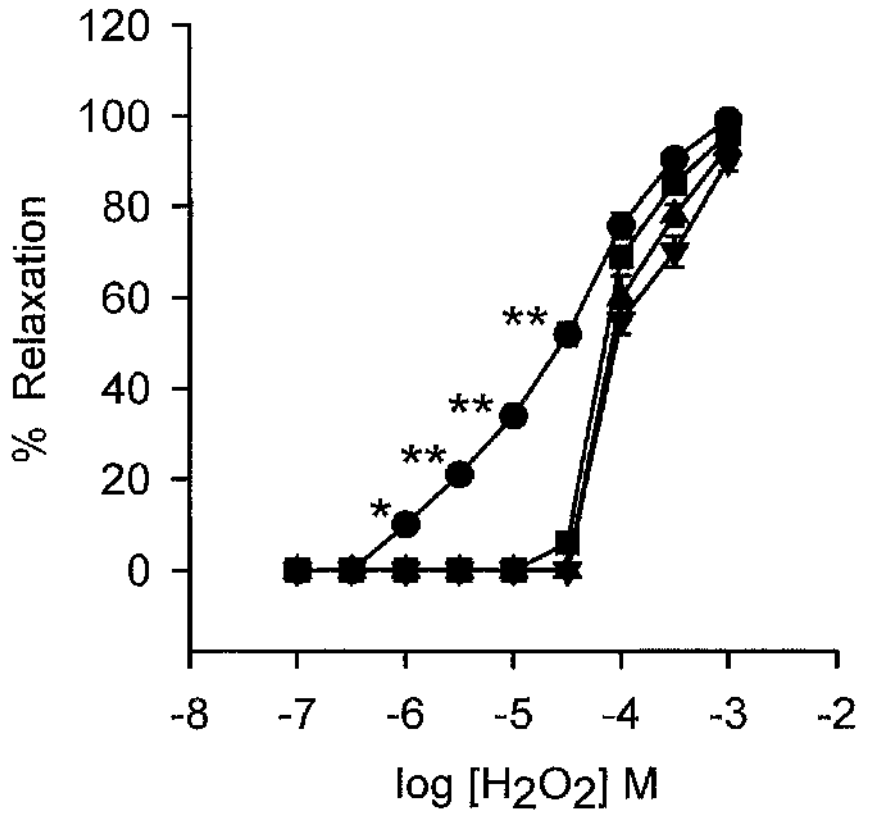


Figure 5.2 Concentration-response curves showing relaxation to hydrogen peroxide (H_2O_2) on phenylephrine-contracted rings of rat aorta: endothelium-containing rings in the absence (●) and presence of N^G-nitro-L-arginine methyl ester (L-NAME, 30 μM, ▲) and endothelium-denuded rings in the absence (■) and presence of L-NAME (30 μM, ▼). Note that L-NAME blocked the endothelium-dependent component of relaxation to hydrogen peroxide but had no effect on the endothelium-independent component. Each point is the mean ± s.e. mean of 7 observations. *P<0.05 and **P<0.005 indicate a significant difference from relaxations obtained in untreated endothelium-denuded rings, respectively.

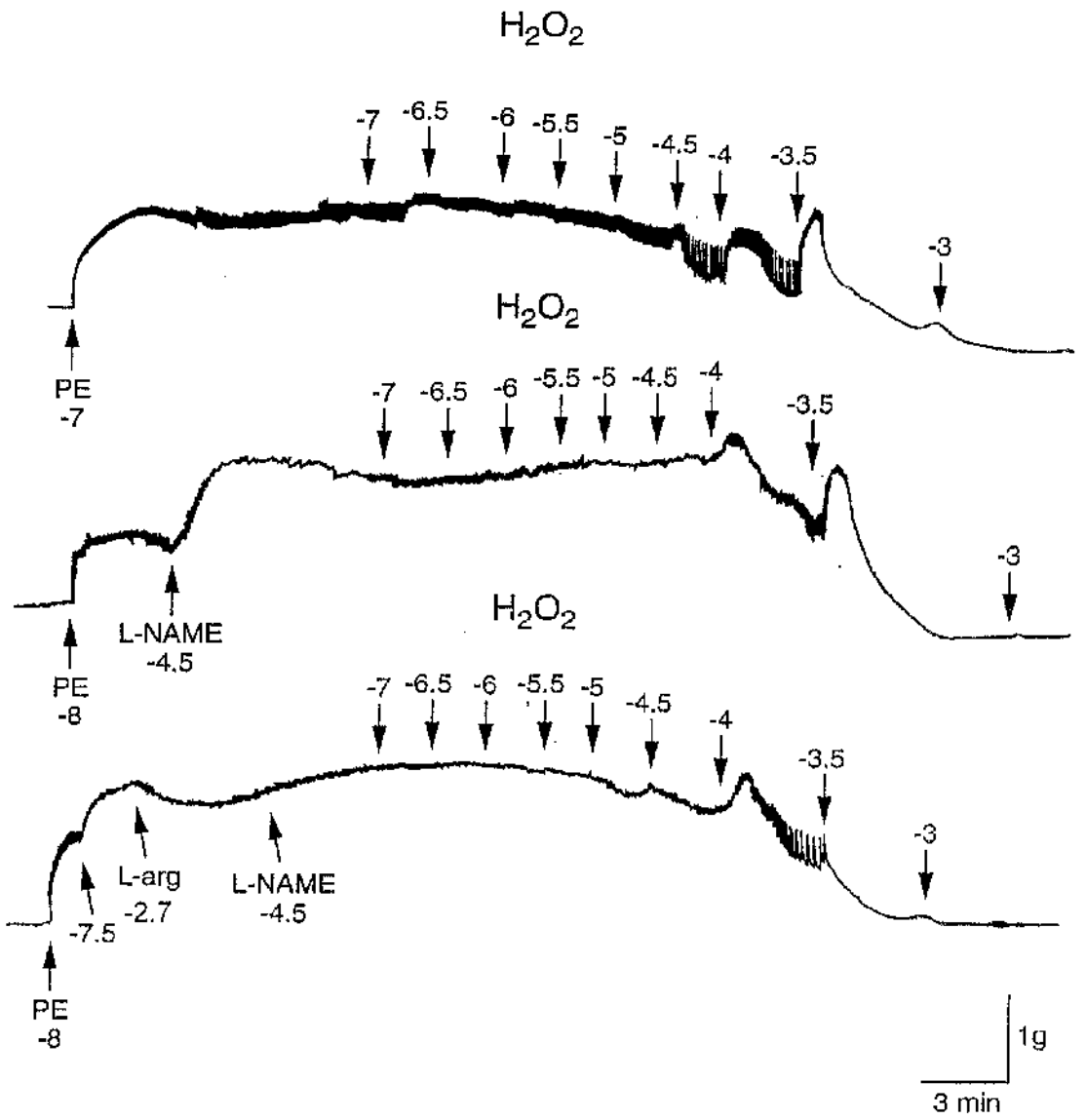


Figure 5.3 Individual experimental tracings showing the relaxation to hydrogen peroxide (H_2O_2) on phenylephrine (PE)-contracted endothelium-containing rings of rat aorta and blockade of the endothelium-dependent component of the relaxation following treatment with N^G-nitro-L-arginine-methyl ester (L-NAME, 30 μM). Furthermore, pretreatment with L-arginine (L-arg, 2 mM) protected against the inhibitory action of L-NAME. Drug concentrations are expressed in log molar units.

5.3 Effects of methylene blue (MeB) on hydrogen peroxide-induced relaxation

In order to investigate if both components of hydrogen peroxide-induced relaxation were mediated via activation of soluble guanylate cyclase, the effects of MeB, an inhibitor of soluble guanylate cyclase (Gruetter *et al.*, 1979; Holzmann, 1982; Martin *et al.*, 1985a), were studied. Both endothelium-containing and endothelium-denuded rings were treated with MeB (30 μM) for 20 min. Under these conditions, in endothelium-containing rings, lower concentrations of PE (3-10 nM) were required to induce a similar degree of tone (1.3 ± 0.1 g, n=7) to that of controls rings (1.5 ± 0.1 g, n=7) contracted with PE (100 nM). This was necessary since MeB potentiated PE-induced tone by blocking the actions of basal NO.

In endothelium-denuded rings the concentration of PE was lowered to 10 nM to induce a similar degree of tone (1.4 ± 0.1 g, n=7) to endothelium-containing rings (1.5 ± 0.1 g, n=7). MeB (30 μM) significantly reduced the relaxation induced by hydrogen peroxide (100 nM-1 mM) at all concentrations. The maximal relaxation induced by hydrogen peroxide at 1 mM in endothelium-containing and endothelium-denuded rings was inhibited by 42.2 ± 4.0 % and 46.3 ± 2.6 %, respectively, (n=7 for both, Figure 5.4). Thus, both the endothelium-dependent and endothelium-independent components of relaxation induced by hydrogen peroxide appear to depend upon the stimulation of soluble guanylate cyclase.

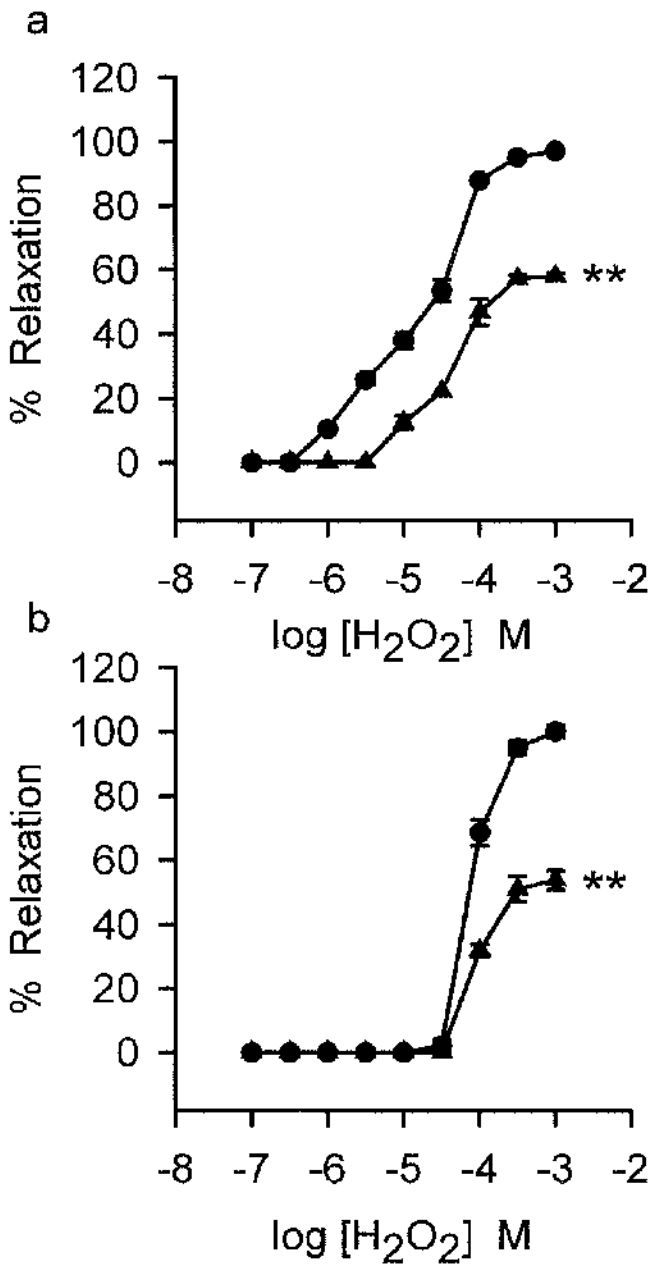


Figure 5.4 Concentration-response curves showing relaxation to hydrogen peroxide (H_2O_2, ●) on phenylephrine-contracted endothelium-containing (a) and endothelium-denuded (b) rings of rat aorta. Pretreatment with methylene blue (30 μM, ▲) inhibited this relaxation. Each point is the mean ± s.e. mean of 7 observations. **P<0.005 indicates a significant difference from maximal relaxation in untreated rings.

5.4 Effects of catalase on hydrogen peroxide-induced relaxation

Pretreatment with catalase (1000 u ml^{-1}), a scavenger of hydrogen peroxide (Halliwell & Gutteridge, 1989), for 20 min powerfully inhibited the relaxation induced by hydrogen peroxide (100 nM-1 mM) at all concentrations. The maximal relaxation induced by hydrogen peroxide at 1 mM in endothelium-containing and endothelium-denuded rings was inhibited by 56.7 ± 6.0 % and 53.5 ± 2.5 %, respectively, (n=6 for both, Figure 5.5). Increasing the concentration of catalase to 3000 u ml^{-1} almost completely abolished the relaxant action of hydrogen peroxide in both endothelium-containing and endothelium-denuded rings (n=6, Figure 5.5) .

5.5 Effects of superoxide dismutase on hydrogen peroxide-induced relaxation

In contrast to the actions of catalase, pretreatment with superoxide dismutase (SOD, 50 u ml^{-1}, 20 min), a scavenger of superoxide anion (Halliwell & Gutteridge, 1989), did not affect the relaxant activity of hydrogen peroxide (100 nM-1 mM) in either endothelium-containing or endothelium-denuded rings (n=6, Figure 5.6).

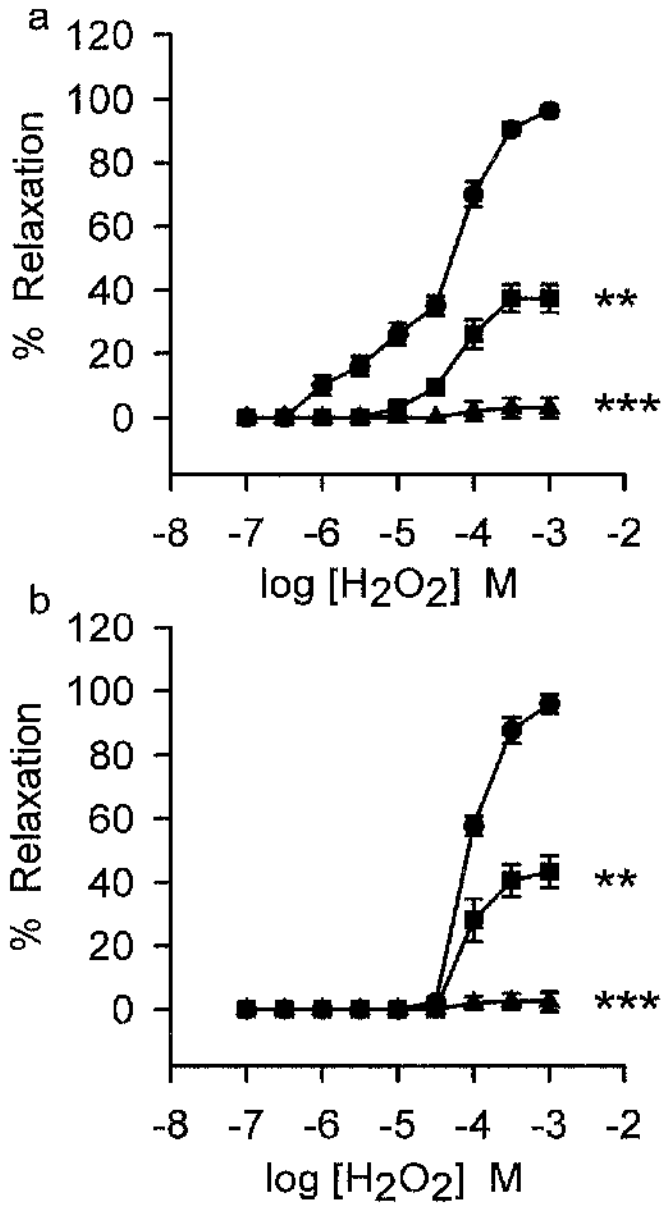


Figure 5.5 Concentration-response curves showing relaxation to hydrogen peroxide (H_2O_2, ●) on phenylephrine-contracted endothelium-containing (a) and endothelium-denuded (b) rings of rat aorta. Pretreatment with catalase at 1000 u ml^{-1} (■) or 3000 u ml^{-1} (▲) inhibited this relaxation. Each point is the mean ± s.e. mean of 6 observations. **P<0.005 and ***P<0.001 indicate a significant difference from maximal relaxation in untreated rings, respectively.

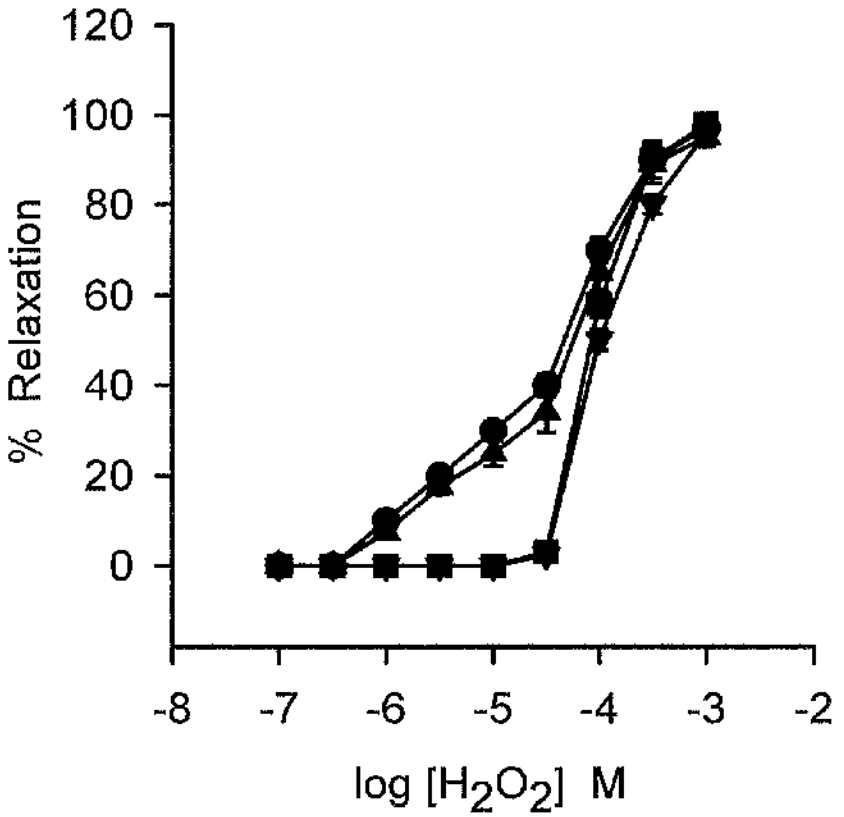


Figure 5.6 Concentration-response curves showing relaxation to hydrogen peroxide (H_2O_2) on phenylephrine-contracted endothelium-containing rings in the absence (●) and presence of superoxide dismutase (SOD, 50 u ml^{-1}, ▲) and endothelium-denuded rings in the absence (■) and presence of SOD (50 u ml^{-1}, ▼). Note that SOD had no effect on relaxation. Each point is the mean ± s.e. mean of 6 observations.

5.6 Relaxant actions of 3-amino-1,2,4-triazole

The effects of the inhibitor of catalase, 3-amino-1,2,4-triazole (AT, Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958), were studied in endothelium-denuded rings. AT (1-100 mM) produced a concentration-dependent relaxation of PE (30 nM)-induced tone. The maximal relaxation induced by AT at 100 mM was 65.1 ± 4.7 % (n=6, Figure 5.7).

In order to investigate if the relaxation induced by AT was due to activation of soluble guanylate cyclase, the effects of MeB were studied. Pretreatment with MeB (30 μM, 20 min) had no effect on the relaxant activity of AT (1-100 mM, n=6, Figure 5.7). The relaxant action of AT is therefore unlikely to be mediated via stimulation of soluble guanylate cyclase.

5.7 Effects of 3 amino-1,2,4-triazole (AT) on the ability of catalase to inhibit hydrogen peroxide-induced relaxation

In order to establish the conditions necessary for optimum inhibition of endogenous catalase, a series of experiments was conducted using AT to inhibit exogenous catalase (bovine liver). Pretreatment of endothelium-denuded rings of rat aorta with catalase (1000 u ml^{-1}) for 30 min led to a 50.4 ± 0.5 % (n=5) reduction in the maximal relaxation induced by hydrogen peroxide (Figure 5.8). When the catalase inhibitor, AT (50 mM, Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958), was present together with catalase during the 30 min incubation, the ability to inhibit hydrogen peroxide relaxation was partially blocked (by 48.8 ± 3.0 %, n=6, Figure 5.8).

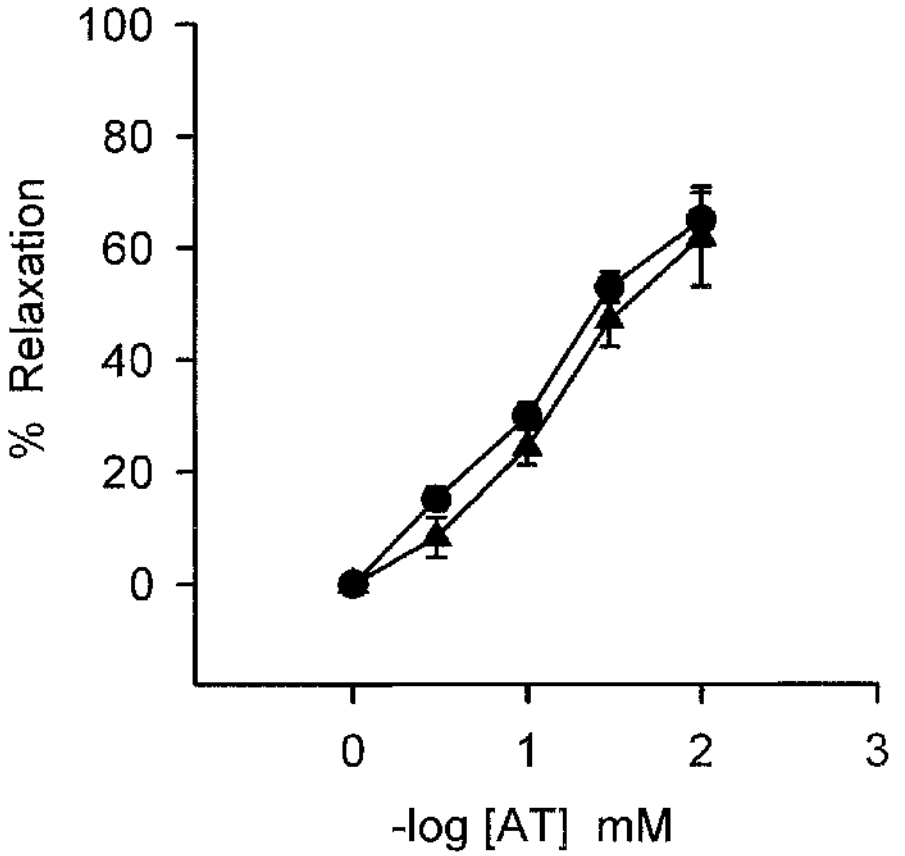


Figure 5.7 Concentration-response curves showing relaxation to 3-amino-1,2,4-triazole (AT, ●) on phenylephrine-contracted endothelium-denuded rings of rat aorta and the lack of effect of methylene blue (30 μM, ▲) on this relaxation. Each point is the mean ± s.e. mean of 6 observations.

Increasing the period of incubation to 90 min, however, led to complete blockade of the ability of catalase to inhibit hydrogen peroxide-induced relaxation (Figure 5.8). It can be concluded that AT does indeed inhibit the activity of catalase and that the onset of inhibition is very slow. Consequently, in all the experiments involving attempted inhibition of endogenous catalase by AT, the tissues were incubated with the drug for 90 min.

5.8 Effects of 3-amino-1,2,4-triazole (AT) on relaxation induced by hydrogen peroxide

Having established the ability of AT (50 mM, 90 min) to inhibit exogenous catalase, we sought to determine the effects of inhibition of endogenous catalase on hydrogen peroxide-induced relaxation. In endothelium-denuded rings pretreatment with AT (50 mM, 90 min) depressed PE (10-30 nM)-induced tone by 31.8 ± 2.2 % (n=15), but we ensured that the level of tone (1.2 ± 0.1 g, n=15) before relaxation was similar to that of untreated preparations (1.3 ± 0.1 g, n=15) by increasing the concentration of PE (to 30-100 nM). Treatment with AT (50 mM, 90 min), however, had no effect on relaxation induced by hydrogen peroxide (10 μM-1 mM, n=12, Figure 5.9). This finding may suggest that metabolism of hydrogen peroxide by catalase or formation of compound I has no role in hydrogen peroxide-induced relaxation.

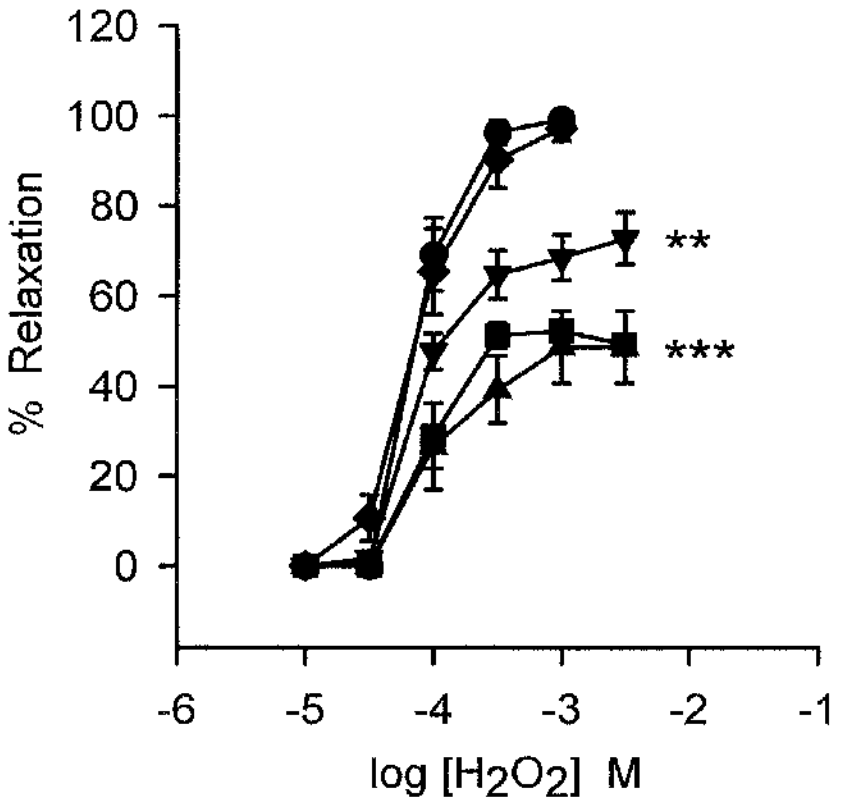


Figure 5.8 Concentration-response curves showing relaxation to hydrogen peroxide (H_2O_2, ●) on phenylephrine-contracted endothelium-denuded rings of rat aorta and blockade of this relaxation following pretreatment with catalase (1000 u ml^{-1}) for 30 min (▲) or 90 min (■). Incubation with 3-amino-1,2,4-triazole (50 mM) for 30 min (▼) partially inhibited the ability of catalase to block H_2O_2-induced relaxation, whereas incubation for 90 min (♦) produced complete inhibition. Each point is the mean ± s.e. mean of 5-6 observations. **P<0.005 and ***P<0.001 indicate a significant difference from maximal relaxation in untreated rings, respectively.

