Nitric oxide, superoxide & peroxynitrite: synthesis, interactions & biological consequences

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ABSTRACT

The control of nitric oxide (NO) production and its interaction with other free radicals were examined *in vitro* and *in vivo*. First, the role of calmodulin in the regulation of inducible NO synthase (iNOS) was assessed. Second, the interaction between NO and superoxide ($O_2^{\cdot-}$) and the product of this reaction peroxynitrite ($ONOO^-$) were investigated. Finally, the role of endothelial NO synthase (eNOS) and its regulation by tetrahydrobiopterin (BH_4) and GTP cyclohydrolase-I (GTPCH-I) were examined in a mouse model of septic shock.

Inducible NOS was expressed in a murine macrophage cell line, J774, and nitrite production measured as an index of NO production. This system was used to examine the effects of calmodulin antagonists on induction and activity of iNOS. Calmodulin antagonists inhibited induction and production of nitrite by J774 cells without significant inhibition of isolated iNOS. The rank order of inhibitory potency of calmodulin antagonists was similar to that on isolated calmodulin activity. Most antagonists exhibited significant cytotoxicity and over a similar concentration range inhibited nitrite production. However, ophiobolin A was identified as a calmodulin antagonist with minimal cytotoxicity that potently inhibited iNOS induction.

Once NO is produced it reacts rapidly with target enzymes and other free radical species such as $O_2^{\cdot-}$. Experiments were designed to determine whether rabbit aortic rings produced $O_2^{\cdot-}$. Under basal conditions, $O_2^{\cdot-}$ measured by chemiluminescence was not detected. Inhibition of endogenous superoxide dismutase (SOD) resulted in detectable basal release, which was further increased, in a concentration-dependent manner, by NADH or NADPH. Incubation of aortic rings with *S*-nitrosoglutathione (GSNO) or sodium nitroprusside (SNP) resulted in concentration-dependent quenching of $O_2^{\cdot-}$ chemiluminescence that was proportional to the amount of NO released. The interaction of NO and $O_2^{\cdot-}$ was assessed by monitoring the formation of $ONOO^-$ by determining protein tyrosine nitration by Western blot analysis. Under basal conditions and in the presence of NADH, a single band immunoreactive to nitrotyrosine was detected. Incubation of rings with GSNO alone or GSNO and NADH resulted in the appearance of additional nitrotyrosine bands. The requirements for protein tyrosine nitration were further examined using an isolated system in which bovine serum albumin was exposed to various combinations of NO or $O_2^{\cdot-}$. Incubation with 3-morpholinosydnonimine

(SIN-1; donor of NO and $O_2^{\cdot-}$) but not GSNO resulted in marked nitration of albumin, which was reduced in the presence of oxyhaemoglobin or SOD. Incubation of albumin with GSNO and pyrogallol ($O_2^{\cdot-}$ generator), resulted in tyrosine nitration. It was concluded that nitrotyrosine formation observed in aortic rings exposed to NO donors is likely to have resulted from the interaction of exogenous NO and basal endogenous $O_2^{\cdot-}$ resulting in the formation of $ONOO^-$.

In conditions such as septic shock induction of iNOS occurs, which results in significant formation of NO and nitrotyrosine. However, in human sepsis, it appears that an up-regulation of eNOS rather than induction of iNOS may occur. To assess the importance of eNOS, a conscious mouse model of sepsis was established using mice deficient in iNOS. Animals were injected with lipopolysaccharide (LPS) and the expression of GTPCH-I measured by Northern blotting. In addition plasma nitrite/nitrate and tissue biopterin levels (as an index of BH_4 production) and GTPCH-I activity were measured. The expression of eNOS was not significantly altered by LPS treatment in wild-type and iNOS mutant mice. However, plasma nitrite/nitrate levels were increased by 14- and 2-fold in wild-type and iNOS mutant mice 12 h after LPS, respectively. This was associated with a significant 2.2- and 2.4-fold increase in heart GTPCH-I activity and a 3.8- and 2.8-fold increase in tissue biopterin levels of wild-type and iNOS mutant mice, respectively. Northern blotting showed the presence of two transcripts corresponding to 1.4 and 3.6 kb GTPCH-I mRNA in liver and heart tissue.

In summary this thesis investigated the control of NO production and its subsequent interaction with $O_2^{\cdot-}$. In conditions such as septic shock where enhanced NO production occurs through the inducible or constitutive pathways, therapies designed to inhibit the NO pathway could result in important therapeutic advances.