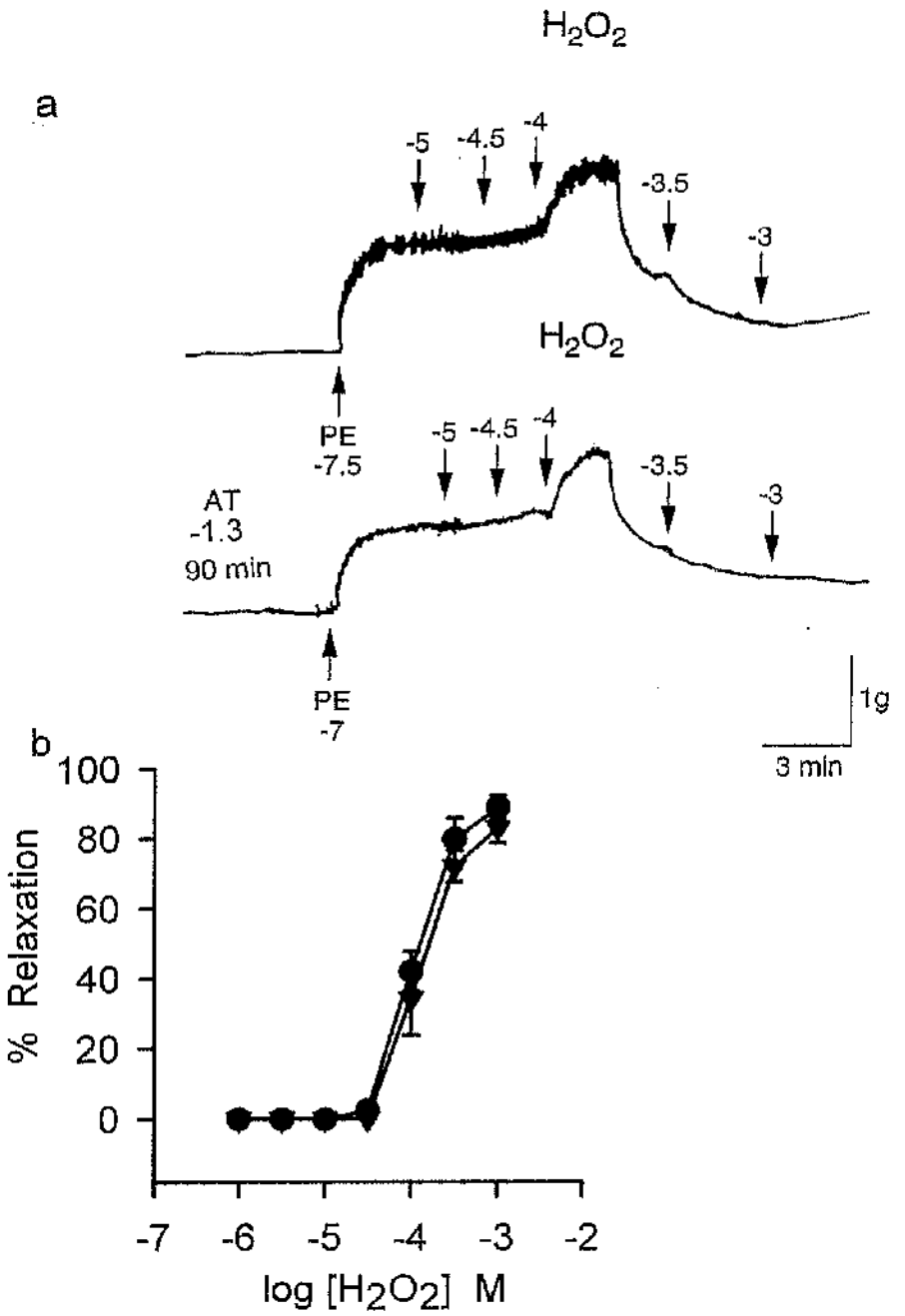


Figure 5.9 Individual experimental traces (a) and concentration-response curves (b) showing relaxation to hydrogen peroxide (H_2O_2, ●) on phenylephrine (PE)-contracted endothelium-denuded rings of rat aorta and the lack of effect of inhibition of endogenous catalase with 3-amino-1,2,4-triazole (AT, 50 mM, 90 min, ▼) on this relaxation. Each point is the mean ± s.e. mean of 8-12 observations.

5.9 Vasodilator actions of sodium azide, hydroxylamine and glyceryl trinitrate

Sodium azide (1-300 nM, Figure 5.10, 5.11, 5.12 and 5.13), hydroxylamine (1-300 nM, Figure 5.10, 5.14 and 5.15) and glyceryl trinitrate (1-100 nM, Figure 5.10 and 5.16) each produced powerful concentration-dependent relaxation of endothelium-denuded rings of rat aorta precontracted with PE (10-30 nM, n=5-8 for each).

5.10 Effects of methylene blue (MeB) on relaxations induced by sodium azide, hydroxylamine and glyceryl trinitrate

In order to investigate if the relaxations induced by sodium azide, hydroxylamine and glyceryl trinitrate were mediated via activation of soluble guanylate cyclase, the effects of MeB, an inhibitor of soluble guanylate cyclase (Gruetter *et al.*, 1979; Holzmann, 1982; Martin *et al.*, 1985a), were studied. Treatment of PE-contracted (10-30 nM) endothelium-denuded rings with MeB (30 μM) for 20 min powerfully blocked relaxations induced by sodium azide (1-300 nM), hydroxylamine (1-300 nM) and glyceryl trinitrate (1-100 nM) at all concentrations. The maximal relaxation induced by each was reduced by 39.0 ± 4.7 %, 27.4 ± 1.1 % and 28.0 ± 1.1 %, respectively (n=6-8 for each, Figure 5.10). Thus, relaxations by all three nitrovasodilators appear to depend upon the stimulation of soluble guanylate cyclase.

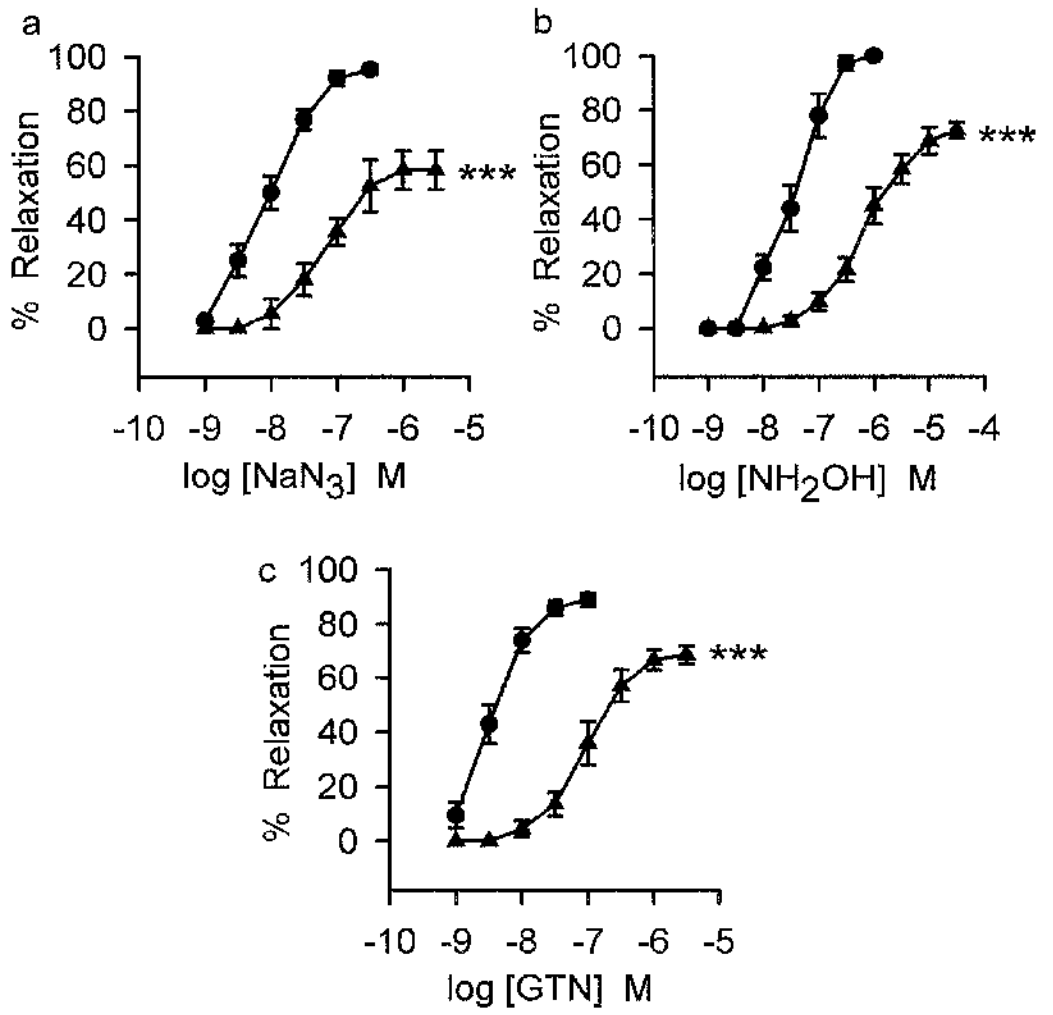


Figure 5.10 Concentration-response curves showing relaxation to (a) sodium azide (NaN_3, ●) (b) hydroxylamine (NH_2OH, ●) and (c) glyceryl trinitrate (GTN, ●) on phenylephrine-contracted endothelium-denuded rings of rat aorta and blockade of this relaxation by methylene blue (30 μM, ▲). Each point is the mean ± s.e. mean of 6-8 observations. ***P<0.001 indicates a significant difference from maximal relaxation in untreated rings.

5.11 Effects of 3-amino-1,2,4-triazole (AT) on relaxations induced by vasodilators

To test the hypothesis that metabolism by catalase is required for vascular relaxation induced by sodium azide and hydroxylamine (Katsuki *et al.*, 1977; Waldman & Murad, 1987; Murad, 1994), but not glyceryl trinitrate which is proposed to be metabolised by a distinct pathway (Armstrong *et al.*, 1980; Feelisch, 1991; Schröder, 1992), the effects of AT were therefore studied on relaxations induced by these nitrovasodilators.

5.11.1 Effects of AT on sodium azide-induced relaxation

If endogenous catalase is important in mediating relaxation induced by sodium azide then its inactivation with AT will be expected to inhibit the relaxant effects of this nitrovasodilator. Pretreatment of endothelium-denuded rings with AT (50 mM) for 30, 60 and 90 min, to inhibit endogenous catalase, produced a time-dependent inhibition of relaxation induced by sodium azide (Figure 5.12), with 90 min incubation being the most effective (n=6 for each).

Furthermore, treatment of endothelium-denuded rings for 90 min with AT (1-50 mM) shifted the concentration-response curve for sodium azide (NaN_3, 1-300 nM) to the right in a concentration-dependent manner: the EC_{50} values were shifted 11.4-, 77.0-, 528- and 1668-fold by AT at concentrations of 1, 5, 10 and 50 mM, respectively (Figure 5.11, 5.12 and 5.13, n=5-8 for each). These results suggested a role for endogenous catalase in sodium azide-induced relaxation.

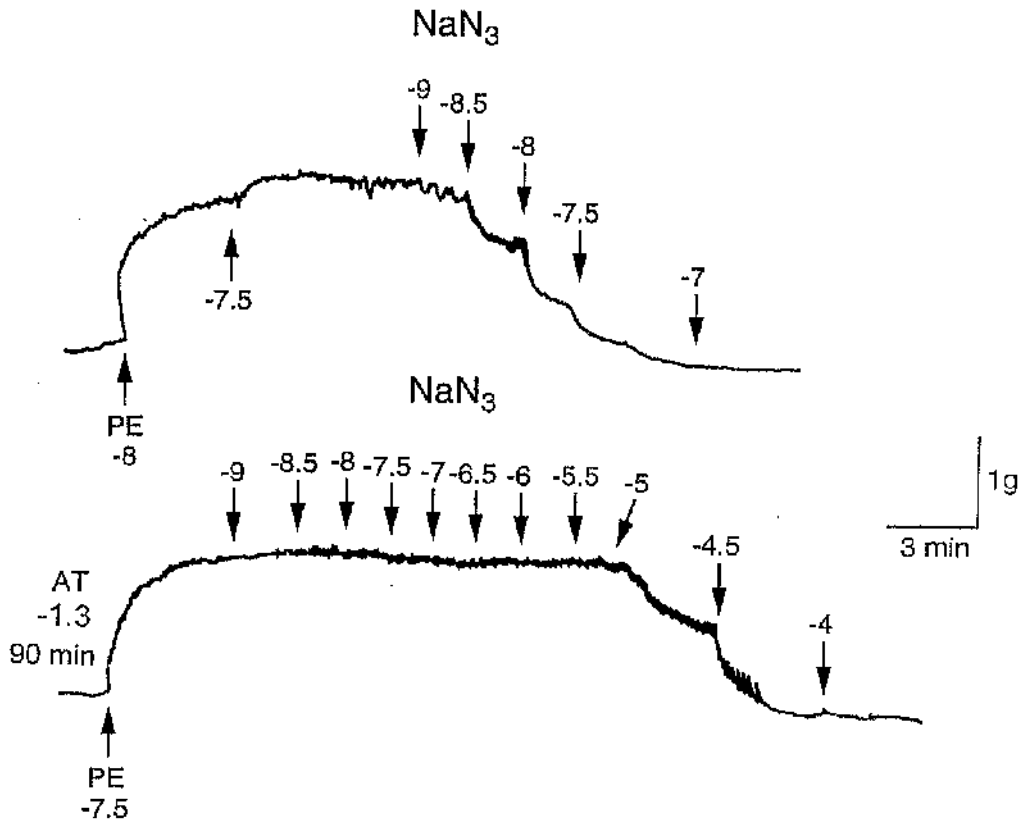


Figure 5.11 Individual experimental tracings showing relaxation to sodium azide (NaN_3) on phenylephrine (PE)-contracted endothelium-denuded rings of rat aorta and the blockade of this relaxation following inhibition of endogenous catalase with 3-amino-1,2,4-triazole (AT, 50 mM, 90 min). Drug concentrations are expressed in log molar units.

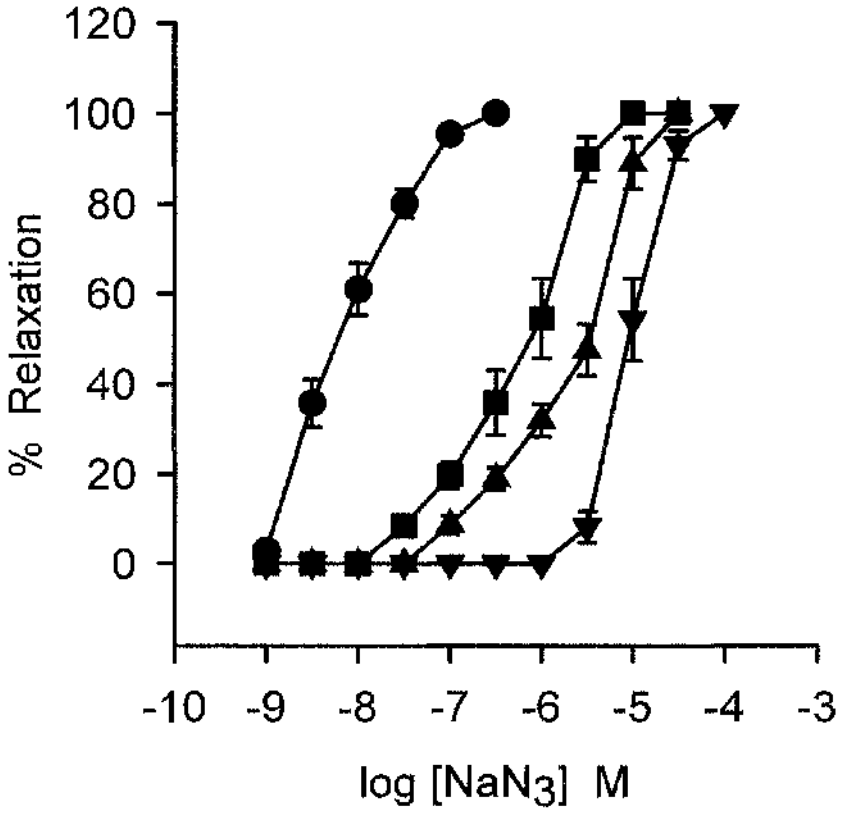


Figure 5.12 Concentration-response curves showing relaxation to sodium azide (NaN_3, ●) on phenylephrine-contracted endothelium-denuded rings of rat aorta. Incubation with 3-amino-1,2,4-triazole (50 mM) produced a time-dependent inhibition of this relaxation: 30 min (■), 60 min (▲) and 90 min (▼). Each point is the mean ± s.e. mean of 6 observations.

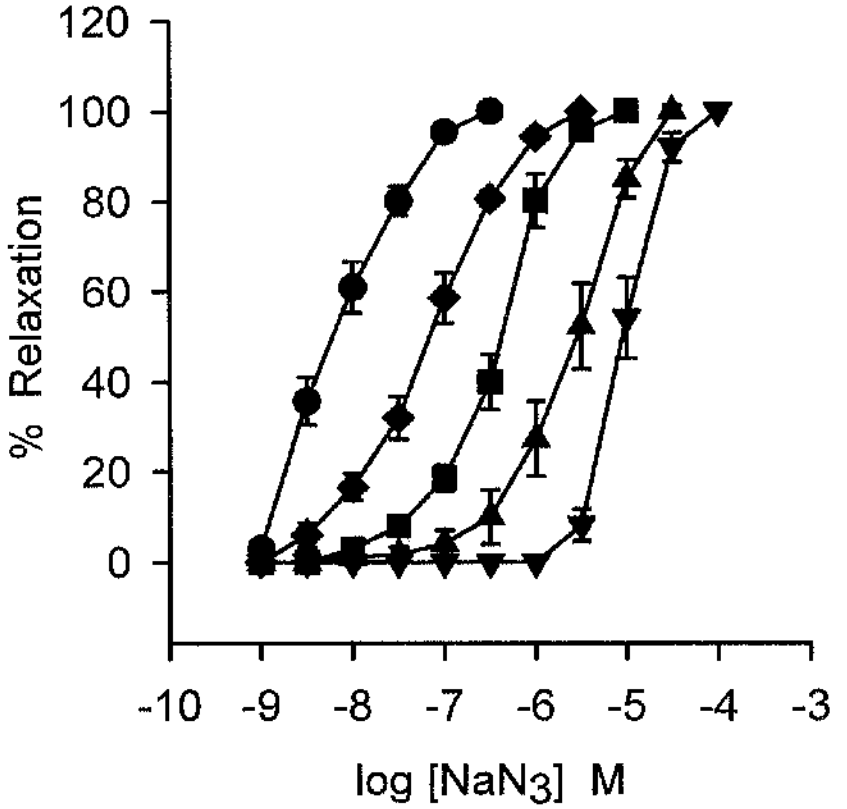


Figure 5.13 Concentration-response curves showing relaxation to sodium azide (NaN_3, ●) on phenylephrine-contracted endothelium-denuded rings of rat aorta and concentration-dependent shifts to the right following inhibition of endogenous catalase with 3-amino-1,2,4-triazole (90 min) at 1 mM (◆), 5 mM (■), 10 mM (▲), and 50 mM (▼). Each point is the mean ± s.e. mean of 5-8 observations.

5.11.2 Effects of AT on hydroxylamine-induced relaxation

If endogenous catalase is important in mediating relaxation induced by hydroxylamine then its inactivation with AT will be expected to inhibit the relaxant actions of this nitrovasodilator. As with sodium azide, treatment of endothelium-denuded rings for 90 min with AT (5-50 mM) shifted the concentration-response curve for hydroxylamine (1-300 nM) to the right in a concentration-dependent manner: the EC_{50} values were shifted 6.4-, 59.9- and 190-fold by AT at concentrations of 5, 10 and 50 mM, respectively (Figure 5.14 and 5.15, n=6 for each). These results suggested a role for endogenous catalase in hydroxylamine-induced ralaxation. AT at all concentrations tested inhibited the actions of sodium azide more powerfully than hydroxylamine.

5.11.3 Effects of AT on glyceryl trinitrate-induced relaxation

In contrast to its ability to block relaxation induced by sodium azide and hydroxylamine, AT (50 mM, 90 min) had no effect on relaxation induced by another nitrovasodilator, glyceryl trinitrate (GTN, 1-100 nM) in endothelium-denuded rings (n=7, Figure 5.16). These results are consistent with the metabolism of GTN by a distinct pathway (Armstrong *et al.*, 1980; Feelisch, 1991; Schröder, 1992).

5.11.4 Effects of AT on acetylcholine-induced relaxation

Pretreatment with AT (50 mM, 90 min) had no effect on the endothelium-dependent relaxation induced by acetylcholine (10 nM-3 μM, n=6, Figure 5.17). This result confirmed the selectivity of AT.

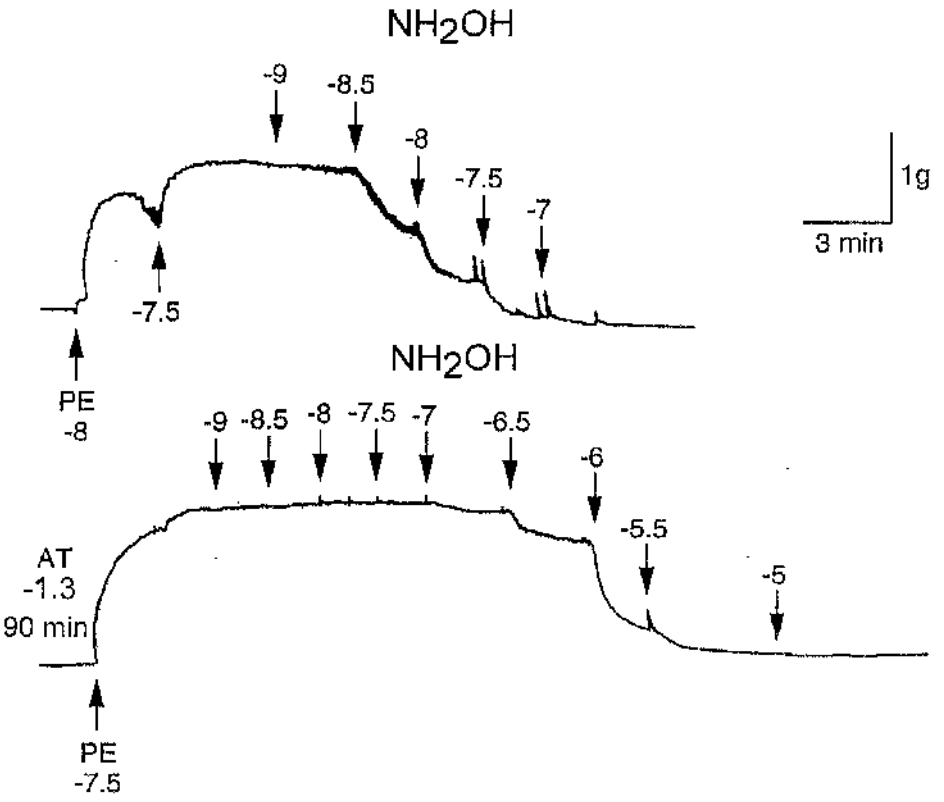


Figure 5.14 Individual experimental tracings showing relaxation to hydroxylamine (NH_2OH) on phenylephrine (PE)-contracted endothelium-denuded rings of rat aorta and the blockade of this relaxation following inhibition of endogenous catalase with 3-amino-1,2,4-triazole (AT, 50 mM, 90 min). Drug concentrations are expressed in log molar units.

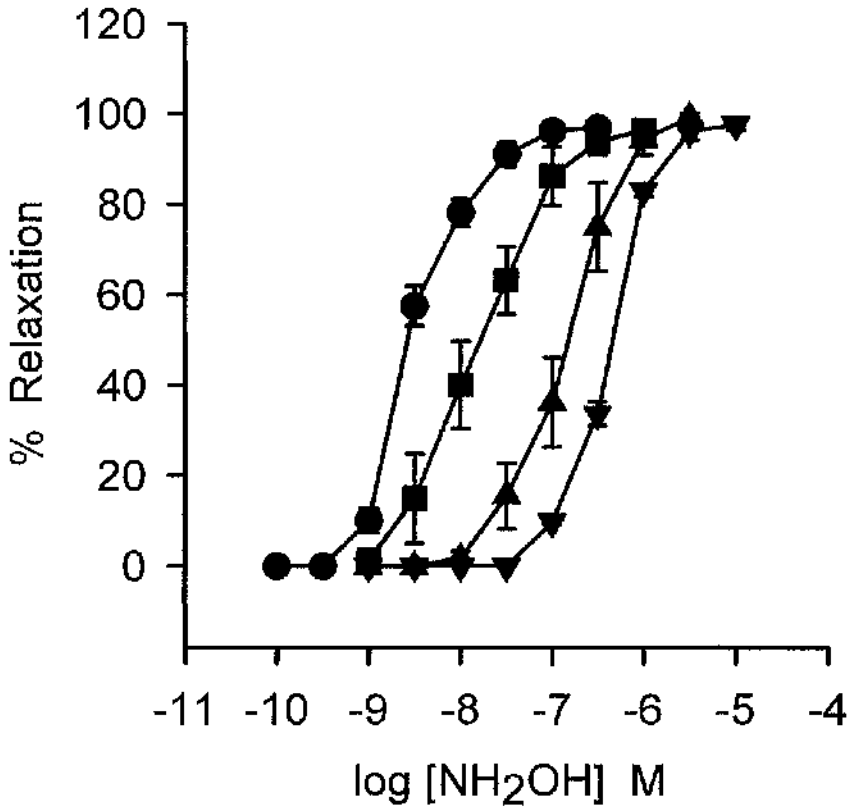


Figure 5.15 Concentration-response curves showing relaxation to hydroxylamine (NH_2OH, ●) on phenylephrine-contracted endothelium-denuded rings of rat aorta and concentration-dependent shifts to the right following inhibition of endogenous catalase with 3-amino-1,2,4-triazole (90 min) at 5 mM (■), 10 mM (▲), and 50 mM (▼). Each point is the mean ± s.e. mean of 6 observations.

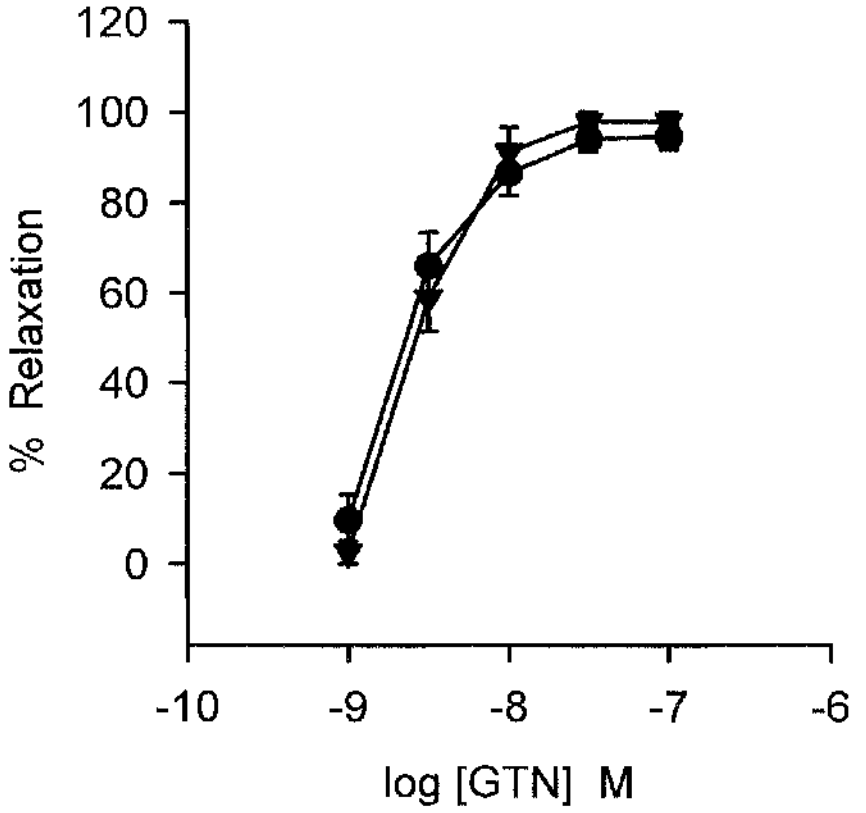


Figure 5.16 Concentration-response curves showing relaxation to glyceryl trinitrate (GTN, ●) on phenylephrine-contracted endothelium-denuded rings of rat aorta and the lack of effect of inhibition of endogenous catalase with 3-amino-1,2,4-triazole (50 mM, 90 min, ▼) on this relaxation. Each point is the mean ± s.e. mean of 6 observations.

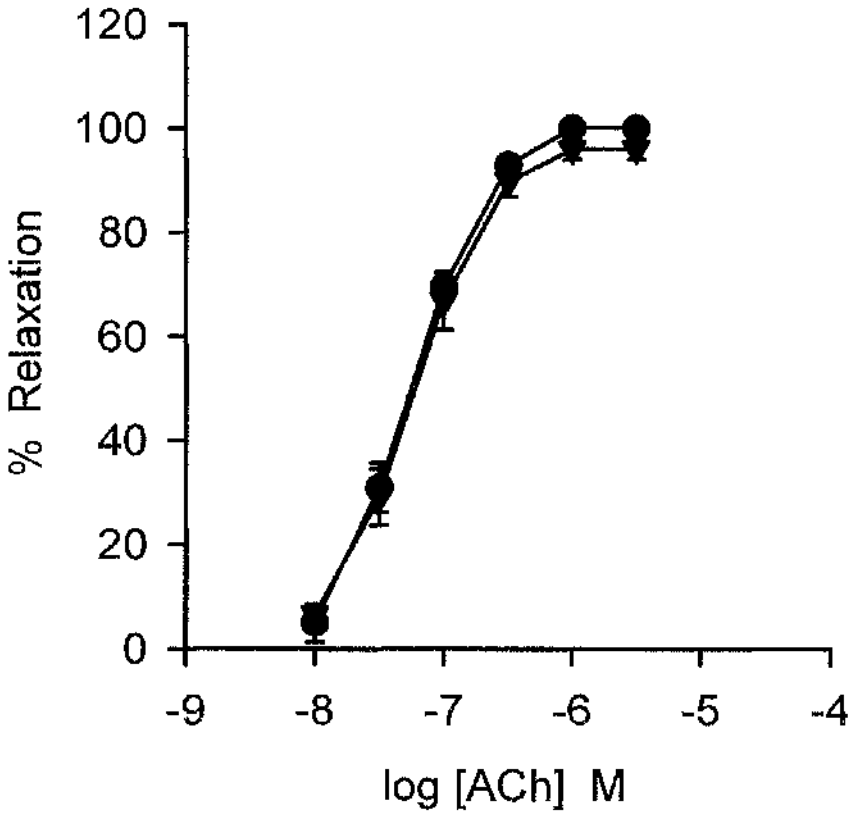


Figure 5.17 Concentration-response curves showing relaxation to acetylcholine (ACh, ●) on phenylephrine-contracted endothelium-containing rings of rat aorta and the lack of effect of inhibition of endogenous catalase with 3-amino-1,2,4-triazole (50 mM, 90 min, ▼) on this relaxation. Each point is the mean ± s.e. mean of 6-7 observations.

Chapter 6

Assessment of the effects of catalase in nitric oxide formation from sodium azide and hydroxylamine

In the previous Chapter (Chapter 5) we indirectly established, by means of functional studies, the role of catalase in relaxations induced by two powerful nitrovasodilators, sodium azide and hydroxylamine. In this Chapter, the role of catalase was investigated further by adopting a more direct approach i.e. by measuring NO formation from sodium azide and hydroxylamine.

It has been proposed that conversion of sodium azide and hydroxylamine to NO requires the presence of low concentrations of hydrogen peroxide and thus occurs as a consequence of the peroxidase activity of catalase (Theorell & Ehrenberg, 1952; Keilin & Hartree, 1954; Nicholls, 1964; Klebanoff & Nathan, 1993).

NO has a short half-life of around 5 seconds under physiological conditions (Griffith *et al.*, 1984). It decays to the more stable end products, nitrite and nitrate, but predominantly the former *in vitro* (see Methods). Consequently, measuring nitrite formation from sodium azide and hydroxylamine was chosen as a convenient way to assess the role of catalase in NO formation from these nitrovasodilators.

6.1 Nitrite formation from sodium azide

6.1.1 Role of hydrogen peroxide

The role of hydrogen peroxide in the conversion of sodium azide to NO by catalase was studied in a cell-free system. In these studies the reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), catalase (bovine liver, 1000 u ml^{-1}), sodium azide (0.3 mM) and hydrogen peroxide (1 μM-10 mM) and were incubated for 1h at 37 °C. Figure 6.1 shows that catalase alone or in combination with sodium azide, failed to produce any nitrite. In contrast, in the presence of hydrogen peroxide a concentration-dependent rise in nitrite formation was seen. Hydrogen peroxide at a concentration of 1 mM was seen to be optimal and increased nitrite formation to 24.5 ± 0.5 μM (n=6, Figure 6.1). These findings suggested that the conversion of sodium azide to NO by catalase required the presence of hydrogen peroxide.

6.1.2 Role of catalase

Having established the optimum concentration of hydrogen peroxide required for the formation of nitrite, the concentration dependence of catalase was investigated. In these studies the reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), sodium azide (0.3 mM), hydrogen peroxide (1 mM) and catalase (100-1000 u ml^{-1}) and were incubated for 1h at 37 °C. Nitrite production increased with increasing concentrations of catalase: at a concentration of 1000 u ml^{-1}, the level of nitrite produced was 23.3 ± 0.6 μM (n=6, Figure 6.2). The intrinsic colour of catalase precluded the use of concentrations of the enzyme higher than 1000 u ml^{-1} in the spectrophotometric assay.

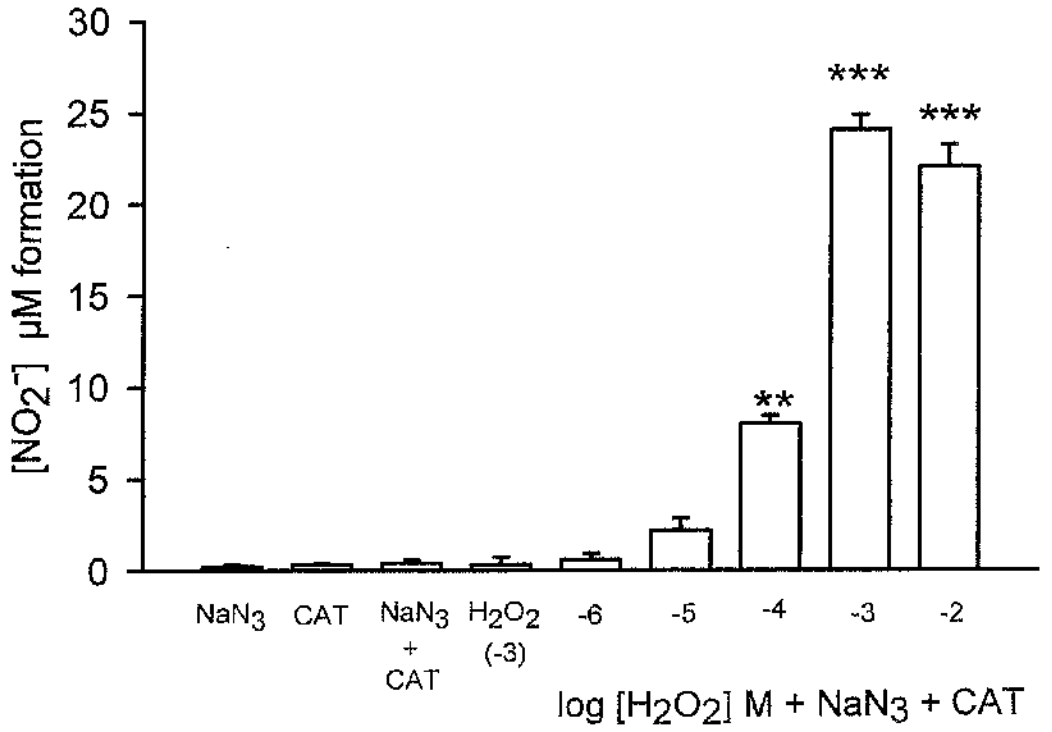


Figure 6.1 The formation of nitrite [NO_2^-] from sodium azide (NaN_3) by catalase (CAT) in a cell-free system requires the presence of hydrogen peroxide (H_2O_2). The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), CAT (1000 u ml^{-1}), NaN_3 (0.3 mM) and H_2O_2 (1 µM-10 mM) in a final volume of 80 µl and were incubated for 1h at 37 °C. Control incubations with each of NaN_3, CAT and H_2O_2 alone and with NaN_3 and CAT were also performed. Each column is the mean ± s.e. mean of 6 observations. **P<0.005 and ***P<0.001 respectively indicate a significant difference from reaction mixtures containing CAT and NaN_3 but not H_2O_2.

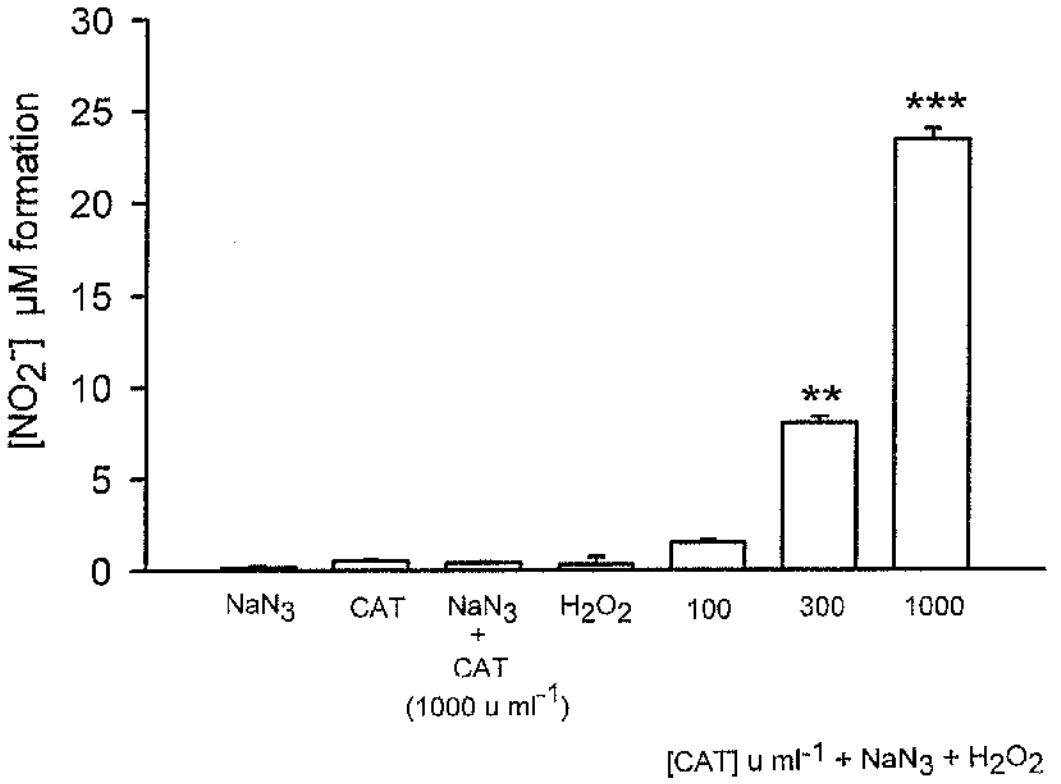


Figure 6.2 Catalase (CAT) produced a concentration-dependent formation of nitrite [NO_2^-] from sodium azide (NaN_3) in the presence of hydrogen peroxide (H_2O_2) in a cell-free system. The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), H_2O_2 (1 mM), NaN_3 (0.3 mM) and CAT (100-1000 u ml^{-1}) in a final volume of 80 µl and were incubated for 1h at 37 °C. Control incubations with each of NaN_3, CAT and H_2O_2 alone and with NaN_3 and CAT were also performed. Each column is the mean ± s.e. mean of 6 observations. **P<0.005 and ***P<0.001 respectively indicate a significant difference from reaction mixtures containing CAT and NaN_3 but not H_2O_2.

6.1.3 Concentration-dependence of sodium azide in the formation of nitrite

Having established the optimum conditions and concentrations of catalase and hydrogen peroxide required for the formation of nitrite in the above experiments, the concentration dependence of sodium azide (10 μM-3 mM) was investigated. As in the experiments above, sodium azide incubated for 1h alone or together with catalase, failed to produce any significant formation of nitrite. However, in the presence of both catalase (1000 u ml^{-1}) and hydrogen peroxide (1 mM), sodium azide (10 μM-3 mM) was converted in a concentration-dependent manner to nitrite: the maximum concentration of nitrite produced was 23.4 ± 0.6 μM from a concentration of 0.3 mM sodium azide (n=6, Figure 6.3). At higher concentrations of sodium azide a slight decrease in nitrite formation was observed.

6.1.4 Time course of nitrite formation

Having established that the presence of hydrogen peroxide was necessary for the formation of nitrite from sodium azide by catalase, the time course of this effect was studied over 1h. Figure 6.4 shows that formation of nitrite was first seen at 3 min (3.1 ± 0.2 μM) and production continued to rise to 23.2 ± 0.7 μM at 60 min (n=5). The production of nitrite throughout this period was almost linear.

All the above findings indicate that catalase metabolises sodium azide to NO and this requires the presence of hydrogen peroxide.

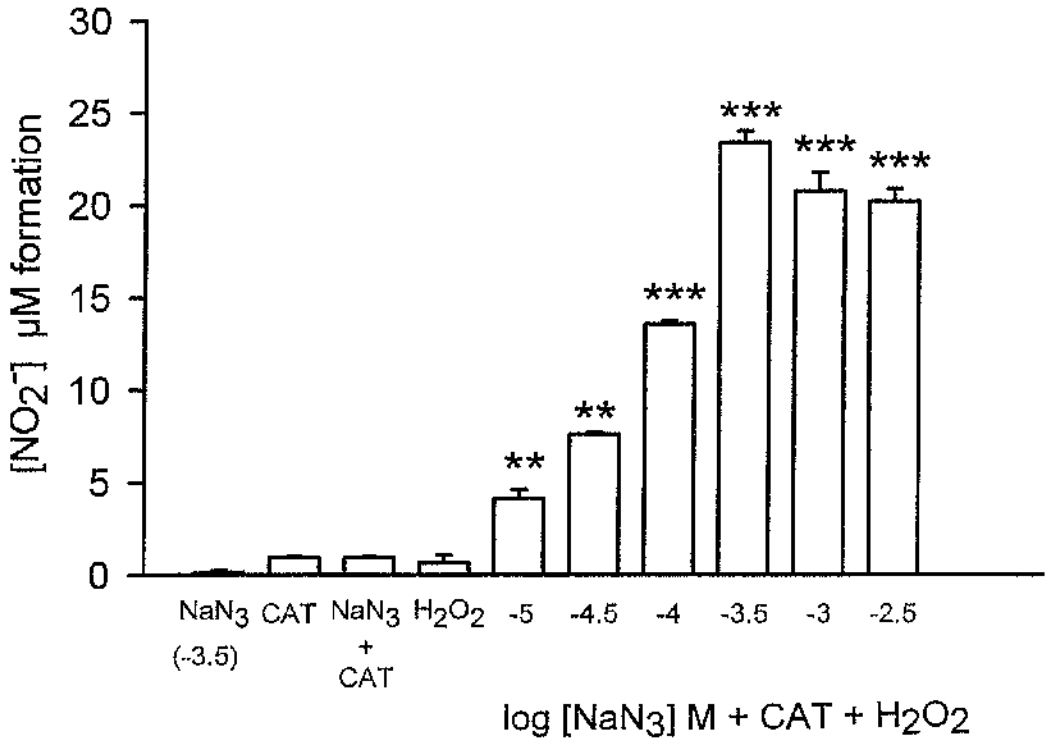


Figure 6.3 The catalase (CAT)-dependent formation of nitrite [NO_2^-] from a range of concentrations of sodium azide (NaN_3) in the presence of hydrogen peroxide (H_2O_2) in a cell-free system. The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), CAT (1000 u ml^{-1}), H_2O_2 (1 mM) and NaN_3 (10 µM-3 mM) in a final volume of 80 µl and were incubated for 1h at 37 °C. Control incubations with each of NaN_3, CAT and H_2O_2 alone and with NaN_3 and CAT were also performed. Each column is the mean ± s.e. mean of 6 observations. **P<0.005 and ***P<0.001 respectively indicate a significant difference from reaction mixtures containing CAT and NaN_3 but not H_2O_2.

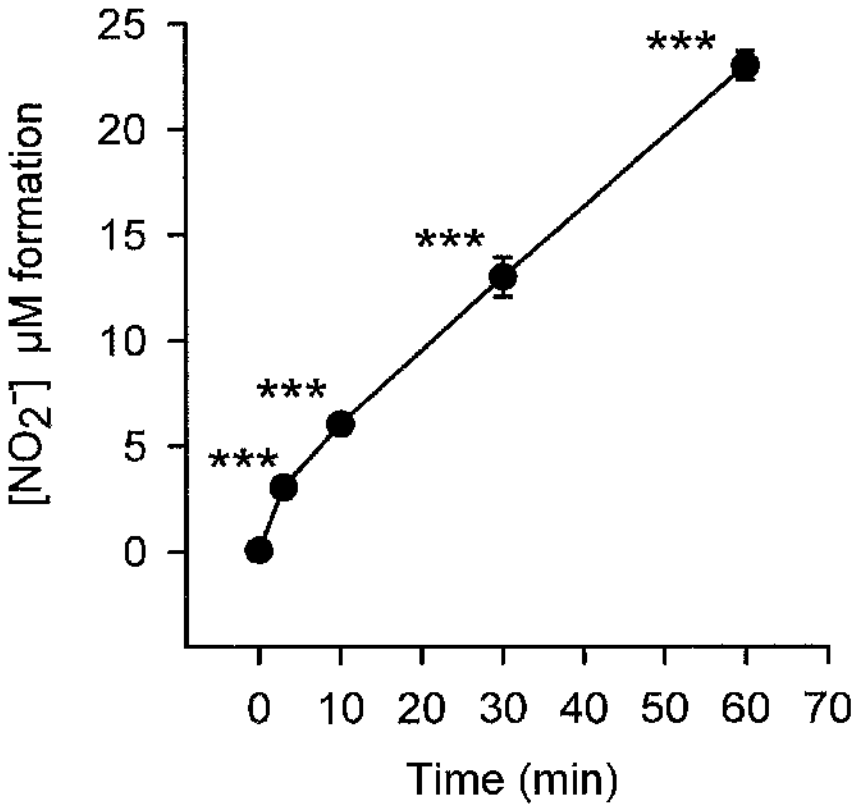


Figure 6.4 Time-course of the catalase (CAT)-dependent formation of nitrite [NO_2^-] from sodium azide (NaN_3) in the presence of hydrogen peroxide (H_2O_2) in a cell-free system. The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), CAT (1000 u ml^{-1}), H_2O_2 (1 mM) and NaN_3 (0.3 mM) in a final volume of 80 µl and were incubated for 0-60 min at 37 °C. Each point is the mean ± s.e. mean of 5 observations. ***P<0.001 indicates a significant increase from nitrite formation at 0 time.

6.2 Nitrite formation from hydroxylamine

Hydroxylamine is another powerful nitrovasodilator which is thought to be converted to NO by catalase. To assess the formation of nitrite from hydroxylamine, the reaction mixtures containing phosphate buffer (pH 5.6, 0.08 M), hydrogen peroxide (1 mM), catalase (1000 u ml^{-1}) and a range of concentrations of hydroxylamine (10 µM-30 mM) were incubated for 1h at 37 °C.

In a similar fashion to sodium azide, hydroxylamine (10 µM-30 mM) was converted in a concentration-dependent manner to nitrite by catalase in the presence but not in the absence of hydrogen peroxide. The maximum concentration of nitrite obtained was 8.7 ± 0.7 µM from a concentration of 10 mM hydroxylamine (n=6-20, Figure 6.5). At a higher concentration of hydroxylamine (30 mM), a considerable reduction in nitrite formation was observed (Figure 6.5).

An attempt was made to determine if nitrite formation from hydroxylamine could be increased by varying the concentration of hydrogen peroxide. In these experiments increasing the concentration of hydrogen peroxide from 1 mM to 100 mM increased the formation of nitrite from hydroxylamine at 3 mM and 10 mM from 5.6 ± 0.1 µM to 9.3 ± 0.3 µM and from 7.6 ± 0.2 µM to 10.0 ± 0.1 µM, respectively (n=6-12, Figure 6.6).

These findings indicate that hydroxylamine, like sodium azide, is converted to NO by catalase in the presence but not in the absence of hydrogen peroxide. They also show that hydroxylamine is a less effective substrate than sodium azide.

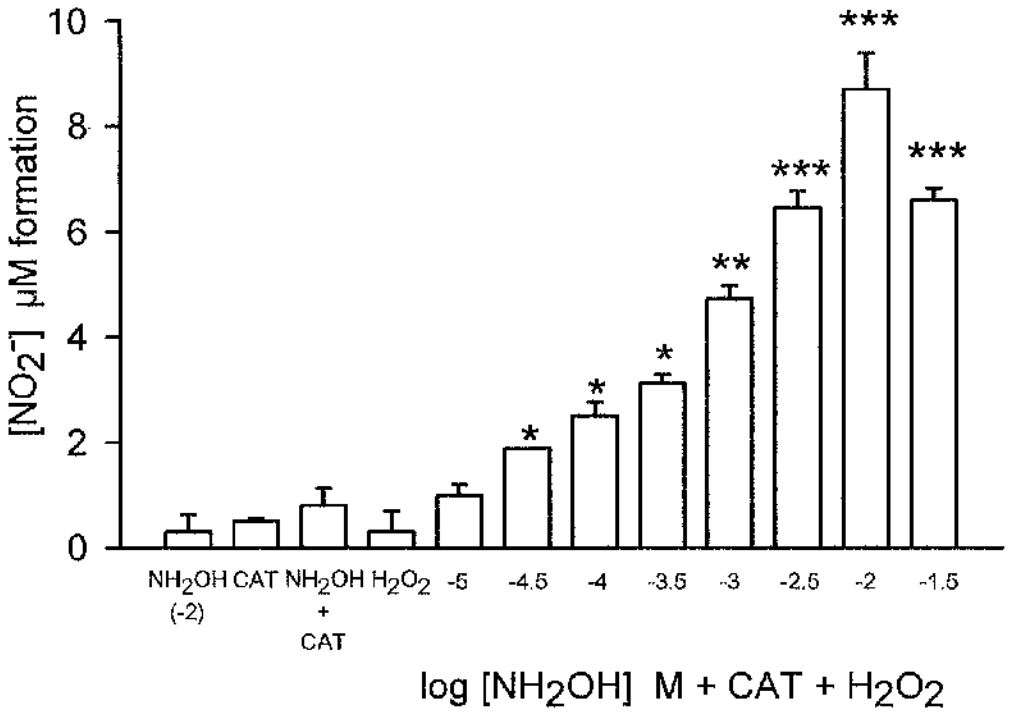


Figure 6.5 The catalase (CAT)-dependent formation of nitrite [NO_2^-] from a range of concentrations of hydroxylamine (NH_2OH) in the presence of hydrogen peroxide (H_2O_2) in a cell-free system. The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), CAT (1000 u ml^{-1}), H_2O_2 (1 mM) and NH_2OH (10 µM-30 mM) in a final volume of 80 µl and were incubated for 1h at 37 °C. Control incubations with each of NH_2OH, CAT and H_2O_2 alone and with NH_2OH and CAT were also performed. Each column is the mean ± s.e. mean of 6-20 observations. *P<0.05, **P<0.005 and ***P<0.001 respectively indicate a significant difference from reaction mixtures containing CAT and NH_2OH but not H_2O_2.

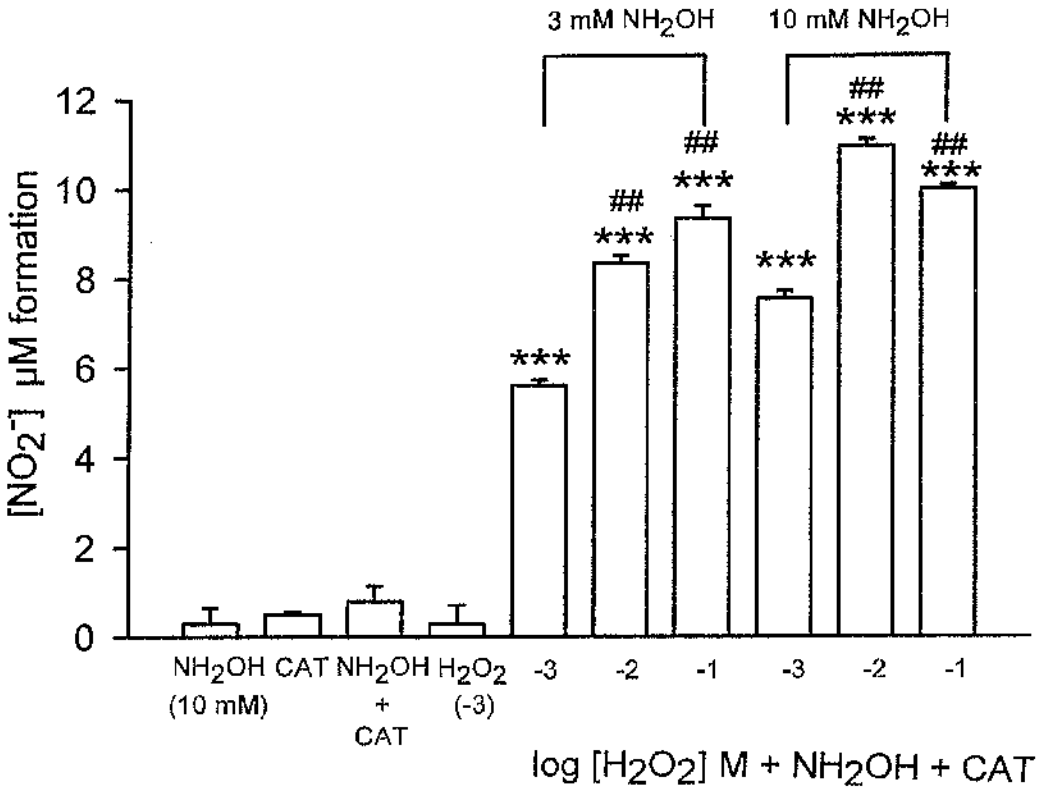


Figure 6.6 The catalase (CAT)-dependent formation of nitrite [NO_2^-] from hydroxylamine (NH_2OH) in the presence of a range of concentrations of hydrogen peroxide (H_2O_2) in a cell-free system. The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), CAT (1000 u ml^{-1}), NH_2OH (3 mM or 10 mM) and H_2O_2 (1-100 mM) in a final volume of 80 µl and were incubated for 1h at 37 °C. Control incubations with each of NH_2OH, CAT and H_2O_2 alone and with NH_2OH and CAT were also performed. Each column is the mean ± s.e. mean of 6-12 observations. ***P<0.001 indicates a significant difference from reaction mixtures containing CAT and NH_2OH but not H_2O_2. ##P<0.005 indicates a significant difference from reaction mixtures containing NH_2OH and CAT and H_2O_2 (1 mM).