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PUBLICATIONS

Work described in this thesis has given rise to the following publications:

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ACh	Acetylcholine
ADP	Adenosine diphosphate
BH_4	Tetrahydrobiopterin
BK	Bradykinin
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine 3',5'-monophosphate
CNS	Central nervous system
DAHP	2,4-Diamino-6-hydroxypyrimidine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
e^-	Electron
ε	Extinction coefficient
EDRF	Endothelium-derived relaxing factor
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GSNO	*S*-nitrosoglutathione
GTN	Glyceryl trinitrate
GTPCH-I	Guanosine triphosphate cyclohydrolase-I
GTP	Guanosine triphosphate
H^+	Hydrogen cation
Hb	Haemoglobin
HCl	Hydrochloric acid
HOCl	Hypochlorous acid

HOONO	Peroxynitrous acid
HPRT	Hypoxanthine phosphoribosyl transferase
H_2O_2	Hydrogen peroxide
IFN-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
i.v.	Intravenous
kb	Kilo bases
KCl	Potassium chloride
kDa	KiloDaltons
KH_2PO_4	Potassium dihydrogen phosphate
l	Path length
LPS	Lipopolysaccharide
MetHb	Methaemoglobin
$MgSO_4$	Magnesium sulphate
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide, phosphate form
Na_2-EDTA	Disodium ethylenediaminetetraacetic acid
$NaHCO_3$	Sodium hydrogen carbonate
Na_2HPO_4	Disodium hydrogen phosphate
NANC	Non-adrenergic, non-cholinergic
$NaNO_2^-$	Sodium nitrite
$NaNO_3^-$	Sodium nitrate
NMDA	N-methyl-D-aspartate
L-NMMA	L-N^G-monomethyl-arginine
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO^+	Nitrosyl cation
NO^-	Nitroxyl anion

NO_2	Nitrogen dioxide
NO_2^+	Nitronium cation
NO_2^-	Nitrite
NO_3^-	Nitrate
NO_2Cl	Nitryl chloride
O_2	Oxygen
$O_2^{\cdot-}$	Superoxide anion
OD	Optical density
OH^-	Hydroxyl anion
$ONOO^-$	Peroxynitrite
P	Probability
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGI_2	Prostacyclin
PNS	Peripheral nervous system
PVDF	Polyrinylidene difluoride
RNA	ribonucleic acid
ROS	Reactive oxygen species
RSNO	*S*-nitrosothiol
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
s.e.m	Standard error of the mean
sGC	Soluble guanylate cyclase
SIN-1	3-morpholinosydnonimine
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SSC	Saline-sodium citrate
TCA	Trichloroacetic acid
U/ml	Units per millilitre
v/v	volume per volume
w/v	weight per volume

CHAPTER 1
INTRODUCTION

1.1. Historical background

A noxious, unstable gas is an unlikely candidate to act as a biological messenger. Twenty years ago nitric oxide (NO) was known to us as a pollutant which appeared in the exhaust from motor cars, caused acid rain, and destroyed the ozone layer. However, this unorthodox second messenger molecule is now widely accepted as an important biological mediator. The discovery of NO emerged from four strands of apparently uncorrelated research. These included the endogenous nitrate and macrophage cytotoxicity; studies on the active metabolite of the vasodilator nitrates; the discovery and characterisation of endothelium-derived relaxing factor (EDRF); and the work on the nature of non-adrenergic, non-cholinergic (NANC) transmission. These four main areas of research intersected in 1987-88 when it was established that all of them were related to the same simple molecule, NO.

In 1916 it was first observed that the body has an endogenous production of nitrate. This observation emerged from dietary balance studies in humans showing that more nitrate was excreted in comparison to its ingestion (Mitchell *et al.*, 1916). Later it was shown that nitrate was biosynthesised mainly outside the intestine (Green *et al.*, 1981), which renewed interest in this area. Also, in a separate study it was observed that immunostimulated macrophages produced high levels of nitrite and nitrate. The nitrogen source of these compounds was found to be one of the terminal nitrogens in the guanidino group of L-arginine (Ivengar *et al.*, 1987). By use of the L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA), the generation of nitrite/nitrate was inhibited. The use of such analogues formed the cornerstone of much subsequent NO research.

The discovery of NO in the vasculature began with studies by Furchgott & Zawadzki (1980) who showed that the vascular relaxations of rabbit aorta induced by acetylcholine (ACh) were dependent upon the presence of a functional endothelium. They postulated that these endothelium-derived relaxations were mediated by a humoral factor, which they called endothelium-dependent relaxing factor (EDRF).

The first evidence for the formation of NO by mammalian cells came from experiments where EDRF released from vascular endothelial cells was detected by bioassay on spiral strips of rabbit aorta superfused in a cascade, and the effluent assessed chemically to identify NO (Palmer *et al.*, 1987). NO can be measured directly as the

chemiluminescent product of its reaction with ozone (Downes *et al.*, 1976). It was shown using this method that the concentrations of bradykinin (BK) required to induce the release of EDRF also caused a concentration-dependent release of NO. Also, sufficient amounts of NO were released by the cells to account for relaxation of vascular strips (Palmer *et al.*, 1987). A detailed comparison of the biological actions of EDRF and NO on vascular strips (Hutchinson *et al.*, 1987; Ignarro *et al.*, 1987; Palmer *et al.*, 1987) also showed that the two compounds were pharmacologically indistinguishable. Both EDRF and NO caused a relaxation of the vascular strips that declined at the same rate during passage down the bioassay cascade (Palmer *et al.*, 1987). Initially, it was thought that compounds such as nitrite, nitrate and hydroxylamine were the source of NO (see Moncada *et al.*, 1991 for a review). In 1988 it was demonstrated that the amino acid L-arginine was the precursor for the synthesis of NO by vascular endothelial cells. Endothelial cells, cultured in the absence of L-arginine for 24 hours before the experiments showed a decrease in the release of NO induced by the calcium ionophore A23187 and BK, which could be restored by reculturing of the cells with L-arginine (Palmer *et al.*, 1988a). Definitive experiments using mass spectrometry techniques and [^{15}N]L-arginine showed the formation of ^{15}NO from the terminal guanidino nitrogen atom of L-arginine when endothelial cells were stimulated with bradykinin (Palmer *et al.*, 1988b).