6.3 Nitrite formation from glyceryl trinitrate

In contrast to sodium azide and hydroxylamine, the formation of NO from the nitrovasodilator, glyceryl trinitrate, is believed not to involve catalase. Instead, conversion is reported to occur by the action of glutathione-S-transferase (Armstrong *et al.*, 1980) or cytochrome P_{450} (Schröder, 1992), or by a direct reduction by tissue thiols (Feelisch, 1991). An attempt was therefore made to determine if catalase was indeed unable to convert glyceryl trinitrate to nitrite in our system. Indeed, no nitrite was generated when glyceryl trinitrate (1 mM) was incubated for 1h at 37 °C with catalase (1000 u ml^{-1}) in the presence of hydrogen peroxide (1 mM, Figure 6.7). In contrast, glyceryl trinitrate (1 mM) but not sodium azide (1 mM) or hydroxylamine (1 mM) was reduced to nitrite by the thiol, L-cysteine (3 mM) following a 1h incubation at 37 °C (n=6, Figure 6.8). The maximum concentration of nitrite obtained from glyceryl trinitrate (1 mM) was 93.7 ± 1.5 μM (Figure 6.8). These data are therefore consistent with a distinct pathway for generation of NO from glyceryl trinitrate.

6.4 Use of the glucose-glucose oxidase system in nitrite formation from sodium azide

The sustained generation of hydrogen peroxide by the glucose/glucose oxidase system has been reported to be more efficient than a single high concentration of hydrogen peroxide in supporting the catalase-dependent formation of NO from sodium azide (Klebanoff & Nathan, 1993). Consequently, in order to investigate this experiments were conducted in which the formation of nitrite from sodium azide by catalase was observed in the presence and absence of the glucose/glucose oxidase system.

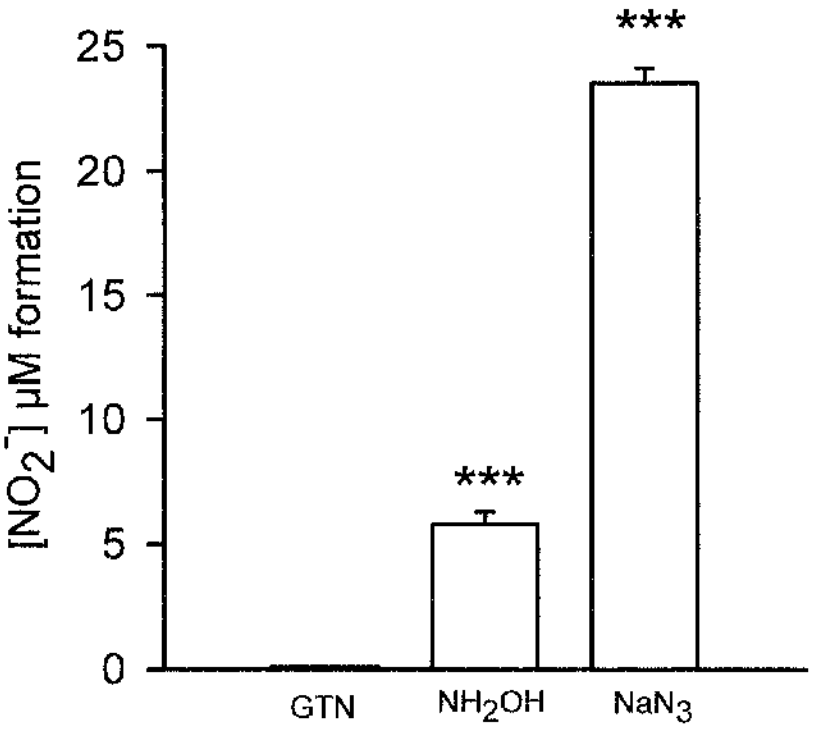


Figure 6.7 The catalase (CAT)-dependent formation of nitrite from sodium azide (NaN_3) and hydroxylamine (NH_2OH) but not glyceryl trinitrate (GTN) in the presence of hydrogen peroxide (H_2O_2) in a cell-free system. The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), CAT (1000 u ml^{-1}), H_2O_2 (1 mM) and NaN_3 (1 mM), NH_2OH (1 mM) or GTN (1 mM) and were incubated for 1h at 37 °C. Each column is the mean ± s.e. mean of 6 observations. ***P<0.001 indicates a significant difference from reaction mixtures containing CAT and H_2O_2 and GTN.

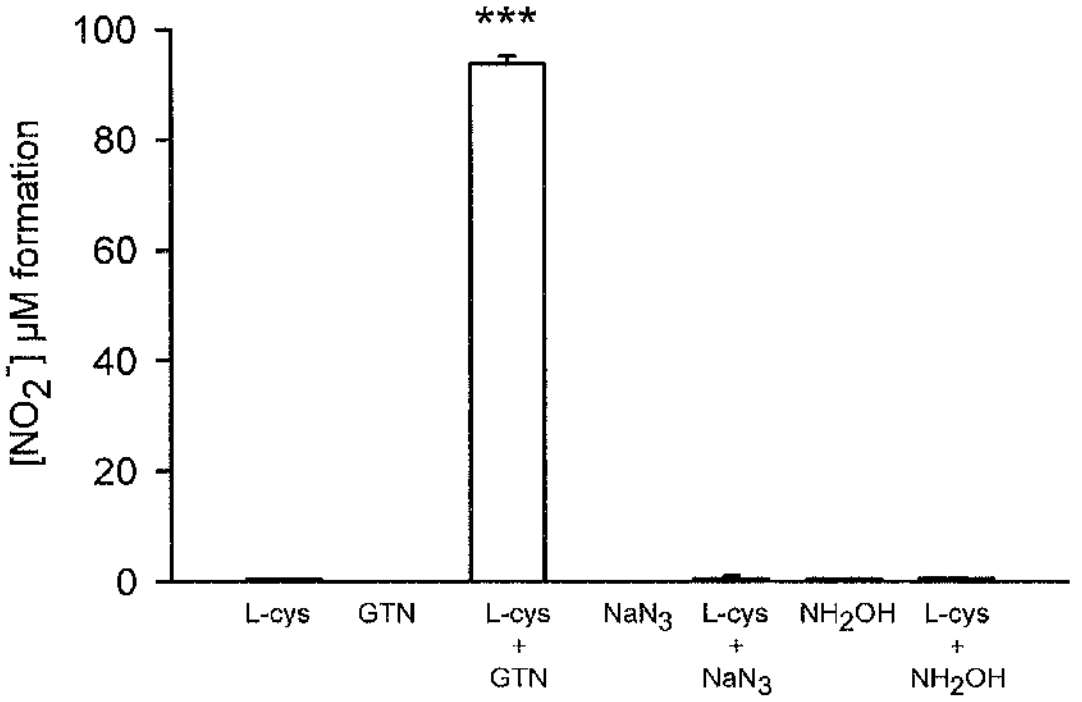


Figure 6.8 The formation of nitrite [NO_2^-] from glyceryl trinitrate (GTN) but not sodium azide (NaN_3) or hydroxylamine (NH_2OH) by L-cysteine (L-cys) in a cell-free system. The reaction mixtures contained HEPES buffer (pH 7.4, 5 mM), NaN_3 (1 mM), NH_2OH (1 mM) or GTN (1 mM) together with L-cys (3 mM) and were incubated for 1h at 37 °C. Control incubations with each of L-cys, GTN, NaN_3 and NH_2OH alone were also performed. Each column is the mean ± s.e. mean of 6 observations. ***$P<0.001$ indicates a significant difference from reaction mixtures containing GTN but not L-cys.

The ability of a range of concentrations of the enzyme, glucose oxidase (1-1000 mu ml^{-1}), together with a fixed concentration of glucose (5 mM) to promote nitrite formation from sodium azide was investigated. The reaction mixtures contained phosphate buffer (pH 5.6. 0.08 M), catalase (1000 u ml^{-1}), sodium azide (0.3 mM), glucose (5 mM) and glucose oxidase (1-1000 mu ml^{-1}) and were incubated for 1h at 37 °C. Figure 6.9 shows that nitrite production increased with increasing concentrations of glucose oxidase: the maximum concentration of nitrite obtained was 45.1 ± 0.4 µM from a concentration of 300 mu ml^{-1} glucose oxidase (n=6).

Having established the optimum concentration of glucose oxidase, the time course of nitrite formation was studied over 1h. Figure 6.10 shows that formation of nitrite was first seen at 3 min (5.2 ± 0.2 µM) and production continued to rise almost linearly to 42.7 ± 1.3 µM at 60 min (n=6).

Having established the optimum conditions in the above experiments, we then studied how nitrite formation varied with the concentration of sodium azide (10 µM-3 mM). In presence of both catalase (1000 u ml^{-1}) and the glucose (5 mM)/glucose oxidase (300 mu ml^{-1}) system, sodium azide (10 µM-3 mM) was converted in a concentration-dependent manner to nitrite during the 1h incubation. The maximum concentration of nitrite obtained was 44.5 ± 0.8 µM from a concentration of 0.3 mM sodium azide (n=6, Figure 6.11), which was about 2-fold greater than that obtained with hydrogen peroxide (1 mM, see Figures 6.1, 6.2 and 6.3). At concentrations of sodium azide ≥ 1 mM, a considerable reduction in nitrite formation was observed (Figure 6.11).

These findings confirmed the slightly greater efficiency of glucose/glucose oxidase system over hydrogen peroxide in facilitating catalase-dependent formation of nitrite from sodium azide (Klebanoff & Nathan, 1993).

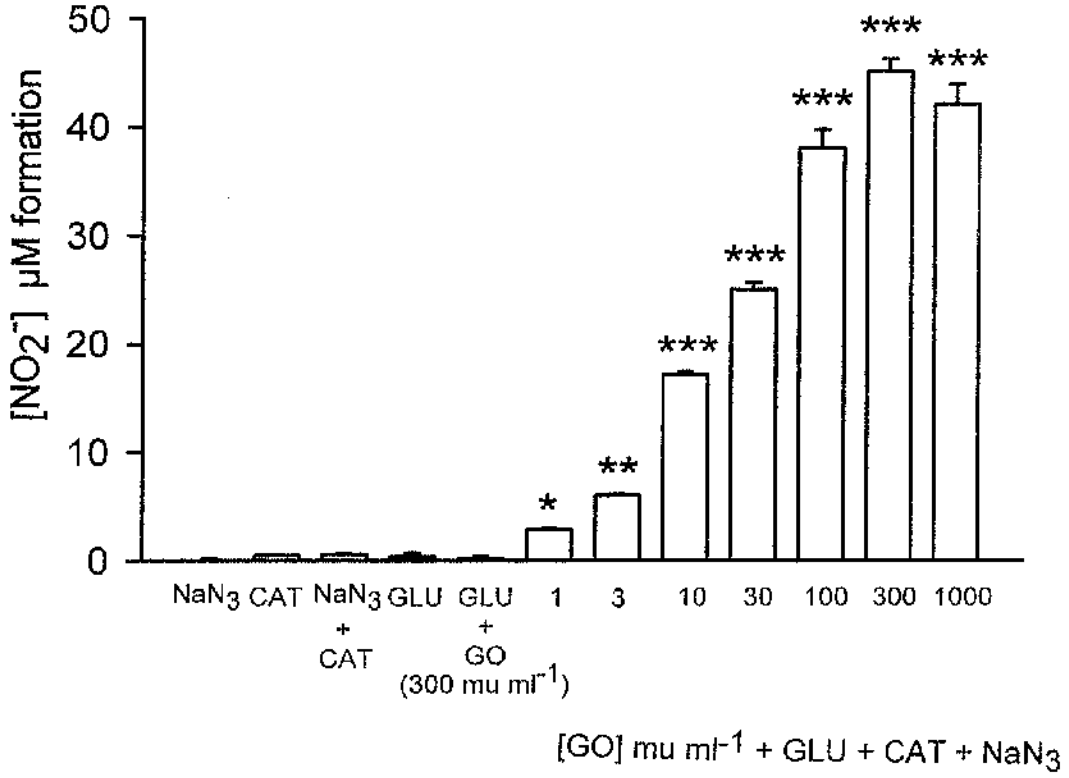


Figure 6.9 The catalase (CAT)-dependent formation of nitrite [NO_2^-] from sodium azide (NaN_3) in the presence of a range of concentrations of glucose (GLU)/glucose oxidase (GO) in a cell-free system. The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), CAT (1000 u ml^{-1}), NaN_3 (0.3 mM) and GLU (5 mM)/ GO (1-1000 mu ml^{-1}) in a final volume of 80 µl and were incubated for 1h at 37 °C. Control incubation with each of NaN_3, CAT and GLU alone and with NaN_3 and CAT, and with GLU and GO were also performed. Each column is the mean ± s.e. mean of 6 observations. *P<0.05, **P<0.005 and ***P<0.001 respectively indicate a significant difference from reaction mixtures containing CAT and NaN_3 but not H_2O_2.

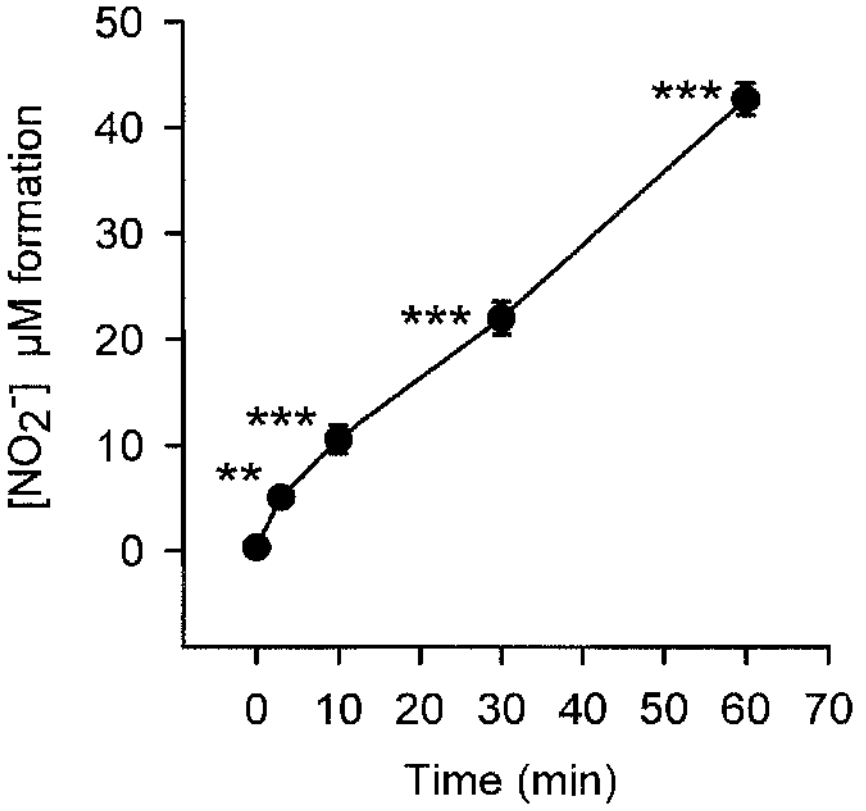


Figure 6.10 Time-course of the catalase (CAT)-dependent formation of nitrite [NO_2^-] from sodium azide (NaN_3) in the presence of glucose (GLU)/glucose oxidase (GO) in a cell-free system. The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), CAT (1000 u ml^{-1}), NaN_3 (0.3 mM) and GLU (5 mM)/GO (300 mu ml^{-1}) in a final volume of 80 µl and were incubated for 0-60 min at 37 °C. Each point is the mean ± s.e. mean of 6 observations. **P<0.005 and ***P<0.001 indicate a significant increase from nitrite formation at 0 time, respectively.

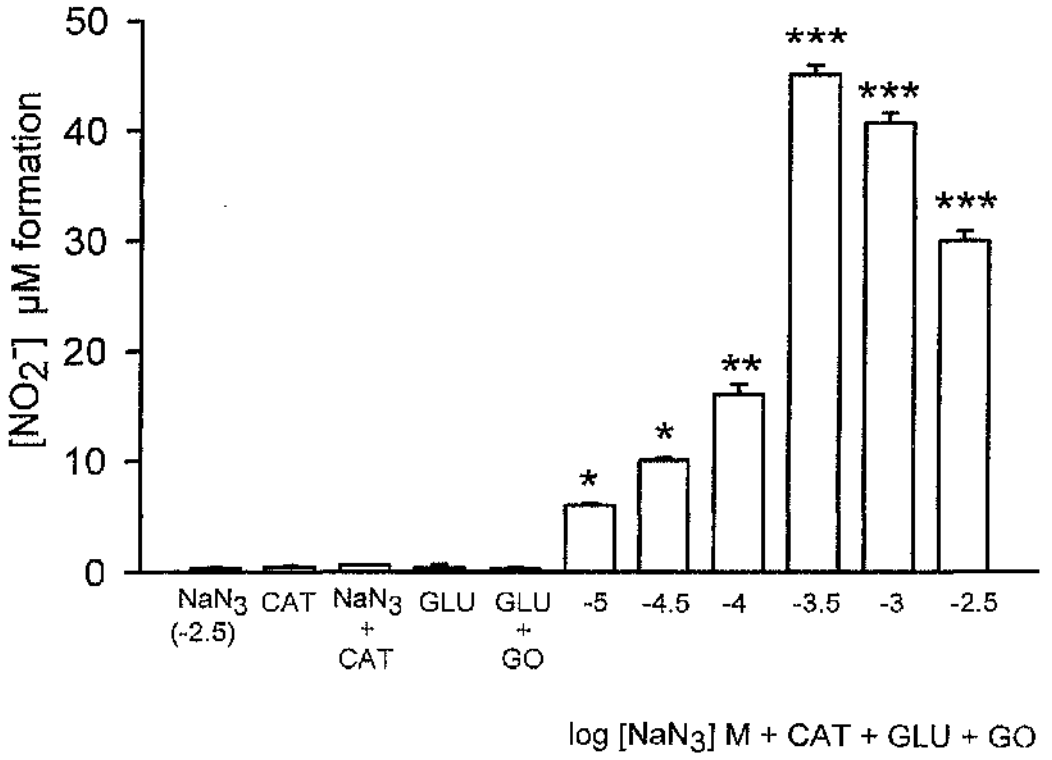


Figure 6.11 The catalase (CAT)-dependent formation of nitrite [NO_2^-] from a range of concentrations of sodium azide (NaN_3) in the presence of glucose (GLU)/glucose oxidase (GO) in a cell-free system. The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), CAT (1000 u ml^{-1}), GLU (5 mM)/GO (300 mu ml^{-1}) and NaN_3 (10 µM-3 mM) in a final volume of 80 µl and were incubated for 1h at 37 °C. Control incubations with each of NaN_3, CAT and GLU alone and with NaN_3 and CAT, and with GLU and GO were also performed. Each column is the mean ± s.e. mean of 6 observations. *P<0.05, **P<0.005 and ***P<0.001 respectively indicate a significant difference from reaction mixtures containing CAT and NaN_3 but not H_2O_2.

6.5 Use of the glucose/glucose oxidase system in nitrite formation from hydroxylamine

Since hydroxylamine was a poor substrate for nitrite formation by catalase in the presence of a fixed concentration of hydrogen peroxide, an attempt was made to determine if formation could be increased by replacing hydrogen peroxide by the glucose/glucose oxidase system. The optimum concentration of glucose (5 mM)/glucose oxidase (300 mu ml^{-1}) found in the previous experiments were incubated with catalase (1000 u ml^{-1}) and a range of concentrations of hydroxylamine (10 μM-30 mM) for 1h at 37 °C. In these experiments the maximum concentration of nitrite obtained was 8.49 ± 0.2 μM from a concentration of 10 mM hydroxylamine (n=6-11, Figure 6.12), which was not significantly different from that obtained with hydrogen peroxide (see Figures 6.5 and 6.6).

Thus, the glucose/glucose oxidase system, despite significantly increasing the catalase-dependent formation of nitrite from sodium azide failed to enhance production from hydroxylamine.

6.6 Inhibition of catalase-dependent formation of nitrite from sodium azide by 3-amino-1,2,4-triazole

In Chapter 5, it was clear that 3-amino-1,2,4-triazole (AT) inhibited the relaxant actions of sodium azide and hydroxylamine in rat aorta indicating an important role for endogenous catalase. Furthermore, the results from this Chapter have shown that NO, assessed as nitrite, can indeed be generated from sodium azide and hydroxylamine by catalase in the presence of hydrogen peroxide. Consequently, it was important to investigate if the formation of nitrite from sodium azide by catalase could be blocked by AT. In these experiments the

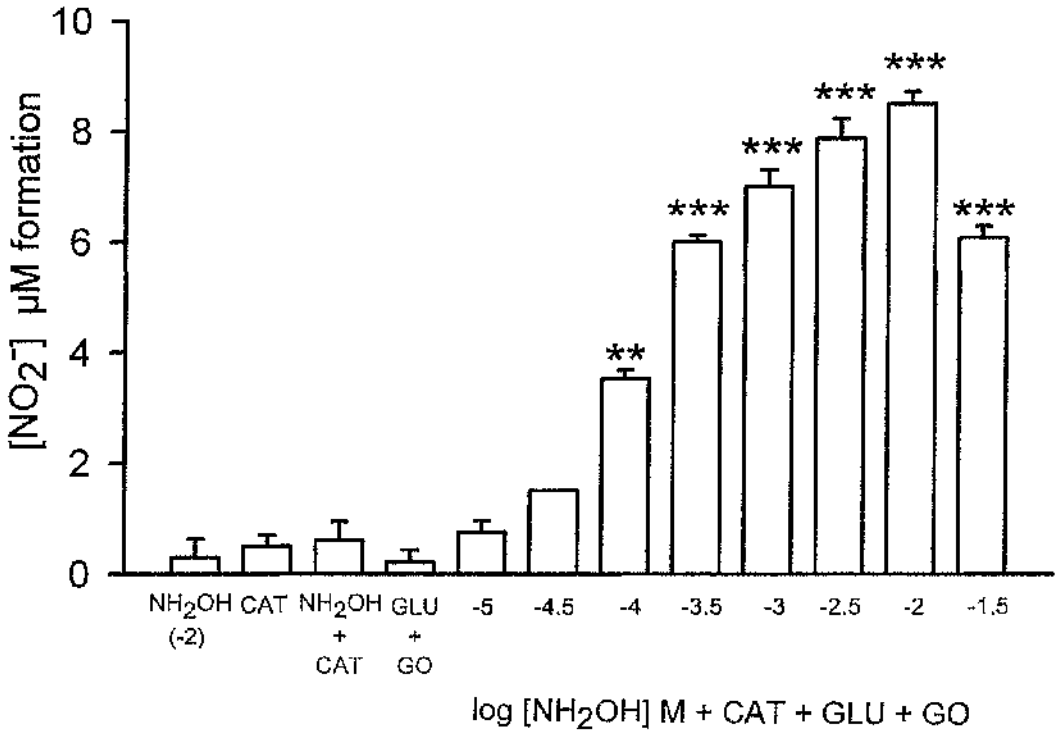


Figure 6.12 The catalase (CAT)-dependent formation of nitrite [NO_2^-] from a range of concentrations of hydroxylamine (NH_2OH) in the presence of glucose (GLU)/glucose oxidase (GO) in a cell-free system. The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), CAT (1000 u ml^{-1}), GLU (5 mM)/ GO (300 mu ml^{-1}) and NH_2OH (10 µM-30 mM) in a final volume of 80 µl and were incubated for 1h at 37 °C. Control incubations with each of NH_2OH and CAT alone and together, and with GLU and GO were also performed. Each column is the mean ± s.e. mean of 6-11 observations. **P<0.005 and ***P<0.001 respectively indicate a significant difference from reaction mixtures containing CAT and NH_2OH but not H_2O_2.

reaction mixtures contained phosphate buffer (pH 6.5, 0.08 M), catalase (300 or 1000 u ml^{-1}), hydrogen peroxide (1 mM), sodium azide (0.3 mM) and AT (0.1-100 mM) and were incubated for 1 or 3h at 37 °C.

When AT (1-100 mM) was present during the 1h incubation, the catalase-dependent formation of nitrite from sodium azide in the presence of hydrogen peroxide was inhibited only slightly: a maximum inhibition of 36.4 ± 1.2 % was obtained with AT at 100 mM (n=6, Figure 6.13). If, however, the duration of the incubation was extended to 3h together with a reduction of the catalase concentration to 300 u ml^{-1}, the ability of AT to block formation of nitrite was enhanced. A maximum inhibition of 77.7 ± 6.3 % was obtained with AT at 100 mM (n=6-20, Figure 6.14).

Since hydroxylamine was a poorer substrate than sodium azide, no experiments were conducted to determine if AT blocked the catalase-dependent formation of nitrite using this agent.

Our biochemical experiments indicate that AT indeed blocks the catalase-dependent conversion of sodium azide to nitrite in the presence of hydrogen peroxide and the blockade is slow, consistent with its known slow onset of action (Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958).

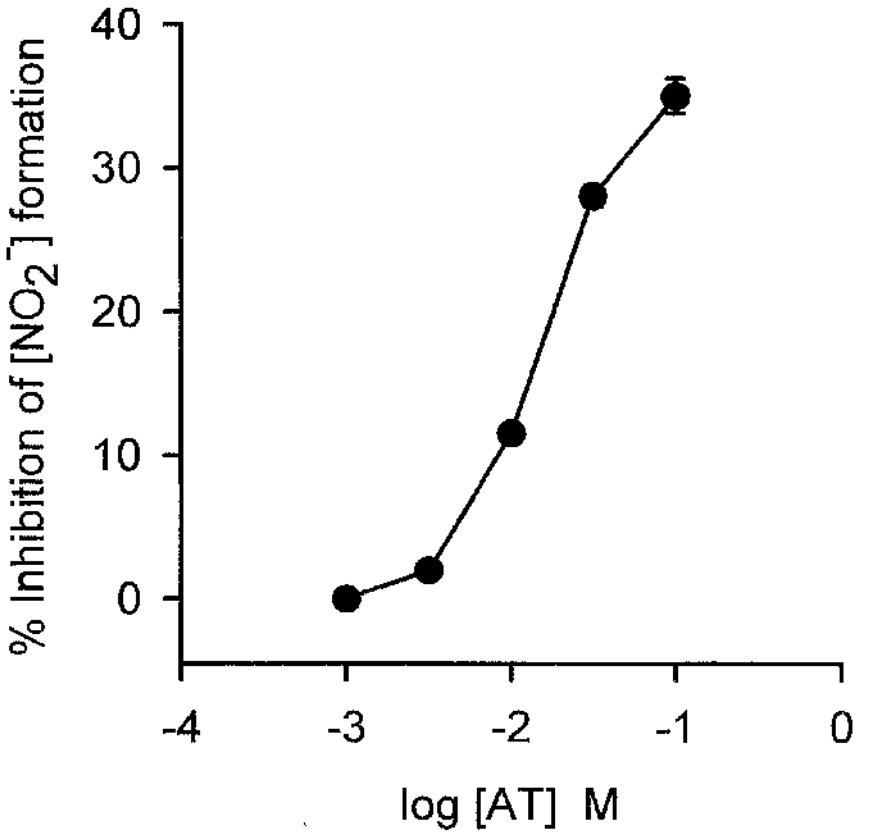


Figure 6.13 Graph showing the inhibition by 3-amino-1,2,4-triazole (AT) of the catalase (CAT)-dependent formation of nitrite [NO_2^-] from sodium azide (NaN_3) in the presence of hydrogen peroxide (H_2O_2). The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), CAT (1000 u ml^{-1}), H_2O_2 (1 mM), NaN_3 (0.3 mM) and AT (1-100 mM) and were incubated for 1h at 37 °C. Each point is the mean ± s.e. mean of 6 observations.

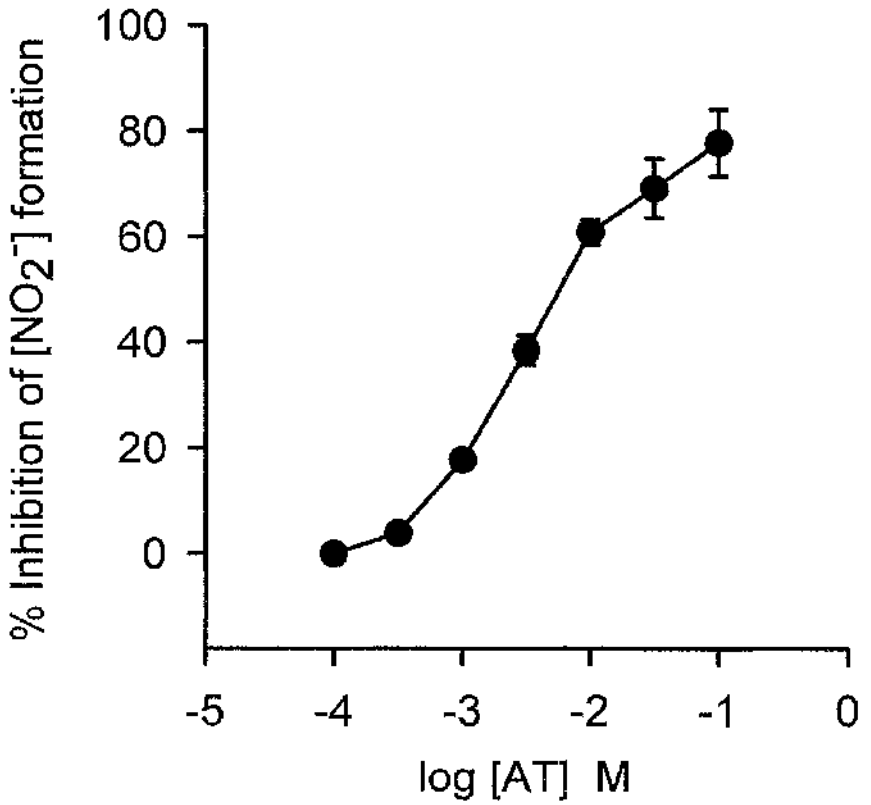


Figure 6.14 Graph showing the inhibition by 3-amino-1,2,4-triazole (AT) of the catalase (CAT)-dependent formation of nitrite [NO_2^-] from sodium azide (NaN_3) in the presence of hydrogen peroxide (H_2O_2). The reaction mixtures contained phosphate buffer (pH 5.6, 0.08 M), CAT (300 u ml^{-1}), H_2O_2 (1 mM), NaN_3 (0.3 mM) and AT (0.1-100 mM) and were incubated for 3h at 37 °C. Each point is the mean ± s.e. mean of 6-20 observations.

Chapter 7

Assessment of the hydrogen peroxide-induced impairment of vascular reactivity and the role of endogenous catalase

Hydrogen peroxide, a powerful oxidant, is a normal metabolite (Chance *et al.*, 1979) formed in all subcellular compartments (Boveris *et al.*, 1972). Mitochondria are the major source of hydrogen peroxide in cells (Boveris & Chance, 1973; Dionisi *et al.*, 1975) where it is formed due to dismutation of superoxide anion (Freeman & Crapo, 1981; 1982).

In this Chapter, we assessed the damage induced by hydrogen peroxide to vascular responsiveness and investigated the role of endogenous catalase in protection against this damage. In order to investigate the role of endogenous catalase, we made a use of the inhibitor of this enzyme, 3-amino-1,2,4-triazole (Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958).

7.1 Effects of incubation with hydrogen peroxide on phenylephrine (PE)-induced contraction

Following induction of PE (10 nM)-induced contraction (1.3 ± 0.1 g, n=12) in endothelium-containing rings, addition of hydrogen peroxide (0.1 mM) produced a powerful long-lasting relaxation (79.5 ± 1.5 %, n=12), as previously reported in Chapter 5. When the incubation was continued for 15, 30 or 60 min and the tissues were washed, subsequent PE (10 nM)-induced contraction was completely unaffected (1.3 ± 0.1 g following 60 min incubation, n=6-12, Figure 7.1a).

In order to assess the effects of higher concentrations of hydrogen peroxide on PE-induced contraction, the concentration of hydrogen peroxide was increased to 1 mM. In these experiments following induction of PE (10 nM)-induced contraction (1.3 ± 0.1 g, n=12) in endothelium-containing rings, addition of hydrogen peroxide (1 mM) produced a powerful long-lasting relaxation (96.2 ± 3.5 %, n=12), as previously reported in Chapter 5. When the incubation was continued for 15, 30 or 60 min and the tissues were washed, subsequent PE (10 nM)-induced contraction was inhibited in a time-dependent manner. Incubation of endothelium-containing rings with hydrogen peroxide (1 mM) for 15 min followed by washout, had no effect on PE (10 nM)-induced contraction (n=12, Figure 7.1b and 7.3). In contrast, incubation for 30 min followed by washout significantly depressed PE (10 nM)-induced contraction (by 36.5 ± 3.1 %, n=12, Figure 7.1b, 7.6b and 7.8). We ensured, however, that in relaxation studies the level of contraction prior to adding acetylcholine was similar (1.2 ± 0.1 g, n=12) to that of untreated preparations (1.3 ± 0.1 g, n=12) by increasing the concentration of PE to 100 nM. Furthermore, incubation with hydrogen peroxide for 60 min followed by washout depressed PE (10 nM)-induced contraction (n=6-12, Figure 7.1b and 7.3) to such an extent that the maximum contraction obtained in these tissues was only 0.6 ± 0.03 g with PE at 3 μM. These findings suggest that hydrogen peroxide (1 mM) induces damage to the vascular smooth muscle .

7.2 Effects of incubation with hydrogen peroxide on acetylcholine (ACh)-induced relaxation

7.2.1 Effects of incubation with hydrogen peroxide (0.1 mM) on ACh-induced relaxation

In order to determine if hydrogen peroxide impairs endothelial function,

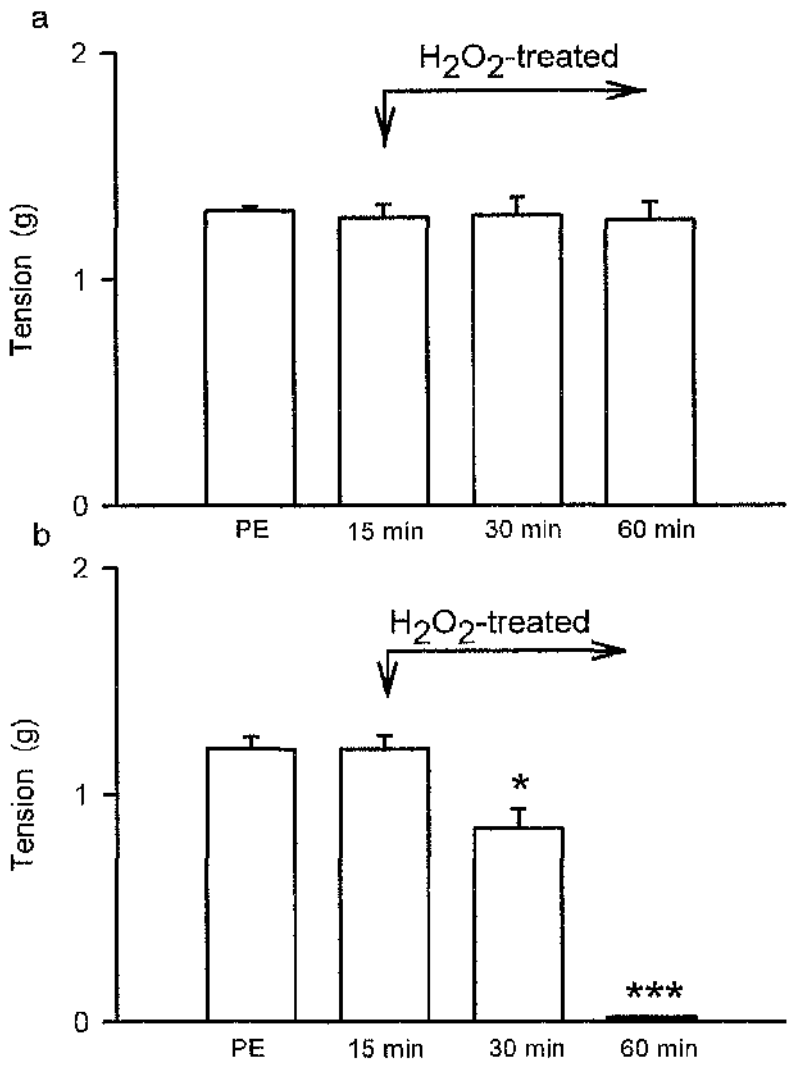


Figure 7.1 (a) Incubation of endothelium-containing rat aortic rings with hydrogen peroxide (H_2O_2, 0.1 mM) for 15, 30 or 60 min followed by washout had no effect on subsequent phenylephrine (PE, 10 nM)-induced contraction. **(b)** Incubation of endothelium-containing rings with H_2O_2 (1 mM) for 15 min followed by washout had no effect on PE (10 nM)-induced contraction. Extending the incubation period to 30 or 60 min followed by washout, however, significantly depressed the contraction generated. Each column is the mean ± s.e. mean of 6-12 observations. *P<0.05 and ***P<0.001 indicate a significant difference from rings receiving PE only, respectively.

the effects of incubation with hydrogen peroxide (0.1 mM) for various times were assessed on ACh (10 nM-3 μM)-induced relaxation. Following induction of PE (10-30 nM)-induced contraction (1.3 ± 0.1 g, n=6) in endothelium-containing rings, incubation with hydrogen peroxide (0.1 mM) for 15, 30 or 60 min followed by washout and re-contraction with PE (10-30 nM), had no effect on subsequent ACh (10 nM-3 μM)-induced relaxation (n=6, Figure 7.2a). Thus, hydrogen peroxide (0.1 mM) did not appear to impair endothelial function.

7.2.2 Effects of incubation with hydrogen peroxide (1 mM) on ACh-induced relaxation

In order to determine if higher concentrations of hydrogen peroxide had any effect on endothelial function, the effects of incubation with hydrogen peroxide (1 mM) for various times were assessed on ACh-induced relaxation.

7.2.2.1 Effects of short exposure to hydrogen peroxide (1 mM) on ACh-induced relaxation

Following induction of PE (10-30 nM)-induced contraction (1.2 ± 0.1 g, n=12), incubation of endothelium-containing rings with hydrogen peroxide (1 mM) for 15 min followed by washout and re-contraction with PE resulted in a profound potentiation of subsequent relaxation at each concentration of ACh (greatest enhancement was seen at 100 nM, n=12, Figure 7.2b, 7.3, 7.4 and 7.5a).

In order to investigate if the potentiation of ACh-induced relaxation seen following a short (15 min) exposure to hydrogen peroxide (1 mM) was sustained, a second concentration-response curve to ACh was constructed

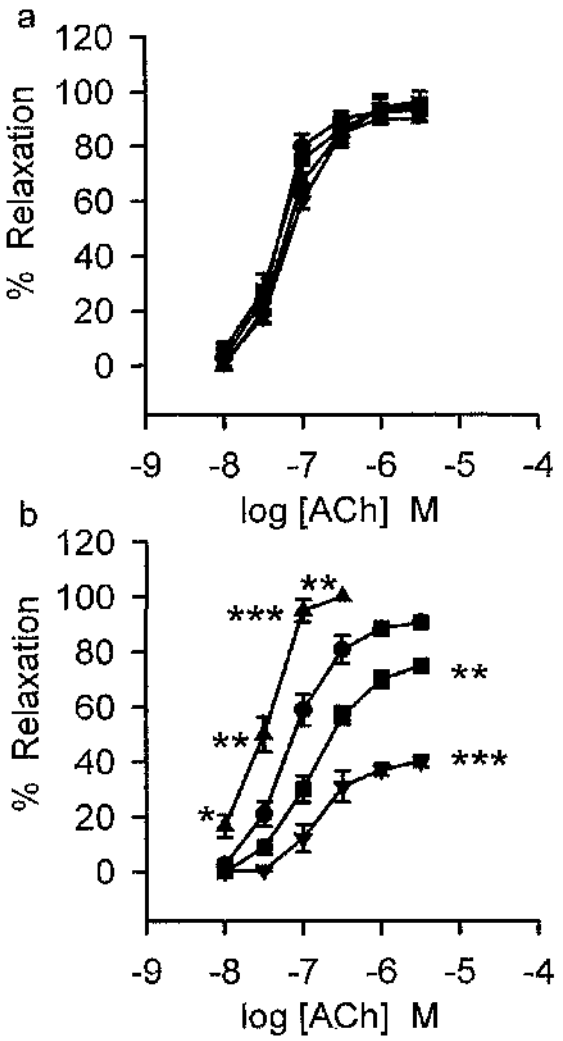


Figure 7.2 (a) Concentration-response curves showing relaxation to acetylcholine (ACh, ●) following incubation with hydrogen peroxide (H_2O_2, 0.1 mM) for 15 min (▲), 30 min (■) or 60 min (▼) followed by washout on phenylephrine-contracted endothelium-containing rings of rat aorta. **(b)** Concentration-response curves showing relaxation to ACh (●) and potentiation of this following incubation with H_2O_2 (1 mM) for 15 min followed by washout (▲). Furthermore, incubation with H_2O_2 (1 mM) for 30 min (■) or 60 min (▼) followed by washout resulted in a significant blockade of ACh-induced relaxation. Each point is the mean ± s.e. mean of 5-12 observations. *P<0.05, **P<0.005 and ***P<0.001 indicate a significant difference from relaxations obtained in untreated rings, respectively.

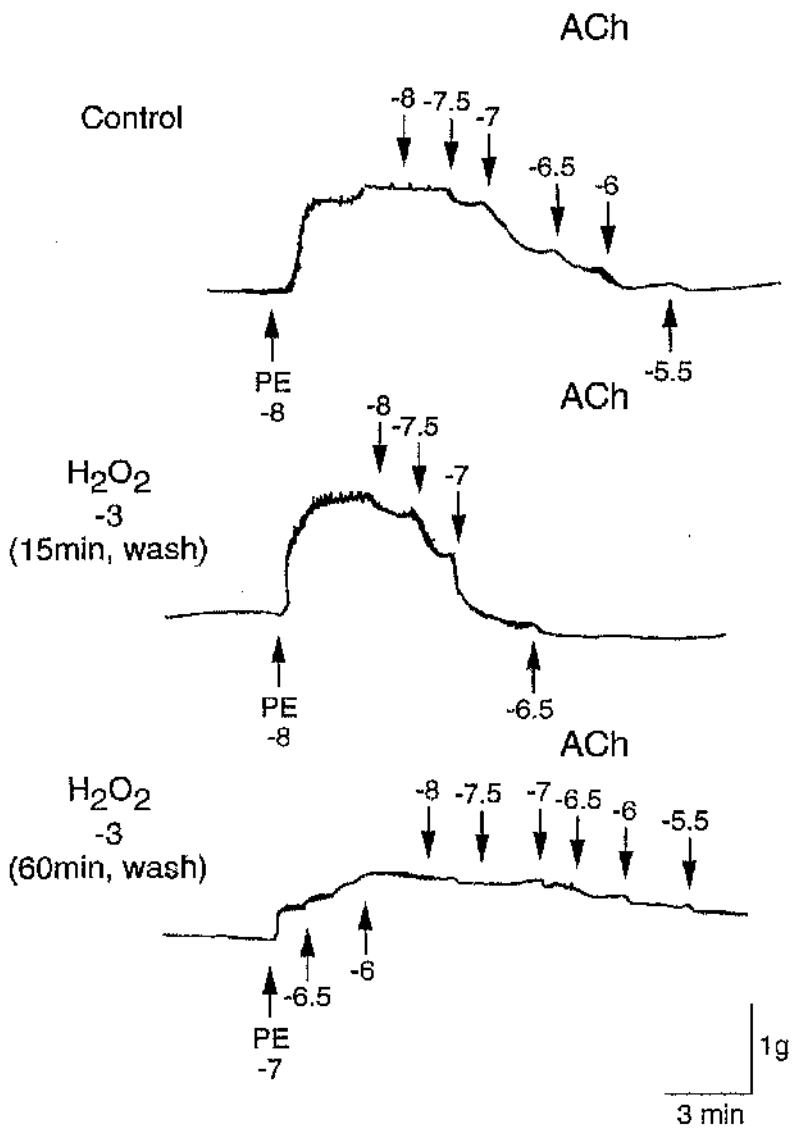


Figure 7.3 Individual experimental traces showing the potentiation of acetylcholine (ACh)-induced relaxation after 15 min incubation with hydrogen peroxide (H_2O_2, 1 mM) followed by washout and blockade of relaxation following incubation with H_2O_2 for 60 min followed by washout on phenylephrine (PE)-contracted endothelium-containing rings of rat aorta. Drug concentrations are expressed in log molar units.

after 2h in the same tissue. This second concentration-response curve to ACh demonstrated a substantial blockade of relaxation: the greatest blockade was seen at 100 nM where the relaxation was reduced from 91.4 ± 3.9 % to 27.4 ± 4.5 % (n=8-9, Figure 7.4).

7.2.2.2 Effects of longer exposure to hydrogen peroxide (1 mM) on ACh-induced relaxation

In contrast to short (15 min) exposure, incubation of endothelium-containing rings, precontracted with PE (10-30 nM), with hydrogen peroxide (1 mM) for 30 and 60 min followed by washout and re-contraction with PE resulted in a profound blockade of ACh-induced relaxation: the maximal relaxation induced by ACh (3 μM) was reduced by 17.3 ± 0.6 % and 55.9 ± 2.7 %, respectively (n=6-12, Figure 7.2b).

The above findings demonstrate that hydrogen peroxide causes a concentration- and time-dependent impairment of ACh-induced relaxation.

7.2.3 Effects of exogenous catalase on impairment of ACh-induced relaxation by hydrogen peroxide

Catalase removes hydrogen peroxide by converting it to water and oxygen (Cheeseman & Slater, 1993). Consequently, the ability of catalase (1000 u ml^{-1}) to protect against the impairment induced by hydrogen peroxide (1 mM) of ACh-induced relaxation was studied. In these experiments endothelium-containing rings were pretreated with catalase (1000 u ml^{-1}) for 15 min prior to the incubation with hydrogen peroxide (1 mM). Pretreatment with catalase completely prevented the ability of hydrogen peroxide (15 min, followed by washout) to potentiate ACh-induced relaxation (n=5, Figure 7.5a).

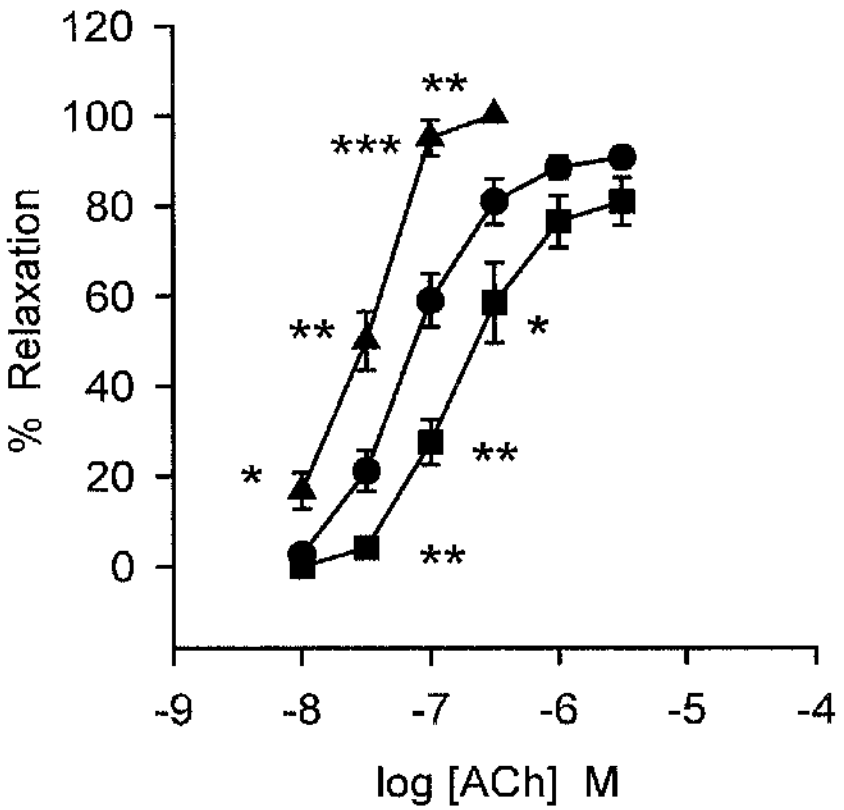


Figure 7.4 Concentration-response curves showing relaxation to acetylcholine (ACh, ●) on phenylephrine-contracted endothelium-containing rings of rat aorta, potentiation of this relaxation following incubation with hydrogen peroxide (H_2O_2, 1 mM) for 15 min followed by washout (▲) and subsequent blockade (■), evident from a second concentration-response curve constructed after 2h in the same tissue. Each point is the mean ± s.e. mean of 8-9 observations. *P<0.05, **P<0.005 and ***P<0.001 indicate a significant difference from relaxations obtained in untreated rings, respectively.

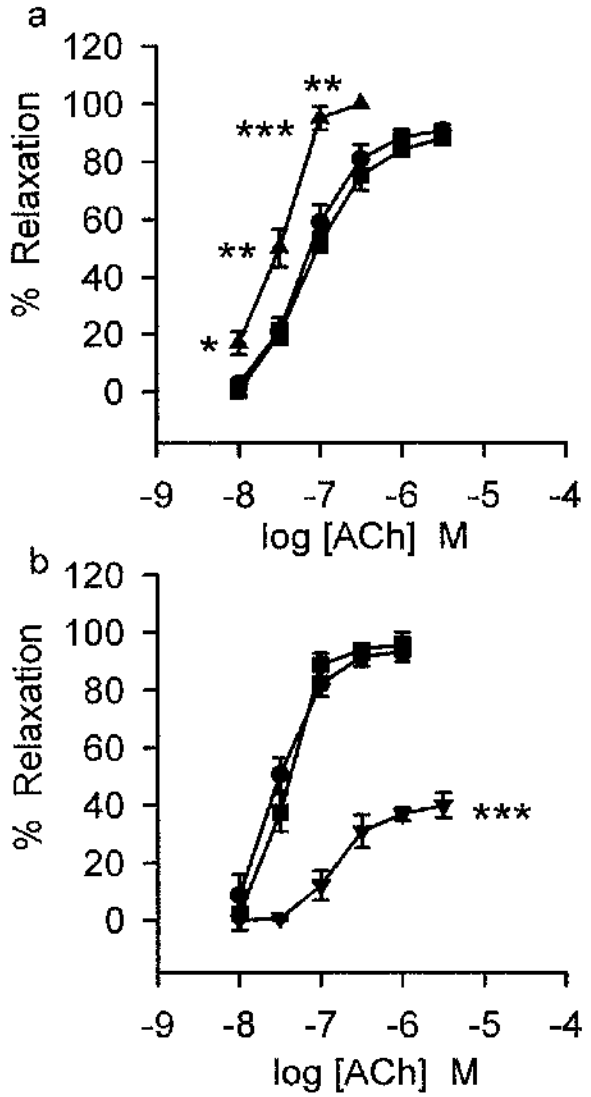


Figure 7.5 Concentration-response curves showing relaxation to acetylcholine (ACh, ●), potentiation of this relaxation after 15 min incubation with hydrogen peroxide (H_2O_2, 1 mM) followed by washout (a, ▲) and blockade of relaxation after 60 min incubation with H_2O_2 (1 mM) followed by washout (b, ▼) on phenylephrine-contracted endothelium-containing rings of rat aorta. Pretreatment with catalase (1000 u ml^{-1}, ■) completely protected against both the augmentation and inhibition produced by H_2O_2. Each point is the mean ± s.e. mean of 5-6 observations. *P<0.05, **P<0.005 and ***P<0.001 indicate a significant difference from relaxations obtained in untreated rings, respectively.

It also completely prevented the blockade of ACh-induced relaxation following 60 min incubation with hydrogen peroxide (n=6, Figure 7.5b).

7.3 Effects of 3-amino-1-2,4,-triazole (AT) on hydrogen peroxide-induced impairment of PE-induced contraction

If endogenous catalase is important in protecting vascular function from hydrogen peroxide, then its inactivation with AT would be expected to potentiate the inhibitory effects of this oxidant. Consequently, experiments were carried out to investigate the effects of AT on the ability of hydrogen peroxide (0.1 or 1 mM) to depress PE-induced contraction. In these experiments, incubation of endothelium-containing rings with AT (50 mM, 90 min, followed by washout) alone, to inhibit endogenous catalase, had no effect on subsequent PE (10 nM)-induced contraction (1.3 ± 0.1 g, n=10, Figure 7.6a and 7.6b). Similarly, incubation of tissues with hydrogen peroxide (0.1 mM, 30 min, followed by washout) alone had no effect on PE (10 nM)-induced contraction (n=6-10, Figure 7.1a, 7.6a). Furthermore, incubation of tissues with AT (50 mM) for 90 min together with hydrogen peroxide (0.1 mM) for the last 30 min followed by washout had no effect on PE (10 nM)-induced contraction (n=6-10, Figure 7.6a).

The effects of AT together with hydrogen peroxide at 1 mM on PE-induced contraction were also studied. In these experiments, incubation of endothelium-containing rings with hydrogen peroxide (1 mM, 30 min, followed by washout) alone significantly depressed subsequent PE (10 nM)-induced contraction (by 36.2 ± 3.7 %, n=10, Figure 7.1b, 7.6b and 7.8). In contrast to its lack of effect when combined with hydrogen peroxide at 0.1 mM, treatment with AT (50 mM, 90 min) markedly potentiated the ability of hydrogen

peroxide at 1 mM (present during the final 30 min of 90 min incubation, followed by washout) to depress PE (10 nM)-induced contraction: depression was increased to 98.0 ± 2.5 % ($P<0.001$, n=6-10, Figure 7.6b). In relaxation studies involving the combination of AT and hydrogen peroxide (1 mM, 30 min), an attempt was made to raise the level of contraction to that of untreated preparations (1.3 ± 0.1 g, n=10) by increasing the concentration of PE, but the maximum contraction obtained in these tissues was only 0.6 ± 0.02 g with PE at 3 μM (n=10, Figure 7.8).

These findings suggest that endogenous catalase is important in protecting vascular smooth muscle against impairment by high (1 mM) concentrations of hydrogen peroxide.

7.4 Effects of inhibiting endogenous catalase with 3-amino-1,2,4-triazole (AT) on hydrogen peroxide-induced impairment of vascular relaxation of rat aorta

7.4.1 Effects of inhibition of endogenous catalase by AT on hydrogen peroxide-induced impairment of endothelium-dependent relaxation

Having established the effects of hydrogen peroxide alone and together with the inhibition of endogenous catalase with AT on PE-induced contraction, the effects of this combined treatment were studied on the endothelium-dependent relaxation induced by ACh.

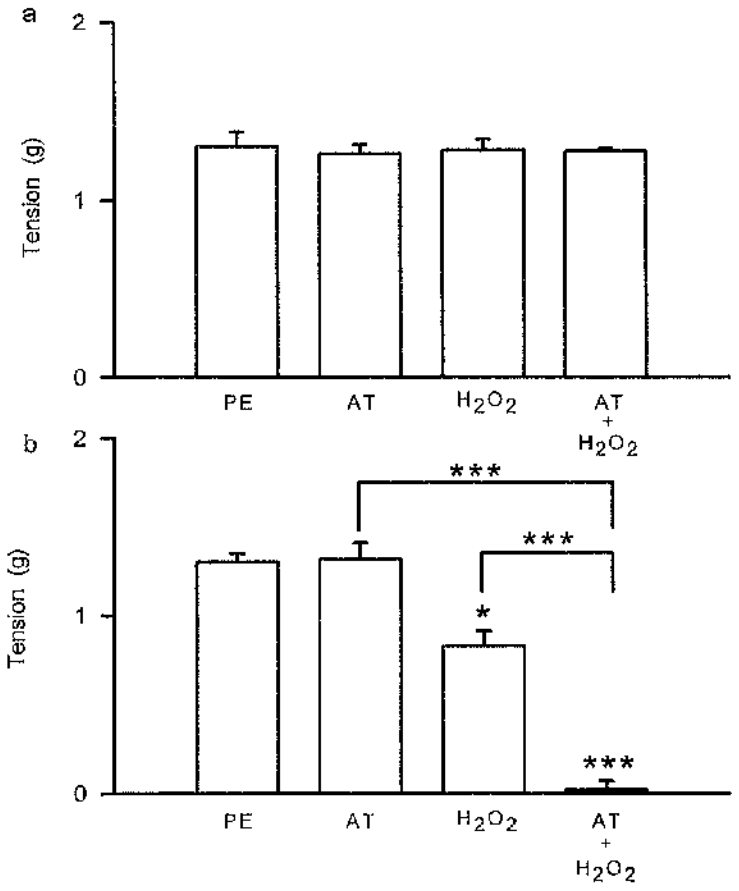


Figure 7.6 (a) Incubation with the inhibitor of catalase, 3-amino-1,2,4-triazole (AT, 50 mM, 90 min, followed by washout) alone, incubation with hydrogen peroxide (H_2O_2, 0.1 mM, 30 min, followed by washout) alone, or the combined treatment with AT (50 mM, 90 min) and H_2O_2 (0.1 mM, present during the last 30 min of the 90 min incubation, followed by washout) had no effect on phenylephrine (PE, 10 nM)-induced contraction in endothelium-containing rings of rat aorta. **(b)** Incubation with H_2O_2 (1 mM, 30 min, followed by washout), however, significantly depressed PE-induced contraction. Furthermore, contraction was depressed further if H_2O_2 (1 mM) was present during the last 30 min of the 90 min incubation with AT. Each column is the mean ± s.e. mean of 6-10 observations. *P<0.05, ***P<0.001 indicate a significant difference from rings receiving PE only, respectively, or between groups joined by brackets.

7.4.1.1 Effects of AT with hydrogen peroxide at 0.1 mM on ACh-induced relaxation

Treatment with AT (50 mM) for 90 min followed by washout, to inhibit endogenous catalase, alone (n=12, Figure 7.7 and 7.8), or incubation with low concentrations of hydrogen peroxide (0.1 mM) alone for 30 min followed by washout had no effect on subsequent ACh-induced relaxation (n=12, Figure 7.2a and 7.7). Furthermore, treatment of endothelium-containing rings with AT (50 mM) for 90 min together with hydrogen peroxide (0.1 mM) for the last 30 min followed by washout had no effect on ACh (10 nM-3 μM)-induced relaxation (n=6, Figure 7.7).

7.4.1.2 Effects of AT with hydrogen peroxide at 1 mM on ACh-induced relaxation

Incubation of endothelium-containing rings with hydrogen peroxide (1 mM) alone for 30 min followed by washout produced a significant inhibition (21.0 ± 0.7 %) of maximal ACh-induced relaxation at 3 μM (Figure 7.2b, 7.8 and 7.9).

In contrast to its lack of effect when combined with hydrogen peroxide at 0.1 mM, treatment with AT (50 mM, 90 min) markedly potentiated the ability of hydrogen peroxide at 1 mM (present during the final 30 min of 90 min incubation, followed by washout) to inhibit ACh (10 nM-3 μM)-induced relaxation. The blockade of maximal relaxation induced by ACh at 3 μM was increased to 68.0 ± 6.2 % ($P<0.001$ for each, n=6-10, Figure 7.8 and 7.9).

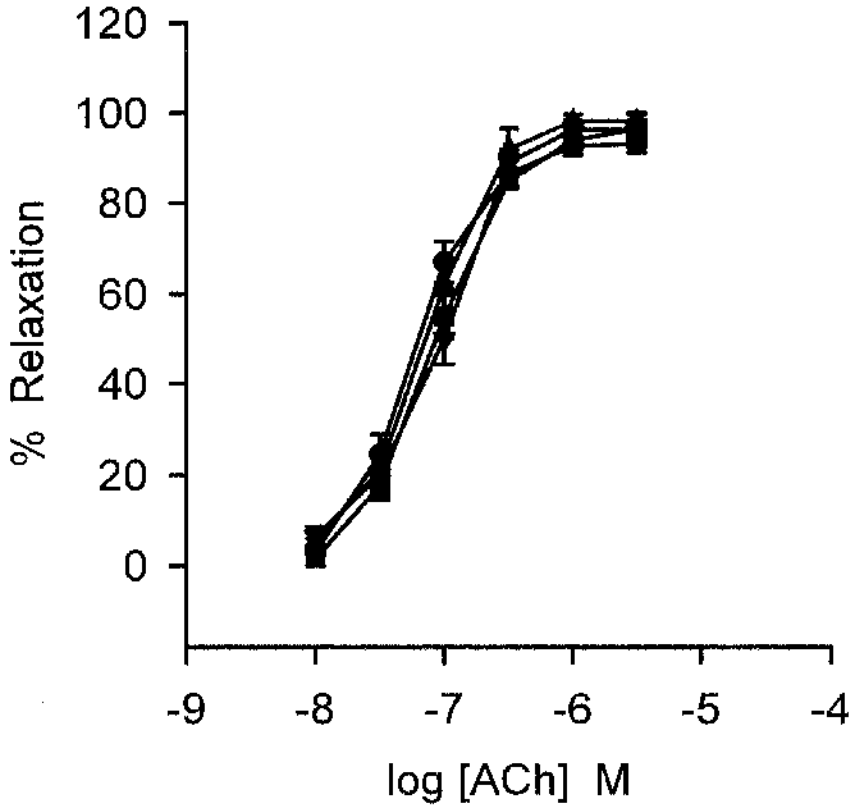


Figure 7.7 Concentration response curves showing relaxation to acetylcholine (ACh, ●) on phenylephrine-contracted endothelium-containing rings of rat aorta and the lack of effect of inhibition of endogenous catalase with 3-amino-1,2,4-triazole (AT, 50 mM, 90 min, followed by washout, ▼) or incubation with hydrogen peroxide (H_2O_2, 0.1 mM, 30 min, followed by washout, ■) on this relaxation. Furthermore, incubation with AT (50 mM, 90 min) together with H_2O_2 (0.1 mM) during the final 30 min followed by washout (▲) also had no effect on this relaxation. Each point is the mean ± s.e. mean of 6-12 observations.

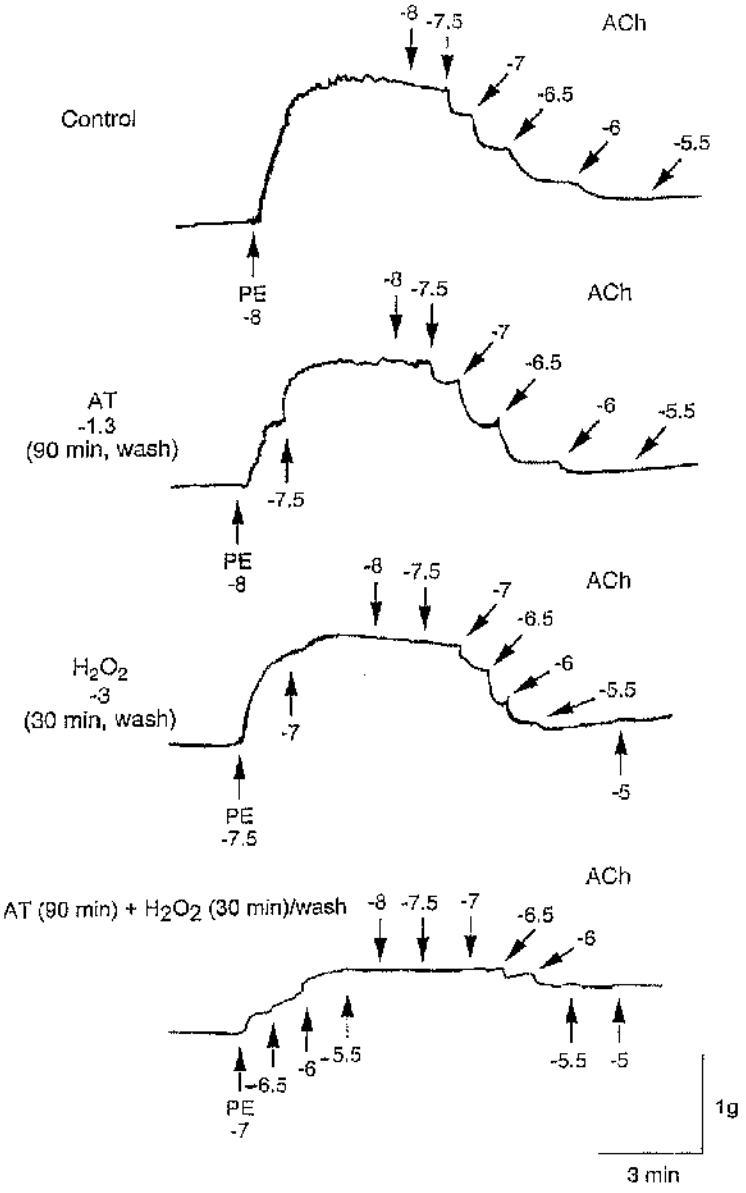


Figure 7.8 Individual experimental traces showing the lack of effect of the inhibitor of catalase, 3-amino-1,2,4-triazole (AT, 50 mM, 90 min, followed by washout), on acetylcholine (ACh)-induced relaxation of phenylephrine (PE)-contracted endothelium-containing rings of rat aorta. Also shown is blockade of ACh-induced relaxation following incubation with hydrogen peroxide (H_2O_2, 1 mM, 30 min, followed by washout), and the potentiation of this blockade following inhibition of endogenous catalase with AT (50 mM, 90 min). H_2O_2 alone or, more profoundly, together with AT also depressed PE-induced contraction. Drug concentrations are expressed in log molar units.

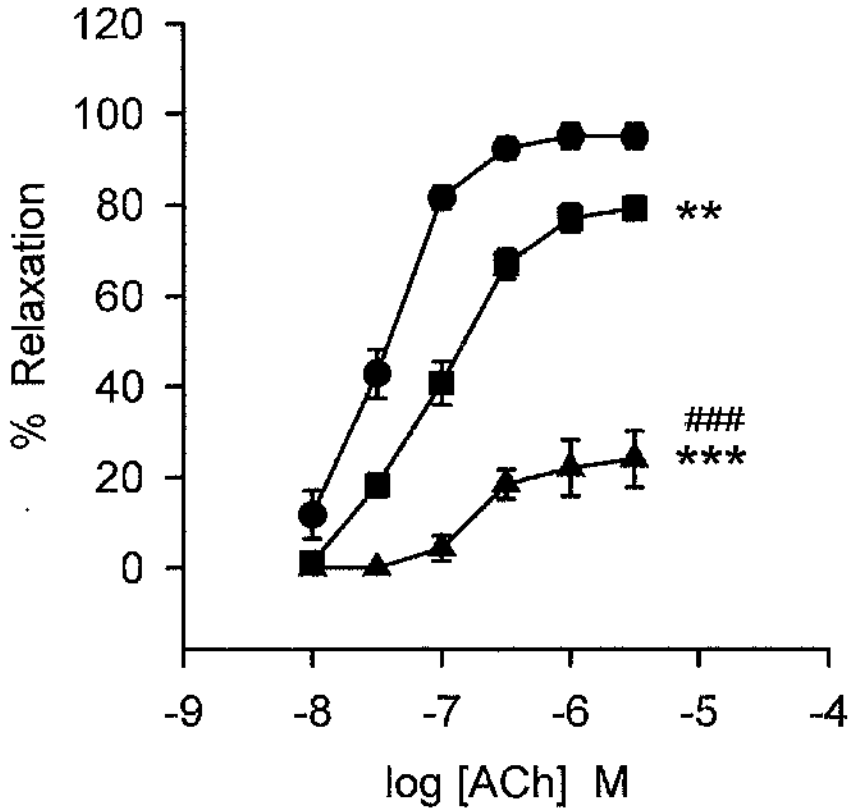


Figure 7.9 Concentration-response curves showing relaxation to acetylcholine (ACh, ●) on phenylephrine-contracted endothelium-containing rings of rat aorta and blockade of relaxation following incubation with hydrogen peroxide (H_2O_2, 1 mM, 30 min, followed by washout, ■). Furthermore, following inhibition of endogenous catalase with 3-amino-1,2,-4-triazole (AT, 50 mM, 90 min) incubation with H_2O_2 (1 mM, present for last 30 min of the 90 min incubation, followed by washout) now produced a much greater blockade (▲) of relaxation induced by ACh. Each point is the mean ± s.e. mean of 6-10 observations. **P<0.005 and ***P<0.001 indicate a significant difference from maximal relaxation in untreated rings, respectively. ###P<0.001 indicates a significantly greater blockade following combined treatment with AT and H_2O_2 than with H_2O_2 alone.

These findings suggest an important role for endogenous catalase in protecting ACh-induced relaxation against impairment by high (1 mM) concentrations of hydrogen peroxide.

7.4.2 Effects of inhibition of endogenous catalase by AT on hydrogen peroxide-induced impairment of endothelium-independent relaxation

Since inhibition of endogenous catalase with AT potentiated the impairment of endothelium-dependent, ACh-induced relaxation of rat aorta following treatment with hydrogen peroxide (1 mM), the effects on relaxation induced by the endothelium-independent relaxants, glyceryl trinitrate and isoprenaline, were examined.

7.4.2.1 Effects of AT with hydrogen peroxide at 0.1 mM on relaxations induced by glyceryl trinitrate and isoprenaline

Incubation of endothelium-denuded rings with AT (50 mM, 90 min, followed by washout), had no effect on relaxations induced by glyceryl trinitrate (GTN, 1-100 nM, n=6-10, Figure 7.10a) or isoprenaline (ISO, 10 nM-3 μM, n=6-10, Figure 7.11a).

Incubation with a low concentration of hydrogen peroxide (0.1 mM, 30 min, followed by washout) alone or in combination with AT (50 mM, 90 min, followed by washout) failed to affect relaxations induced by GTN or ISO (n=10, Figure 7.10a and 7.11a).

7.4.2.2 Effects of AT with hydrogen peroxide at 1 mM on relaxations induced by glyceryl trinitrate and isoprenaline

Incubation of endothelium-denuded rings with a higher concentration of hydrogen peroxide (1 mM) for 30 min followed by washout did, however, inhibit maximal relaxation induced by GTN and ISO by 28.6 ± 1.9 % and 29.9 ± 1.3 %, respectively, (n=6, Figure 7.10b and 7.11b). Furthermore, as was seen with ACh, inhibition of endogenous catalase with AT (50 mM, 90 min) significantly enhanced the ability of hydrogen peroxide (1 mM, present during the final 30 min of 90 min incubation, followed by washout) to block relaxation induced by GTN or ISO: the blockade of maximal relaxation induced by GTN and ISO was increased to 73.1 ± 7.1 % and 64.0 ± 5.5 %, respectively ($P<0.001$ for each, n=6, Figure 7.10b and 7.11b).

The data presented in this Chapter suggest that hydrogen peroxide produces non-selective damage to the vascular smooth muscle resulting in loss of contractile and relaxant actions, and that endogenous catalase plays an important role in protection against this damage.

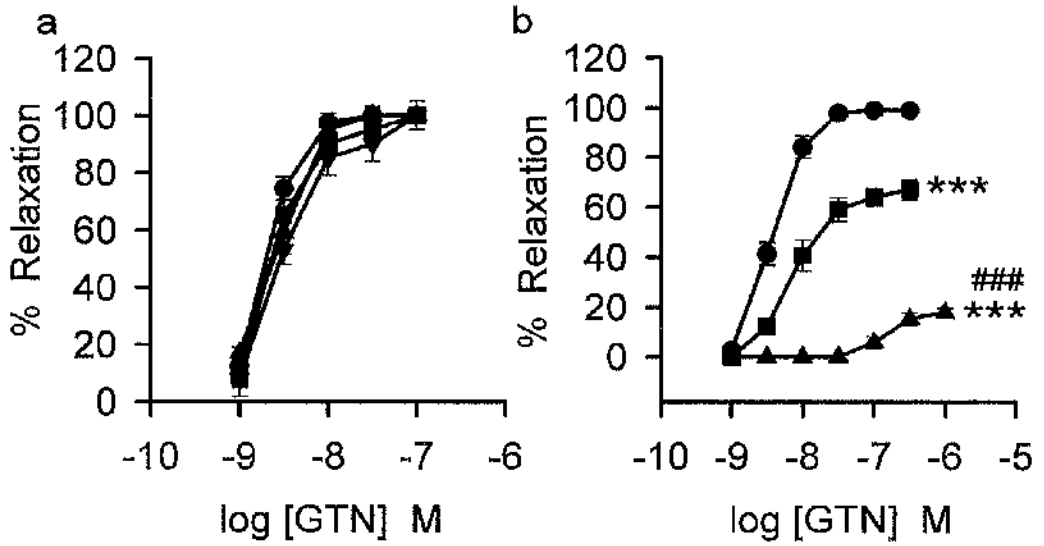


Figure 7.10 (a) Concentration-response curves showing relaxation to glyceryl trinitrate (GTN, ●) on phenylephrine (PE)-contracted endothelium-denuded rings of rat aorta and the lack of effect of inhibition of endogenous catalase with 3-amino-1,2,4-triazole (AT, 50 mM, 90 min, followed by washout, ▼) or incubation with hydrogen peroxide (H_2O_2, 0.1 mM, 30 min, followed by washout, ■) on this relaxation. Furthermore, incubation with AT (50 mM, 90 min) together with H_2O_2 (0.1 mM) during the final 30 min followed by washout (▲) also had no effect on this relaxation. **(b)** Concentration-response curves showing relaxation to GTN (●) on PE-contracted endothelium-denuded rings and blockade of relaxation following incubation with H_2O_2 (1 mM, 30 min, followed by washout, ■). Furthermore, following inhibition of endogenous catalase with AT (50 mM, 90 min) incubation with H_2O_2 (1 mM, present for last 30 min of the 90 min incubation, followed by washout) now produced a much greater blockade (▲) of relaxation. Each point is the mean ± s.e. mean of 6-10 observations. ***$P<0.001$ indicates a significant difference from maximal relaxation in untreated rings. ###$P<0.001$ indicates a significantly greater blockade following combined treatment with AT and H_2O_2 than with H_2O_2 alone.

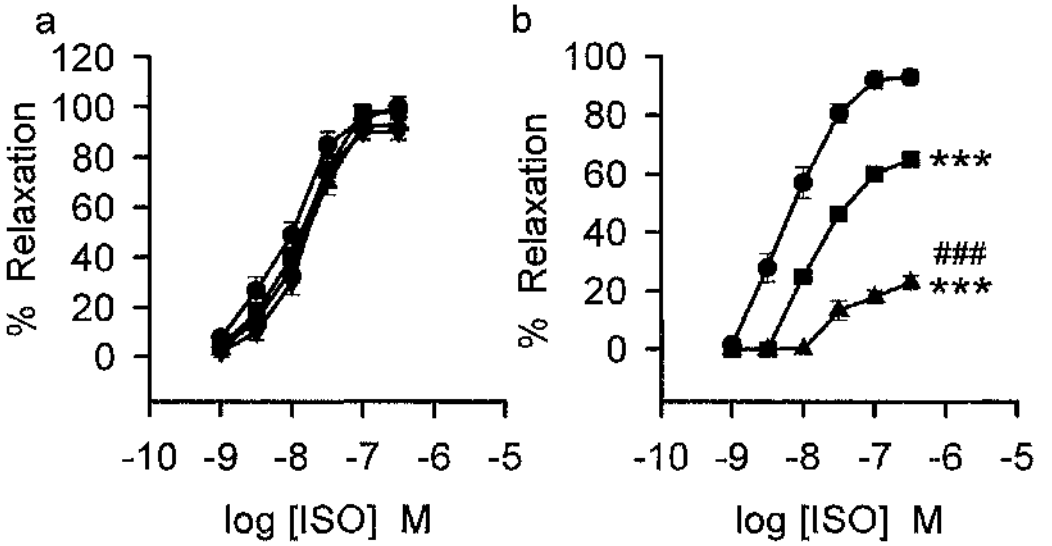


Figure 7.11 (a) Concentration-response curves showing relaxation to isoprenaline (ISO, ●) on phenylephrine (PE)-contracted endothelium-denuded rings of rat aorta and the lack of effect of inhibition of endogenous catalase with 3-amino-1,2,4-triazole (AT, 50 mM, 90 min, followed by washout, ▼) or incubation with hydrogen peroxide (H_2O_2, 0.1 mM, 30 min, followed by washout, ■) on this relaxation. Furthermore, incubation with AT (50 mM, 90 min) together with H_2O_2 (0.1 mM) during the final 30 min followed by washout (▲) also had no effect on this relaxation. **(b)** Concentration-response curves showing relaxation to ISO (●) on PE-contracted endothelium-denuded rings and blockade of relaxation following incubation with H_2O_2 (1 mM, 30 min, followed by washout, ■). Furthermore, following inhibition of endogenous catalase with AT (50 mM, 90 min) incubation with H_2O_2 (1 mM, present for last 30 min of the 90 min incubation, followed by washout) now produced a much greater blockade (▲) of relaxation. Each point is the mean ± s.e. mean of 6-10 observations. ***$P<0.001$ indicates a significant difference from maximal relaxation in untreated rings. ###$P<0.001$ indicates a significantly greater blockade following combined treatment with AT and H_2O_2 than with H_2O_2 alone.

DISCUSSION

Chapter 8

8.1 Effects of superoxide anion on basal and agonist-stimulated activity of NO

The importance of the endothelium in the regulation of vascular tone has become increasingly apparent over the last decade. The demonstration of the phenomenon of "endothelium-dependent relaxation", in 1980, by Furchgott and Zawadzki and its mediation by a labile factor termed endothelium-derived relaxing factor (EDRF), has revolutionised our understanding of how local vascular tone is regulated. This has led to a re-examination of the mechanisms by which various vasoactive mediators produce their effects. Many vasoactive substances have now been classified as acting entirely through the endothelium to produce vasorelaxation, whereas other agents exert their effects on the smooth muscle. EDRF-mediated vasorelaxation is associated with enhanced levels of cGMP in the smooth muscle cells, resulting from the stimulation of soluble guanylate cyclase (Holzmann, 1982; Rapoport & Murad, 1983). Similarly, nitrovasodilators produce vasorelaxation by the formation of NO and enhancement in cGMP levels (Murad, 1994), suggesting a common mechanism of action. Indeed, in 1987, Palmer *et al.* demonstrated that NO accounts for the biological actions of EDRF and therefore EDRF may be considered as an endogenous nitrovasodilator. EDRF is derived from either of the guanidino nitrogens of L-arginine by the constitutively expressed enzyme, NO synthase (Rees *et al.*, 1989; 1990a; Mülsch & Busse, 1990).

In addition to NO, endothelial cells are a potential source of superoxide anion (Rosen & Freeman, 1984). Recently, the interaction between NO and superoxide anion has received a great deal of attention. This interaction leads

to various consequences: destruction of the vasodilator effects of EDRF and authentic NO in cascade bioassay systems (Gryglewski *et al.*, 1986b; Rubanyi & Vanhoutte, 1986a; Furchgott *et al.*, 1990); induction of endothelium-dependent vasoconstriction in arterial rings from loss of the dilator actions of basally produced NO (Katusic & Vanhoutte, 1989; Ohlstein & Nichols, 1989); and production of a cytotoxic oxidant, peroxynitrite (Beckman *et al.*, 1990). This widespread potential for loss of NO-induced vasodilatation and production of peroxynitrite following the reaction of NO with superoxide anion has led to the suggestion that this process contributes to a number of pathological situations such as hypertension, ischemia-reperfusion damage, hypercholesterolemia/atherosclerosis, diabetes and cytotoxic brain injury (Wei *et al.*, 1985; Downey, 1990; Minor *et al.*, 1990; Mügge *et al.*, 1991a; Langenstroer & Pieper, 1992; Lipton *et al.*, 1993).

In vascular endothelium, the production of NO is subject to complex control; it is stimulated by a large number of mediators such as acetylcholine (ACh; Furchgott & Zawadzki, 1980a) and by the physical shearing force of the flowing blood (Pohl *et al.*, 1986). In the present study, agonist-stimulated activity of NO was determined by assessing ACh-induced relaxation. In addition, there is a basal production of NO which exerts a tonic vasodilator action on endothelium-containing arterial rings (Eglème *et al.*, 1984; Martin *et al.*, 1986; Martin, 1988). Basal activity of NO was assessed indirectly by measuring the endothelium-dependent depression of phenylephrine (PE)-induced vasoconstriction (Martin *et al.*, 1986). This endothelium-dependent depression of vasoconstriction would be expected to be blocked or enhanced by agents which inhibit or enhance the activity of NO, respectively.

Recent reports have suggested differences in the effects of drugs on basal and agonist-stimulated activity of NO. For example, the sulphydryl alkylating agent, N-ethylmaleimide, selectively inhibits the activity of NO stimulated by agonists from cultured endothelial cells, but has no effect on basal activity of NO (Siegle *et al.*, 1989). Furthermore, in the perfused vascular bed of the rabbit ear, N^G-nitro-L-arginine methyl ester (L-NAME) inhibits both basal and agonist-stimulated activity of NO, but only in the former case is blockade reversed by L-arginine (Randall & Griffith, 1991). Moreover, in the isolated coronary artery of the greyhound, the inhibitor of acetyl-coA lysolecithin acyltransferase, thimerosal, has a complex action, transiently producing endothelium-dependent relaxation and then blocking agonist-stimulated but not basal production of NO (Crack & Cocks, 1992). In addition to these data, work from our own laboratory has shown in the rat aorta, that N^G-monomethyl-L-arginine (L-NMMA) selectively inhibits basal but not agonist-stimulated activity of NO (Frew *et al.*, 1993). Such studies may suggest a difference in the chemical nature of basal and agonist-stimulated EDRF.

In this part of the study, we sought to determine if basal and ACh-stimulated activity of NO in rat aorta were equally sensitive to destruction by superoxide anion. These experiments were considered important in relation to the reported ability of superoxide dismutase to potentiate the effects of basal NO but to have no effect on the relaxant actions of ACh in arterial rings (Abrahamsson *et al.*, 1992), suggesting differential actions of superoxide anion on basal and agonist-stimulated activity. In our experiments the effects of superoxide anion were assessed either by generating the free radical using hypoxanthine/xanthine oxidase or pyrogallol (Halliwell & Gutteridge, 1989), or by increasing the background level of the free radical by inhibiting the endogenous Cu-Zn form of superoxide dismutase with diethyldithiocarbamate (DETCA, Heikkila *et al.*, 1976; Cocco *et al.*, 1981; Halliwell & Gutteridge, 1989).

8.1.1 Effects of superoxide dismutase on basal and ACh-stimulated activity of NO

Superoxide dismutase (SOD) scavenges superoxide anion ($^{\bullet}O_2^-$) by converting it to hydrogen peroxide (H_2O_2) and oxygen (Halliwell & Gutteridge, 1989; Cheeseman & Slater, 1993).

$$^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

It is well established that exogenously added SOD potentiates both basal and agonist-stimulated activity of NO in cascade bioassay systems (Gryglewski *et al.*, 1986b; Rubanyi & Vanhoutte, 1986a). In isolated arterial rings SOD also appears to potentiate basal activity (Ohlstein & Nichols, 1989; Langenstroer & Pieper, 1992) but, surprisingly has no effect on agonist-stimulated activity of NO (Abrahamsson *et al.*, 1992). Clearly, the apparent differential effects of SOD on basal and agonist-stimulated activity of NO need to be explained.

We found that SOD (Cu-Zn-containing isoform, from bovine erythrocytes, 1-300 u ml^{-1}) produced powerful concentration-dependent relaxation of phenylephrine (PE)-contracted endothelium-containing rings of rat aorta but had no effect on endothelium-denuded rings. The relaxation induced by SOD appeared to be mediated by NO since pretreatment of endothelium-containing rings with N^G-nitro-L-arginine (L-NOARG, 30 μM), an inhibitor of NO synthesis (Moore *et al.*, 1990), completely blocked this effect. The relaxant effects of SOD are therefore likely to have arisen from removal of superoxide anions generated either within the tissue or the oxygenated Krebs' solution, which were destroying basally produced NO. Indeed, endothelial cells in culture have been shown to generate and release superoxide anion (Rosen & Freeman,

1984). The Krebs' solution oxygenated with gas mixtures containing 95 % oxygen provides a ready source for superoxide anion generation. Having established that exogenous SOD induced endothelium-dependent relaxation, almost certainly through potentiation of basal activity of NO, the effects of this enzyme on ACh-stimulated activity of NO were determined. In keeping with Abrahamsson and co-workers (1992), we found that a concentration of SOD (50 u ml^{-1}) which induced almost maximal potentiation of basal activity of NO had absolutely no effect on ACh-induced relaxation. The background levels of superoxide anion therefore appeared to be exerting a selective destructive action on basal but not agonist-stimulated activity of NO. An explanation for this was not obvious, but in order to gain further insight into the mechanisms that may be responsible for this difference, the effects of generators of superoxide anion were examined.

8.1.2 Effects of hypoxanthine/xanthine oxidase on basal and ACh-stimulated activity of NO

The hypoxanthine/xanthine oxidase (HX/XO) system is commonly used for the generation of superoxide anions. HX/XO is a simple enzymic system where XO catalyses the conversion of HX to xanthine and superoxide anion. Xanthine is further converted to uric acid by XO with concomitant release of another superoxide anion. The biological significance of this superoxide anion generating system becomes increasingly apparent under pathological conditions such as ischemia-reperfusion injury where levels of hypoxanthine increase as does conversion of xanthine dehydrogenase to xanthine oxidase (Kloner *et al.*, 1989). The validity of this system to generate superoxide anion has been well established using the known ability of the radical to reduce oxidised cytochrome C (Fridovich, 1970; Berman & Martin, 1993). This reduction is associated with an increase in absorbance at 550 nm. Using this

technique, it was shown that HX and XO when present in combination led to reduction of oxidised cytochrome C, indicating the successful generation of superoxide anion.

Following induction of tone with PE, HX (0.1 mM)/XO (16 mu ml^{-1}) produced two effects on rings of rat aorta. The first of these effects was an immediate and profound rise in PE-induced tone in endothelium-containing rings. This effect, however, was absent in endothelium-denuded rings. It was also absent in endothelium-containing rings pretreated with the NO synthase inhibitors, L-NMMA (30 μM, Rees *et al.*, 1989; 1990a) or L-NOARG (30 μM, Moore *et al.*, 1990). The absolute dependence of the HX/XO-elicited rise of tone in PE-contracted rings upon the presence of endothelium suggests that it is due to destruction of basal NO by superoxide anion. This conclusion was indeed confirmed in experiments in which SOD (35 u ml^{-1}) produced an immediate reversal of the potentiation of PE-induced tone produced by HX/XO. Furthermore, the increase in PE-induced tone was not seen if the tissues were pretreated with SOD (250 u ml^{-1}). These results are therefore consistent with the previously reported endothelium-dependent vasoconstrictor action of superoxide anion (Katusic & Vanhoutte, 1989; Ohlstein & Nichols, 1989) mediated through destruction of basal NO.

These experiments were complicated by the fact that superoxide anion generating systems also give rise to other reactive species. Indeed, HX/XO was seen to produce a secondary effect, consisting of a delayed (about 5 min) fall in tone which was seen in both endothelium-containing and endothelium-denuded rings. It was thought likely that this relaxation resulted from the spontaneous dismutation of superoxide anion to form hydrogen peroxide. This, in fact, was confirmed with the use of catalase (CAT) which removes hydrogen peroxide by converting it to water and oxygen (Halliwell & Gutteridge, 1989; Cheeseman & Slater, 1993) as follows:

$$\xrightarrow{CAT} 2H_2O + O_2$$

Specifically, we found that the delayed relaxant action of HX/XO in both endothelium-containing and endothelium-denuded rings was abolished by the addition of catalase, confirming the involvement of hydrogen peroxide. Moreover, in presence of catalase (3000 u ml^{-1}), HX/XO was seen to produce only one effect; an immediate and sustained rise in PE-induced tone in endothelium-containing but not endothelium-denuded rings. Hydrogen peroxide is known to promote relaxation by two means, i.e. via an increased production of NO (Gryglewski *et al.*, 1986b) and by a direct effect on vascular smooth muscle (Burke & Wolin, 1987; Furchgott, 1991; Zembowicz *et al.*, 1993).

Since both superoxide anion and hydrogen peroxide are present and, provided there is a sufficient source of ferric iron, it is possible that hydroxyl radicals could also be formed by the Fenton reaction (Cross *et al.*, 1987; Halliwell & Gutteridge, 1989) as follows:

$$Fe^{3+} + {}^{\bullet}O_2^- \rightarrow Fe^{2+} + O_2$$

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

Also, it is feasible that endothelial NO reacts with superoxide anion to generate peroxynitrite anion which itself is a powerful oxidant and which can also lead to formation of hydroxyl radical by an iron-independent pathway (Beckman *et al.*, 1990) as shown below.

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

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

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

These reactive oxygen species generated from superoxide anion can damage the endothelium and thereby impair endothelium-dependent relaxations (Kvietys *et al.*, 1989; Beckman *et al.*, 1990; Todoki *et al.*, 1992; Dowell *et al.*, 1993). Since we wished to examine specifically the interaction between NO and superoxide anion, catalase (3000 u ml^{-1}) was included in all experiments in order to remove hydrogen peroxide.

A major novel finding of this study was that despite almost completely blocking basal activity of NO, as indicated by the profound enhancement of PE-induced tone in endothelium-containing rings, HX (0.1 mM)/XO (16 mu ml^{-1}) had no effect on the relaxation produced by ACh at any concentration. Thus, these results suggest a greater sensitivity of basal than ACh-stimulated activity of NO to destruction by superoxide anion.

8.1.3 Effects of pyrogallol on basal and ACh-stimulated activity of NO

Pyrogallol is known to generate superoxide anion by autooxidation. It is believed to autooxidise to its semiquinone which then reacts reversibly with oxygen to form quinone and superoxide anion (Halliwell & Gutteridge, 1989). We utilised pyrogallol as another source of superoxide anion, to determine if it affected basal and ACh-stimulated activity of NO in a similar way to HX/XO.

Like HX/XO, pyrogallol (0.1 mM) produced an immediate and profound rise in PE-induced tone in endothelium-containing rings of rat aorta. However, unlike with HX/XO exogenous SOD (250 u ml^{-1}) only partially reversed the augmentation of tone by pyrogallol, suggesting that only part of this rise (approx. 26 %) is mediated through generation of superoxide anion. Also unlike HX/XO, which enhanced PE-induced tone in endothelium-containing

rings only, pyrogallol also augmented tone in endothelium-denuded rings. However, the rise obtained was less than that obtained in endothelium-containing rings. The origin of endothelium-independent augmentation of tone by pyrogallol is unclear, but it is unrelated to the ability of the drug to generate superoxide anion since it was unaffected by SOD (250 u ml^{-1}). The endothelium-dependent component is, however, likely to be due to the destruction of basal NO by superoxide anion.

Like HX/XO, pyrogallol also produced a delayed (about 5 min) fall in tone in both endothelium-containing and endothelium-denuded rings which was blocked by catalase and is therefore likely to have resulted from the build up of hydrogen peroxide. In view of this, all subsequent experiments were conducted in the presence of catalase (3000 u ml^{-1}) to prevent the accumulation of hydrogen peroxide.

The effects of pyrogallol were then assessed on ACh-induced relaxation. In common with HX (0.1 mM)/XO (16 mu ml^{-1}), pyrogallol (0.1 mM), despite substantially blocking basal activity of NO, had no effect on ACh-induced relaxation at any concentration in endothelium-containing rings.

Thus, the results obtained with HX/XO and pyrogallol, together with those demonstrating SOD-induced relaxation, clearly highlighted a greater sensitivity of basal than agonist-stimulated activity of NO to destruction by superoxide anion. What was less clear at this stage, however, was whether the data suggested a chemical difference between basal and agonist-stimulated NO. Indeed, it has been hypothesised that EDRF is a mixture of NO and a stable NO-releasing molecule such as an S-nitrosothiol (Myers *et al.*, 1990). Specifically, it has been proposed that basal EDRF is free NO whereas the enhanced formation of EDRF elicited by endothelium-dependent relaxants such as ACh may be attributed to Ca^{2+}-dependent exocytosis of recently

formed S-nitrosothiol (Myers *et al.*, 1990; Ignarro, 1991; Cocks & Angus, 1991). Furthermore, Kukreja *et al.* (1993) compared the effects of NO and S-nitrosocysteine on cerebral arteriolar caliber in relation to the associated increment in nitrite concentration in the effluent and concluded that S-nitrosocysteine resembled EDRF more closely than did authentic NO.

Although our results, so far, indicated a difference in the susceptibility of basal and ACh-stimulated NO to destruction by superoxide anion, it was not yet clear if the difference was absolute or one of degree. Consequently, the level of superoxide anion generation was increased by employing higher concentrations of HX/XO and pyrogallol and the effects on ACh-induced relaxation were examined.

8.1.4 Effects of higher concentration of HX/XO and pyrogallol on ACh-induced relaxation

Increasing the concentration of HX to 1 mM (keeping XO at 16 mu ml^{-1}) and pyrogallol to 0.3 mM, however, now led in each case to a profound blockade of ACh-induced relaxation in PE-contracted endothelium-containing rings. In addition, the duration of relaxation induced by ACh was affected dramatically; in control tissues relaxation to high concentrations of ACh (> 0.1 μM) was sustained but in the presence of each of the superoxide generators, relaxation was transient. As in previous studies (Wei *et al.*, 1985; Rubanyi & Vanhoutte, 1986b; Abrahamsson *et al.*, 1992), the inhibition of ACh-induced relaxation was likely to have occurred as a consequence of destruction of agonist-stimulated NO by superoxide anion. This was confirmed in our experiments in which pretreatment with exogenous SOD (250 u ml^{-1}) completely protected against the inhibitory effects of HX/XO or pyrogallol. Thus, it may be concluded from these results that the differential sensitivity of basal and

agonist-stimulated NO is one of a degree rather than being absolute and that higher concentrations of superoxide anion are required to destroy agonist-stimulated NO than basal NO. It was still not clear why basal activity of NO should be more sensitive to destruction by superoxide anion than agonist-stimulated.

8.1.5 Effects of diethyldithiocarbamate on basal and ACh-stimulated activity of NO

It was possible that the greater sensitivity of basal than ACh-stimulated NO to destruction might reflect differential protection by endogenous SOD. This hypothesis was tested by inhibiting the enzyme.

Mammalian cells contain two major forms of SOD; a Cu-Zn-containing form which is located both extracellularly and intracellularly and a Mn-containing form which resides mainly in the mitochondria (Hassan, 1988). Cu-Zn SOD, but not Mn-SOD, is irreversibly inactivated by diethyldithiocarbamate (DETCA, Heikkila *et al.*, 1976; Cocco *et al.*, 1981; Halliwell & Gutteridge, 1989). DETCA irreversibly binds Cu at the active site and removes the metal from the enzyme. The copper ion appears to function in the enzymic reaction, whereas the zinc stabilises the enzyme (Taniguchi, 1992). DETCA inhibits Cu-Zn SOD both intracellularly and extracellularly (Kelner *et al.*, 1989), leading to increased levels of superoxide anion, as detected by lucigenin-elicited chemiluminescence (Cherry *et al.*, 1990; Omar *et al.*, 1991). Previous studies have shown that treatment of cultured endothelial cells in a cascade bioassay with DETCA leads to loss of NO activity and this was likely to have resulted from destruction rather than reduced synthesis of NO since the release of total nitrogen oxides, as measured by chemiluminescence, was unaffected (Mügge *et al.*, 1991b).

In this study, we found that treatment with DETCA (0.1 mM) for 1h followed by extensive washing, to remove any non-specific effects of the drug, enhanced the sensitivity of endothelium-containing rings to PE. Furthermore, L-NMMA (30 μM) which would ordinarily augment PE-induced tone in endothelium-containing rings through blocking synthesis of basal NO, failed to do so in DETCA-treated tissues. The effects of pretreatment with DETCA were also studied on endothelium-denuded rings. In contrast to the augmentation of tone in endothelium-containing rings, DETCA had no effect on the sensitivity of endothelium-denuded rings to PE. Thus, the augmentation of PE-induced tone in DETCA-treated, endothelium-containing tissues was likely to have occurred from the complete loss of basal activity of NO.

The restoration of basal activity of NO following addition of exogenous SOD (Cu-Zn-containing isoform from bovine erythrocytes, 250 u ml^{-1}) to DETCA-treated tissues, albeit reduced in comparison to control tissues, indicates that the loss had occurred as a consequence of destruction by the greater steady-state levels of superoxide anion. Thus, the effects of DETCA suggest that endogenous Cu-Zn SOD plays a vital role in protecting basal activity of NO from destruction by superoxide anion. Furthermore, in these experiments where treatment with DETCA had led to a complete loss of basal activity of NO, ACh-induced relaxation was blocked only slightly, further strengthening the view that basal and agonist-stimulated activity are differentially sensitive to destruction by superoxide anion.

Others have reported that increasing the concentration of DETCA above 0.1 mM can lead to almost complete loss of ACh-induced relaxation (Mügge *et al.*, 1991b; Omar *et al.*, 1991) and we indeed confirmed this. We found that DETCA (0.1-3 mM, 1h, followed by washout) produced a concentration-dependent inhibition of ACh-induced relaxation. We also investigated the

ability of exogenous SOD (250 u ml^{-1}) to reverse the inhibitory effect of DETCA on ACh-induced relaxation and found that it could not. This might suggest that this particular action of DETCA occurs by an action independent of inhibition of SOD. Alternatively, it has been concluded that this action results from the ability of DETCA to inhibit intracellular Cu-Zn SOD (Mügge *et al.*, 1991b; Omar *et al.*, 1991), the activity of which cannot be restored by the membrane impermeant nature of exogenous SOD. Consistent with this proposed intracellular site of action is the ability of DETCA to inhibit relaxations induced by nitrovasodilators such as glyceryl trinitrate and nitroprusside, which promote relaxation through the generation of NO inside smooth muscle cells (Omar *et al.*, 1991). Only by restoring intracellular SOD activity and examining whether relaxation is restored will definitive proof of the proposed intracellular actions of DETCA be obtained. This could be achieved by using membrane permeant forms of SOD such as polyethylene glycol-conjugated SOD (Liu *et al.*, 1989), liposome encapsulated SOD (Freeman *et al.*, 1985) or low molecular weight SOD-like mimetics such as tiron (Pagano *et al.*, 1993). To date, however, this has not been attempted.

In this study we reasoned that if Cu-Zn SOD was important in protecting agonist-stimulated activity of NO then its inactivation with DETCA would be expected to potentiate the inhibitory effects of the superoxide generators, HX/XO and pyrogallol. Consequently, experiments were carried out to investigate the effects of DETCA on the ability of HX/XO and pyrogallol to inhibit ACh-induced relaxation. In our experiments we found that following pretreatment with a low concentration of DETCA (0.1 mM, 1h, followed by washout), subsequent treatment with concentrations of HX (0.1 mM)/XO (16 mu ml^{-1}) or pyrogallol (0.1 mM) that had no effect on ACh-induced relaxation by themselves in control tissues each now produced profound blockade of 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 (250 u ml^{-1}). Clearly, therefore, endogenous Cu-Zn SOD protects agonist-stimulated NO from destruction by superoxide anion and this can be abolished following treatment with DETCA. Moreover, since exogenous SOD, which cannot enter cells, fully restores ACh-induced relaxation in DETCA-treated tissues, it is likely that the enhanced blocking ability of HX/XO and pyrogallol results from inhibition of extracellular SOD. With HX/XO this is not surprising since XO too is unable to enter cells due to its large size and is therefore only likely to generate superoxide anion extracellularly. Pyrogallol on the other hand can enter cells and is likely to generate superoxide anion both extracellularly and intracellularly via autooxidation (Halliwell & Gutteridge, 1989).

It is not easy to account for the greater apparent sensitivity of basal over agonist-stimulated activity of NO to destruction by superoxide anion. Our proposed explanation, however, is that under normal circumstances, endogenous Cu-Zn SOD activity lowers levels of superoxide anion, which may originate from endothelial cells or from the oxygenated Krebs' solution, to such an extent that only the low levels of NO produced under basal conditions can be destroyed but high levels produced following stimulation by ACh cannot. However, if the levels of superoxide anion are increased further, either by generation of the free radical or by reducing its breakdown by inhibiting Cu-Zn SOD with DETCA, the higher levels of NO produced following agonist stimulation can now be destroyed. This explanation may also account for the inability of exogenous SOD to potentiate agonist-stimulated activity of NO in arterial rings (Abrahamsson *et al.*, 1992; Mian & Martin, 1995a), since there is already sufficient endogenous SOD present. In contrast, SOD is probably able to potentiate the activity of basal and agonist-stimulated NO in a cascade bioassay system (Gryglewski *et al.*, 1986b; Rubanyi & Vanhoutte, 1986a)

because there is no SOD in the intervening space to protect it from the destructive action of superoxide anion.

In conclusion, our findings suggest that both basal and agonist-stimulated activity of NO can be destroyed by superoxide anion. The apparently greater sensitivity of the former may not necessarily indicate a chemical difference from agonist-stimulated NO but may simply reflect differences in concentration. Furthermore, the importance of the extracellular form of Cu-Zn SOD in protecting endothelial NO was firmly established, whereas only circumstantial evidence for the importance of the intracellular form of the enzyme was obtained.

8.2 Role of catalase in relaxations induced by hydrogen peroxide and nitrovasodilators

As already discussed above, it was clearly established that the delayed relaxant effect of superoxide anion generators in rat aorta is mediated by hydrogen peroxide since exogenously added catalase blocks this effect.

It is also well established that hydrogen peroxide induces vascular relaxation by both endothelium-dependent and endothelium-independent mechanisms. The endothelium-dependent component is thought to be mediated via enhanced synthesis of NO, whilst the endothelium-independent component is due to a direct stimulatory effect on smooth muscle soluble guanylate cyclase (Burke & Wolin, 1987; Furchgott, 1991; Zembowicz *et al.*, 1993). The mechanism by which hydrogen peroxide stimulates soluble guanylate cyclase is not fully elucidated but it has been proposed to occur through a process dependent upon the metabolism of hydrogen peroxide by catalase (Burke & Wolin, 1987; Wolin & Burke, 1987). Specifically, these authors have proposed

that the active species is compound I, i.e. the catalase-hydrogen peroxide complex formed as an intermediate in the conversion of hydrogen peroxide to H_2O and O_2.

Catalase not only promotes the decomposition of hydrogen peroxide (Chance *et al.*, 1952), but also exhibits peroxidase activity, i.e. it catalyses the oxidation of various substrates in the presence of low concentrations of hydrogen peroxide (Keilin & Hartree, 1945; Theorell & Ehrenberg, 1952; Nicholls, 1964). Two well characterised substrates for peroxidation are sodium azide and hydroxylamine which undergo oxidation to nitrous oxide, nitrogen and NO (Theorell & Ehrenberg, 1952; Keilin & Hartree, 1954; Nicholls, 1964). The NO thus produced is believed to underly the ability of sodium azide and hydroxylamine to activate soluble guanylate cyclase and promote vascular relaxation (Katsuki *et al.*, 1977; Waldman & Murad, 1987; Murad, 1994).

In this part of the study we utilised an inhibitor of catalase, 3-amino-1,2,4-triazole (AT, Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958) which acts by binding to compound I thereby inhibiting catalytic activity, in rat aorta to test the hypothesis that metabolism by catalase is necessary to express the relaxant activity of hydrogen peroxide, sodium azide and hydroxylamine. For comparison, the effects of AT were also examined on relaxation produced by another nitrovasodilator, glyceryl trinitrate which is converted to NO not by catalase but by glutathione-S-transferase (Armstrong *et al.*, 1980) or cytochrome P_{450} (Schröder, 1992), or by a direct reduction by tissue thiols (Feelisch, 1991).

8.2.1 Hydrogen peroxide-induced relaxations in endothelium-containing and endothelium-denuded rings and the role of catalase

We examined the nature of hydrogen peroxide-induced relaxation prior to addressing the more complex topic of the role of catalase. We found that low concentrations (100 nM-30 μM) of hydrogen peroxide selectively produced concentration-dependent relaxation in endothelium-containing rings. Hydrogen peroxide also relaxed endothelium-denuded rings, but in these higher concentrations (0.1-1 mM) were required to produce relaxation. These results indicate that in rat aorta, the relaxation induced by hydrogen peroxide has endothelium-dependent and endothelium-independent components, and are thus consistent with previous studies (Furchgott, 1991; Zembowicz *et al.*, 1993).

The effects of scavengers of hydrogen peroxide and superoxide anion, i.e. catalase and SOD, respectively, were investigated on the relaxations induced by hydrogen peroxide. As expected, hydrogen peroxide-induced relaxations in both endothelium-containing and endothelium-denuded rings were significantly reduced if the tissues had been pretreated with catalase (1000 or 3000 u ml^{-1}). In contrast, SOD (50 u ml^{-1}) did not change the relaxant activity of hydrogen peroxide in either endothelium-containing or endothelium-denuded rings, confirming that hydrogen peroxide, but not other reactive oxygen species such as superoxide anion, is involved in these relaxations.

The effects of the NO synthase inhibitor, L-NAME (Rees *et al.*, 1990a), were investigated in order to determine if NO, derived from L-arginine/NO pathway, mediated the endothelium-dependent component of hydrogen peroxide-induced relaxation. The results of these experiments were clear; pretreatment of endothelium-containing rings with L-NAME (30 μM) abolished endothelium-dependent component of hydrogen peroxide-induced relaxation. Furthermore, the inhibitory action of L-NAME on relaxation induced by hydrogen peroxide at low concentrations (100 nM-30 μM) was prevented in the presence of the

substrate for NO synthesis, L-arginine (2 mM). These results supported the conclusion of earlier studies that the endothelium-dependent component is mediated via enhanced production of NO (Furchgott, 1991; Zembowicz *et al.*, 1993). In contrast, L-NAME had no effect on relaxations induced by hydrogen peroxide (0.1-1 mM) in endothelium-denuded rings, indicating that this occurs via a mechanism that is independent of NO. Addition of hydrogen peroxide to both endothelium-containing and endothelium-denuded rings was seen to elicit a biphasic response: a brief contraction which was more pronounced for concentrations $\geq$ 0.1 mM followed by a powerful sustained relaxation. A previous study (Zembowicz *et al.*, 1993) observed this effect only in endothelium-containing rings of rabbit aorta, and reported that L-NAME abolished it. In our experiments, however, we found L-NAME to have absolutely no effect. The mechanism by which hydrogen peroxide induces these contractions is not clear, but it has been proposed that it may involve activation of a serine esterase and/or phospholipase C (Sheehan *et al.*, 1993).

In order to investigate the mechanism by which hydrogen peroxide produces vascular relaxation, the effects of an inhibitor of soluble guanylate cyclase, methylene blue (MeB, Gruetter *et al.*, 1979; Holzmann, 1982; Martin *et al.*, 1985a), were examined. In contrast to L-NAME, MeB (30 μM) blocked relaxations induced by hydrogen peroxide in both endothelium-containing and endothelium-denuded rings. Thus, both the endothelium-dependent and endothelium-independent components appear to depend upon the stimulation of soluble guanylate cyclase. This conclusion is supported by a previous study (Zembowicz *et al.*, 1993) showing the ability of hydrogen peroxide to enhance the levels of cGMP in both endothelium-containing and endothelium-denuded rings of rabbit aorta. In any event, the endothelium-dependent component mediated via NO, would be expected to occur via elevation of cGMP. Furthermore, biochemical studies have shown that low concentrations of

hydrogen peroxide activate soluble guanylate cyclase in tissue homogenates, although higher concentrations lead to inhibition of the enzyme (White *et al.*, 1976), presumably as a result of its oxidant actions, thus mimicking the well characterised inhibitory actions of the oxidants, MeB and potassium ferricyanide (Gruetter *et al.*, 1979; Holzmann, 1982). The mechanism by which hydrogen peroxide stimulates soluble guanylate cyclase remains obscure, but the ability of catalase to potentiate this action has led to the suggestion that an intermediate in its metabolism, perhaps compound I (i.e. the catalase-hydrogen peroxide complex), is the active species (Burke & Wolin, 1987; Wolin & Burke, 1987). Consistent with this hypothesis is the inhibition of hydrogen peroxide-induced relaxation of bovine pulmonary artery by ethanol (Burke-Wolin & Wolin, 1990), which decreases formation of the compound I species of catalase. It was therefore possible that the mechanism of endothelium-independent relaxation induced by hydrogen peroxide in rat aorta also involved the catalase-dependent activation of soluble guanylate cyclase. Consequently, we attempted to test the role of catalase by employing AT, an established inhibitor of this enzyme which acts irreversibly by binding to compound I (Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958).

The experiments with AT were complicated by the fact that it produced powerful concentration-dependent relaxation of PE-contracted endothelium-denuded rings. In order to investigate if the relaxation induced by AT (1-100 mM) was due to activation of soluble guanylate cyclase, the effects of MeB were studied. MeB (30 μM), however, had no effect on the relaxant activity of AT, suggesting that it is unlikely to be mediated via stimulation of soluble guanylate cyclase.

In order to establish the conditions necessary for optimum inhibition of endogenous catalase, a series of experiments was conducted using AT to

inhibit exogenous catalase (bovine liver). Pretreatment of endothelium-denuded rings with catalase (1000 u ml^{-1}) for 30 min led to a profound reduction in the maximal relaxation induced by hydrogen peroxide. When the catalase inhibitor, AT (50 mM, Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958), was present together with catalase during the 30 min incubation, the ability to inhibit hydrogen peroxide-induced relaxation was partially blocked (by 48.8 ± 3.0 %). Increasing the incubation period to 90 min, however, led to complete blockade of the ability of catalase to inhibit hydrogen peroxide-induced relaxation. From these results it was concluded that AT does indeed inhibit the activity of catalase and that the onset of inhibition is slow. This slow onset of action is supported by previous studies and is thought to result from the ability of AT to bind to the intermediate compound I, which will exist only at low concentration, rather than to catalase (Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958).

Having established the ability of AT to inhibit exogenous catalase, we felt confident to address the proposed role of catalase in hydrogen peroxide-induced relaxation in endothelium-denuded rings. In fact, in our experiments, treatment with AT (50 mM, 90 min) to inhibit endogenous catalase failed to have any effect on hydrogen peroxide-induced relaxation of rat aortic rings. Contrary to previous reports (Burke & Wolin, 1987; Wolin & Burke, 1987; Burke-Wolin & Wolin, 1990), our findings thus suggested that metabolism of hydrogen peroxide or formation of compound I is not required for expression of the relaxant activity of hydrogen peroxide. An alternative explanation is therefore required to account for the requirement for catalase in the activation of soluble guanylate cyclase by hydrogen peroxide (Burke & Wolin, 1987; Wolin & Burke, 1987; Burke-Wolin & Wolin, 1990). Although speculative at this stage, in view of the dual actions of hydrogen peroxide, i.e. stimulant at low concentrations and inhibitory at high concentrations (White *et al.*, 1976), it is

possible that catalase, by destroying hydrogen peroxide, will lower its concentration to such an extent that only the stimulant and not the inhibitory actions are seen. Such a conclusion rests heavily on our assumption that AT did indeed inhibit intracellular catalase in rat aorta and evidence to support this was sought by examining the effects of the agent on relaxation induced by a range of nitrovasodilators.

8.2.2 Vasodilator actions of sodium azide, hydroxylamine and glyceryl trinitrate

The three nitrovasodilators employed, sodium azide, hydroxylamine and glyceryl trinitrate all produced powerful, concentration-dependent relaxation in endothelium-denuded rings of rat aorta. It is well established that the relaxant actions of these nitrovasodilators proceed through activation of soluble guanylate cyclase and elevation of smooth muscle cGMP content (Arnold *et al.*, 1977; Katsuki *et al.*, 1977; Waldman & Murad, 1987; Murad, 1994). Consistent with this view was our ability to inhibit relaxation by all three nitrovasodilators using MeB, an inhibitor of soluble guanylate cyclase (Gruetter *et al.*, 1979; Holzmann, 1982; Martin *et al.*, 1985a).

In order for the nitrovasodilators to stimulate soluble guanylate cyclase they must first must be chemically modified to yield the active principle, NO (Katsuki *et al.*, 1977; Waldman & Murad, 1987; Murad, 1994). For glyceryl trinitrate, this has been proposed to occur via metabolism by glutathione-S-transferase (Armstrong *et al.*, 1980) or cytochrome P_{450} (Schröder, 1992), or by a direct reduction by tissue thiols (Feelisch, 1991). In contrast, the seminal work of Murad and his colleagues (Mittal *et al.*, 1975) showed that the activation of soluble guanylate cyclase in tissue homogenates by sodium azide and hydroxylamine required the presence of high molecular weight factors, later identified as catalase and other peroxidase enzymes (Miki *et al.*, 1976).

Although biochemical evidence supporting the role of catalase in the activation of soluble guanylate cyclase by sodium azide and hydroxylamine is strong (Miki *et al.*, 1976; Katsuki *et al.*, 1977; Craven *et al.*, 1979), direct proof of the involvement of this enzyme in vascular relaxation is lacking. We reasoned that if endogenous catalase is important in mediating relaxation induced by sodium azide and hydroxylamine then its inactivation with AT will be expected to inhibit the relaxant effect of these agents.

8.2.3 Effects of AT on relaxations induced by sodium azide, hydroxylamine and glyceryl trinitrate

We found that pretreatment of endothelium-denuded rings with AT (1-50 mM) for 30, 60 and 90 min produced a concentration- and time-dependent inhibition of the relaxation induced by sodium azide. Incubation for 90 min was found to be the most effective in inhibiting sodium azide-induced relaxation and this is consistent with our previous results demonstrating the slow inhibition of exogenous catalase by AT. These data thus suggest a role for endogenous catalase in this relaxation. Similarly, pretreatment of endothelium-denuded rings with AT (5-50 mM) for 90 min shifted the concentration-response curve for hydroxylamine to the right in a concentration-dependent manner. The action of AT on relaxation induced by sodium azide and hydroxylamine was highly selective since relaxation induced by glyceryl trinitrate was completely unaffected, consistent with its metabolism by a separate pathway (Armstrong *et al.*, 1980; Feelisch, 1991; Schröder, 1992).

Although AT at all concentrations tested inhibited the relaxant actions of both sodium azide and hydroxylamine, it inhibited the actions of the former more powerfully than the latter. The maximum shifts in EC_{50} values for sodium azide and hydroxylamine obtained with AT at 50 mM were 1668-fold and 190-

fold, respectively. Despite this difference, sodium azide and hydroxylamine were almost equipotent as relaxants of rat aorta. These results perhaps suggest a possible additional catalase-independent activation pathway for the latter agent. Nevertheless, the ability of AT to inhibit relaxation induced by sodium azide and hydroxylamine provides firm evidence that the agent does indeed inhibit endogenous catalase in rat aorta. This finding thus strengthens our conclusion (section 8.2.1) that endogenous catalase is not involved in the relaxant actions of hydrogen peroxide.

In a related theme, It has been proposed that hydroxylamine may be generated as an intermediate in the endothelial production of NO from L-arginine (DeMaster *et al.*, 1989; Schmidt *et al.*, 1990). This raised the possibility that the last step in the formation of NO by endothelium might involve the metabolism of hydroxylamine by endogenous catalase. We tested this hypothesis by studying the effects of inhibiting endogenous catalase with AT on endothelium-dependent relaxation induced by ACh in rat aortic rings. We found, however, that AT (50 mM, 90 min) had absolutely no effect on ACh-induced relaxation, thus arguing against the above proposal. These experiments do, however, provide further support for the selectivity of AT in blocking relaxation induced by sodium azide and hydroxylamine.

In the above experiments we have established the role of catalase in relaxations induced by two powerful nitrovasodilators, sodium azide and hydroxylamine. It has been proposed that conversion of sodium azide and hydroxylamine to NO requires the presence of low concentrations of hydrogen peroxide and thus occurs as a consequence of the peroxidase activity of catalase (Theorell & Ehrenberg, 1952; Keilin & Hartree, 1954; Nicholls, 1964; Klebanoff & Nathan, 1993). Consequently, we investigated the role of catalase further by adopting a more direct approach, i.e. by measuring the production of

NO from sodium azide, hydroxylamine and glyceryl trinitrate. In these experiments, NO production was assessed by measuring the formation of nitrite, its major stable *in vitro* product (Stuehr & Marletta, 1987a,b). A series of experiments was carried out to establish suitable reagent concentrations, reaction conditions and an appropriate time-course for the generation of nitrite.

8.2.4 Measurement of nitrite formation from sodium azide, hydroxylamine and glyceryl trinitrate

The Greiss reaction has been widely used to establish an assay for measuring accumulation of nitrite in biological systems. The assay is based on diazotization, where NO, generated from acidified nitrite, combines with sulphanilic acid and the resulting diazo product couples with N-(1-naphthyl)-ethylenediamine to produce an intense pink colour (Green *et al.*, 1982). In this study, neither hydrogen peroxide (1 mM) nor catalase (1000 u ml^{-1}) was found to lead to formation of nitrite from sodium azide during a 60 min incubation. In contrast, when all three were present in combination, a rapid development of pink colour (measured quantitatively at 550 nm) was observed indicating that nitrite was being produced from sodium azide. The production, in fact, appeared to be almost linear during the 60 min incubation suggesting a simple one-step enzymic reaction, unaffected by substrate depletion. Our finding that catalase catalysed the formation of nitrite from sodium azide only in the presence of hydrogen peroxide suggested that the reaction occurred by the peroxidase activity of the enzyme, and is consistent with previous studies (Theorell & Ehrenberg, 1952; Keilin & Hartree, 1954; Nicholls, 1964; Klebanoff & Nathan, 1993).

A recent study (Markert *et al.*, 1994) failed to detect nitrite formation from hydroxylamine by catalase in the presence of hydrogen peroxide, but under the conditions of our experiments (higher concentrations of hydroxylamine, catalase and hydrogen peroxide), we found that it could indeed act as a substrate. The maximum levels of nitrite generated, however, were lower (8.7 ± 0.7 μM) than those obtained from sodium azide (23.4 ± 0.6 μM). An attempt was made to determine if the levels of nitrite generated from hydroxylamine could be increased by increasing the concentration of hydrogen peroxide. In these experiments, increasing the concentration of hydrogen peroxide from 1 mM to 100 mM increased slightly the levels of nitrite generated (10.0 ± 0.1 μM), but these were still less than those obtained using sodium azide, suggesting that hydroxylamine is a less effective substrate than sodium azide. These findings are consistent with our earlier results demonstrating the greater inhibition by AT of relaxation induced by sodium azide than by hydroxylamine, perhaps again suggesting an additional catalase-independent route of metabolism of the latter to NO in vascular smooth muscle.

In contrast to findings with sodium azide and hydroxylamine, no nitrite was generated when glyceryl trinitrate (1 mM) was incubated with catalase in the presence of hydrogen peroxide, thus highlighting the selectivity of the enzyme. Moreover, glyceryl trinitrate (1 mM) but not sodium azide (1 mM) or hydroxylamine (1 mM) was reduced to nitrite by the thiol, L-cysteine (3 mM) during a 60 min incubation. These findings are therefore consistent with distinct pathways for the generation of NO from glyceryl trinitrate in comparison with sodium azide and hydroxylamine.

8.2.5 Use of the glucose/glucose oxidase system in the generation of nitrite from sodium azide and hydroxylamine by catalase

The low, sustained generation of hydrogen peroxide by the glucose/glucose oxidase system has been reported to be more efficient than a single high concentration of hydrogen peroxide in supporting the catalase-dependent formation of NO from sodium azide (Klebanoff & Nathan, 1993). Consequently, in some experiments the glucose (5 mM)/glucose oxidase (300 mu ml^{-1}) system was utilised and the effects on nitrite formation from sodium azide and hydroxylamine by catalase compared with those obtained using a high standard concentration (1 mM) of hydrogen peroxide. We found that sodium azide (10 μM-3 mM) was converted to nitrite in a concentration-dependent manner by catalase in the presence of the glucose/glucose oxidase system. Moreover, the maximum concentration obtained with sodium azide at 0.3 mM was some 2-fold greater (44.5 ± 0.8 μM) if the glucose/glucose oxidase system was used instead of hydrogen peroxide. These findings thus confirm the greater efficiency of this system over hydrogen peroxide in facilitating the catalase-dependent formation of nitrite from sodium azide (Klebanoff & Nathan, 1993).

Since hydroxylamine was a poor substrate for nitrite formation by catalase in the presence of a fixed concentration of hydrogen peroxide, an attempt was made to determine if formation could be increased by replacing hydrogen peroxide by the glucose/glucose oxidase system. We found, however, that the glucose/glucose oxidase system, despite significantly increasing the catalase-dependent formation of nitrite from sodium azide, failed to enhance nitrite production from hydroxylamine.

8.2.6 Effects of AT on catalase-dependent formation of nitrite from sodium azide

So far, we have established that AT inhibits the relaxant action of sodium azide and hydroxylamine in rat aorta indicating an important role for endogenous catalase. Furthermore, we have shown that NO, assessed as nitrite, can indeed be generated from sodium azide and hydroxylamine by catalase in the presence of hydrogen peroxide. Consequently, it was important to investigate if the formation of nitrite from sodium azide by catalase could be blocked by AT. Indeed, we found that AT (0.1-100 mM) produced a concentration-dependent blockade of the catalase-dependent conversion of sodium azide (0.3 mM) to nitrite in the presence of hydrogen peroxide. The blockade was only slight during a 1h incubation and the maximum inhibition obtained was 36.4 ± 1.2 % at 100 mM AT. If, however, the duration of the incubation was extended to 3h together with a reduction of the catalase concentration (from 1000 to 300 u ml^{-1}), the ability of AT to block formation of nitrite was enhanced: a maximum inhibition of 77.7 ± 6.3 % was obtained at 100 mM AT. These results indicate that AT indeed blocks the catalase-dependent conversion of sodium azide to nitrite in the presence of hydrogen peroxide and that the blockade is slow, consistent with its known slow onset of action (Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958).

Since hydroxylamine was a poorer substrate than sodium azide, no experiments were conducted to determine if AT blocked the catalase-dependent formation of nitrite from this agent.

Our study using AT, an inhibitor of catalase, thus provides direct proof that metabolism of sodium azide and hydroxylamine to NO by catalase underlies the relaxant actions of these nitrovasodilators. In contrast, our study lends no

support to the proposed concept that metabolism of hydrogen peroxide by catalase is necessary for expression of its relaxant activity (Burke & Wolin, 1987; Wolin & Burke, 1987).

8.3 Hydrogen peroxide-induced impairment of vascular reactivity of rat aorta

The vascular endothelium is constantly exposed to reactive oxygen species including hydrogen peroxide generated from blood cells such as neutrophils, platelets and monocytes (Verkerk & Jondkind, 1992). The reaction of hydrogen peroxide with membrane lipids of endothelial cells may have damaging effects on the vessel wall. Hydrogen peroxide can also exert damaging effects on vascular smooth muscle cells (Verkerk & Jondkind, 1992). Endothelial cells and vascular smooth muscle cells, however, are not passive in this process of peroxidation. They express endogenous antioxidant defence systems such as glutathione peroxidase and catalase to detoxify hydrogen peroxide (Halliwell & Gutteridge, 1989; Cheeseman & Slater, 1993).

In this part of the study we investigated the damage induced by hydrogen peroxide to vascular responsiveness and explored the role of endogenous catalase in protection against this damage. In order to investigate this role of endogenous catalase, we again made use of the inhibitor of catalase, AT (Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958).

8.3.1 Effects of incubation with hydrogen peroxide on PE-induced contraction and endothelium-dependent relaxations

As discussed above (section 8.2.1) we found that incubation with hydrogen peroxide (1 mM) resulted in profound relaxation of endothelium-containing and endothelium-denuded rings of rat aorta. When present for 15, 30 or 60 min

followed by washout, the ability of the tissues to contract in response to PE was severely depressed in a time-dependent manner. This action, in contrast to the relaxant action of hydrogen peroxide was not reversed by treatment with catalase and was essentially irreversible. It was also not produced by a lower concentration of hydrogen peroxide (0.1 mM) which also induces powerful relaxation. It has been demonstrated previously that superoxide anion by oxidising catecholamines abolishes their contractile effects (Wolin & Belloni, 1985). It was considered possible therefore that hydrogen peroxide degraded PE in our experiments thereby inhibiting its contractile actions. This seems unlikely, however, since a recent report (Iesaki *et al.*, 1994) demonstrated that PE, unlike noradrenaline and adrenaline, is not degraded by hydrogen peroxide. These authors suggested that hydrogen peroxide may reduce vasoactive amine-induced contractions of vascular smooth muscle by adversely affecting the signal transduction pathways which increase the intracellular Ca^{2+} concentrations of vascular smooth muscle cells (Iesaki *et al.*, 1994). Furthermore, hydrogen peroxide has been shown to impair glycolytic substrate metabolism in cardiac myocytes (Josephson *et al.*, 1991), leading to ATP depletion which in turn decreases the ability of cells to respond to agonists, as energy is required for cellular activity. Such findings therefore suggest that the ability of hydrogen peroxide to depress subsequent contraction to PE results from a damaging action of the oxidant.

The effects of incubation with various concentrations of hydrogen peroxide for different times were also investigated on the endothelium-dependent relaxation of rat aorta induced by ACh. We found that following induction of PE-induced tone, incubation of endothelium-containing rings with hydrogen peroxide (0.1 mM) for 15, 30 or 60 min followed by washout and re-contraction with PE, failed to produce any apparent damage to ACh-induced relaxation. In contrast, incubation with a higher concentration (1 mM) of hydrogen peroxide

for 15, 30 or 60 min followed by washout, did produce powerful effects on ACh-induced relaxation. Specifically, we found that incubation with hydrogen peroxide (1 mM) for 15 min, followed by washout, led to an unexpected potentiation of relaxation at each concentration of ACh. This potentiation, however, was not sustained as was evident from a second concentration-response curve to ACh constructed after 2h in the same tissue. This second concentration-response curve to ACh, in fact, demonstrated a substantial blockade of relaxation. Thus, the initial potentiation appeared to be a prelude to more permeant blockade by hydrogen peroxide. In contrast to the short exposure, incubation of endothelium-containing rings with hydrogen peroxide (1 mM) for 30 or 60 min, followed by washout, led to ACh-induced relaxation being greatly inhibited. As expected, pretreatment with catalase (3000 u ml^{-1}) completely prevented the ability of hydrogen peroxide to initially increase and then impair ACh-induced relaxation. In contrast, once blockade was established it could not be reversed either by washing or by subsequent treatment with catalase. These findings thus demonstrate an overall concentration- and time-dependent, and essentially irreversible impairment of endothelium-dependent relaxation by hydrogen peroxide.

In view of the ability of hydrogen peroxide to depress contraction to PE (see above), it was not clear if the depression of ACh-induced endothelium-dependent relaxation resulted from damage to the endothelium or vascular smooth muscle, but evidence from the literature does show clear impairment of endothelial function by hydrogen peroxide. For example, work from our own laboratory has shown that hydrogen peroxide impairs the barrier function of porcine aortic endothelial monolayers (Berman & Martin, 1993). It has been proposed that hydrogen peroxide acts by rapidly diffusing into cells where it comes into contact with Fe^{2+} resulting in the formation of hydroxyl radicals which may finally be responsible for endothelial cell damage (Kvietys *et al.*,

1989). This, however, was not found to be the case for porcine endothelial monolayers (Berman & Martin, 1993), since catalase which removes hydrogen peroxide but not scavengers of hydroxyl radical such as mannitol and dimethylthiourea, was able to protect against the hydrogen peroxide-induced impairment of endothelial function.

An alternative explanation proposed for the damaging actions of hydrogen peroxide involves actions on intracellular Ca^{2+} homeostasis. It has been reported that exposure of cardiac myocytes (Josephson *et al.*, 1991), renal tubular cells (Ueda & Shah, 1992), vascular endothelial cells (Hirosumi *et al.*, 1988; Franceschi *et al.*, 1990; Geeraerts *et al.*, 1991), and vascular smooth muscle cells (Krippeit-Drews *et al.*, 1995) to hydrogen peroxide leads to sustained increases in intracellular Ca^{2+} levels resulting in tissue injury. The mechanisms by which these increases in intracellular Ca^{2+} might cause tissue injury include lipid peroxidation (Hirosumi *et al.*, 1988; Geeraerts *et al.*, 1991), activation of Ca^{2+}-dependent proteases (Geeraerts *et al.*, 1991), and alterations in the cytoskeleton (Hinshaw *et al.*, 1989). Hydrogen peroxide may thus have impaired endothelium-dependent relaxation and depressed PE-induced contraction via widespread Ca^{2+}-dependent damage to the endothelium and the vascular smooth muscle.

The potential role of endogenous catalase in protecting vascular reactivity against hydrogen peroxide-induced damage was investigated with the use of AT (Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958). In these experiments, treatment of endothelium-containing rings with AT (50 mM, 90 min, followed by washout) alone had no effect on subsequent PE-induced contraction showing that the drug did not produce any non-selective action. In contrast, this treatment with AT (50 mM, 90 min) potentiated the ability of hydrogen peroxide at 1 mM but not at 0.1 mM (present during the final 30 min

of 90 min incubation, followed by washout) to depress PE-induced contraction. This finding indeed suggested a potential role for endogenous catalase in protecting vascular smooth muscle cells from damage induced by high (1 mM) but not low (0.1 mM) concentrations of hydrogen peroxide .

The effects of inhibiting endogenous catalase with AT together with hydrogen peroxide were also examined on ACh-induced relaxation. In these experiments, treatment of endothelium-containing rings with AT (50 mM, 90 min, followed by washout) alone had no effect on ACh-induced relaxation. Furthermore, treatment with AT (50 mM, 90 min) together with hydrogen peroxide (0.1 mM, present for the final 30 min of 90 min incubation, followed by washout) also had no effect on ACh-induced relaxation. These findings suggest that endogenous catalase has no role in protection against low concentrations of hydrogen peroxide. In contrast, treatment with AT (50 mM, 90 min) markedly potentiated the ability of hydrogen peroxide at 1 mM (present during the final 30 min of 90 min incubation, followed by washout) to block ACh-induced relaxation. These results together with those showing enhanced impairment of PE-induced contraction by hydrogen peroxide in AT-treated tissues clearly suggest an important role for endogenous catalase in protecting vascular cells from the effects of high concentrations of hydrogen peroxide. Consistent with these findings are previous studies (Cohen & Hochstein, 1963; Nicholls, 1972; Jones *et al.*, 1981; Dobrina & Patriarca, 1986; Verkerk & Jondkind, 1992) demonstrating in many cell types that catalase acts as a high capacity, but low affinity scavenger of hydrogen peroxide and is more important at high concentrations of the oxidant. They also show, in contrast, that glutathione peroxidase acts as a high affinity, low capacity scavenger, preferentially metabolising low concentrations of hydrogen peroxide.

8.3.2 Effects of incubation with hydrogen peroxide on endothelium-independent relaxations

Since it was not clear whether the enhanced ability of hydrogen peroxide to impair endothelium-dependent, ACh-induced relaxation of rat aorta following treatment with AT was due to endothelial or smooth muscle damage, the effects on relaxation induced by the endothelium-independent relaxants, glyceryl trinitrate and isoprenaline, were examined. Consistent with the findings with ACh, incubation of endothelium-denuded rings with hydrogen peroxide (0.1 mM, 30 min, followed by washout) alone, incubation with AT (50 mM, 90 min, followed by washout) alone, or incubation with AT (50 mM, 90 min) together with hydrogen peroxide (0.1 mM, present during the final 30 min of 90 min incubation, followed by washout) had no effect on relaxations induced by glyceryl trinitrate or isoprenaline. In contrast, increasing the concentration of hydrogen peroxide to 1 mM (30 min, followed by washout) produced a substantial blockade of relaxation induced by glyceryl trinitrate or isoprenaline. Furthermore, this blockade by hydrogen peroxide was significantly enhanced in tissues in which endogenous catalase had been inhibited with AT. The ability of hydrogen peroxide either alone, or in combination with AT, to inhibit the endothelium-independent relaxations induced by glyceryl trinitrate and isoprenaline clearly suggest a damaging action on the vascular smooth muscle.

In conclusion, our findings suggest that the oxidant, hydrogen peroxide, produces a non-selective damaging action on the vascular smooth muscle resulting in loss of the contractile and relaxant actions. Potentiation of these damaging actions by AT is consistent with a role for endogenous catalase in protecting vascular smooth muscle function from oxidant damage by hydrogen peroxide. The possibility that a component of the impairment of ACh-induced

relaxation by hydrogen peroxide involved an additional damaging action on the endothelium was suspected but not proven.

8.4 General conclusions

Endothelial cells possess the antioxidant machinery necessary to protect themselves against toxic reactive oxygen species. In normal situations a delicate equilibrium exists between free radical production and these antioxidant systems. However, in different pathological processes, such as ischemia-reperfusion, atherosclerosis, diabetes, or during inflammatory responses, the generation of free radicals is increased and the balance is modified, contributing to the endothelial damage associated with these processes (Halliwell, 1982; Downey, 1990; Minor *et al.*, 1990; Langenstroer & Pieper, 1992). Our work with DETCA has established that the extracellular, and perhaps also the intracellular isoform of Cu-Zn SOD is important in protecting endothelium-derived NO from destruction by superoxide anion. These isoforms of SOD are likely to play an important role in many pathological conditions where involvement of free radicals has been implicated. The ability to restore functional SOD activity would thus be expected to offer enormous therapeutic potential in a large number of vascular diseases. SOD itself is unsuitable for therapeutic purposes because of its large size and inability to enter cells. Consequently, more research needs to be focused on the development of low molecular weight, membrane-permeant SOD-like agents as therapeutic tools in the treatment of vascular disease. In addition, our work with AT has confirmed the important role of endogenous catalase in protecting vascular function against the damage induced by the oxidant, hydrogen peroxide. The role of endogenous catalase in the expression of the relaxant activity of certain nitrovasodilators was also established.

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Chapter 9

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