1.2. Diverse roles of nitric oxide

Under atmospheric temperature and pressure, NO exists as a gas due to its free radical structure. It possesses an unpaired electron in its atomic configuration that makes it highly chemically reactive and labile. Synthesis of NO from the semi-essential amino acid L-arginine is implicated in many physiological and pathophysiological processes including regulation of the cardiovascular system, central and peripheral nervous systems, and a variety of homeostatic mechanisms (Moncada & Higgs, 1995). Some of the systems in which NO plays vital roles are described below.

1.2.1. Cardiovascular system

NO is a potent vasodilator which also inhibits adhesion of platelets and leukocytes to the vascular endothelium (Radomski *et al.*, 1987a; Sneddon & Vane, 1988; Kubes *et al.*, 1991), which occurs through the activation of guanylate cyclase and a consequent increase in the intracellular concentration of cyclic guanosine monophosphate (cGMP; Furchgott, 1984; Radomski *et al.*, 1987b).

Endothelial cells release NO in response to several stimuli, e.g. ACh, thrombin, ADP, serotonin, calcium ionophore A23187, phorbol esters or shear stress (Furchgott, 1984; Griffith *et al.*, 1984; Buga *et al.*, 1991). However, endothelial cells are not the only source of NO in blood vessels. The adventitia of blood vessels are also innervated with NO-releasing nerves (Rand, 1992). Furthermore, in pathophysiological conditions related to the release of cytokines, vascular smooth muscle cells also synthesise NO via an inducible NO synthase (see later), which leads to vasodilation and hypotension (Busse & Mülsch, 1990; Knowles *et al.*, 1990).

1.2.2. Immune system

NO displays two properties in the defence of mammals against pathogenic organisms. NO released from immunostimulated macrophages can inhibit growth of bacteria, fungi, parasites and viruses (Adams *et al.*, 1991; Alspaugh & Granger, 1991; Croen, 1993). Further, peripheral vasodilation and vascular hyporeactivity to vasoconstrictor agents occurs. This results from induction in vascular smooth muscle cells of NOS in response to lipopolysaccharide (LPS) or cytokines (Thiemermann & Vane, 1990).

Apart from the antimicrobial actions of NO in the immune system, NO modulates the release of histamine from mast cells (Masini *et al.*, 1991) and also acts as an immune modulator by inhibiting lymphocyte proliferation.

1.2.3. Nervous system

NO participates in the development and physiological or pathophysiological functioning of the central nervous system (CNS) and peripheral nervous system (Bredt & Snyder, 1994; Schmidt & Walter, 1994). NO regulates the development of synaptic connections in the CNS, and has also been implicated in sensory processing, synaptic plasticity and learning. In central neurones, NO formation is coupled to the activation of the excitatory N-methyl-D-aspartate (NMDA)-type glutamate receptor (Garthwaite *et al.*, 1988) resulting in an influx of extracellular calcium, which under physiological conditions leads to the diffusion of NO out of the presynaptic neurone into the postsynaptic neurone thereby elevating cGMP levels. NO-releasing nerves in the peripheral nervous system (PNS) are responsible for NANC neurotransmission in contractile and secretory tissues, e.g. gastrointestinal or respiratory (Rand, 1992; Vallance & Moncada, 1994).

1.3. Nitric oxide synthase isoforms

NO synthase exists in at least 3 distinct isoforms, all of which have been purified and characterised. Molecular cloning has shown these isoforms share 50-60 % homology. There are two constitutive forms. Neuronal NOS (nNOS), whose activity is regulated by calcium and calmodulin, and which is found in neural tissue, both centrally and peripherally. The other, calcium/calmodulin-requiring, constitutive enzyme (endothelial NOS; eNOS) is present in vascular endothelial cells. A third, calcium-insensitive isoform (inducible NOS; iNOS) has been isolated from a variety of cells following induction with inflammatory mediators and bacterial products. Whilst most iNOS enzymes characterised so far exhibit functional calcium/calmodulin-insensitivity, iNOS from rabbit chondrocytes (Palmer *et al.*, 1992, 1993) and rat liver (Evans *et al.*, 1992) is calcium-dependent and inhibited by calmodulin antagonists. The NO synthase enzyme isoforms have been cloned and analysis of the deduced amino acid sequence of nNOS, suggested a bidomain protein structure (Bredt *et al.*, 1991). Dimerisation to homodimers, each of approximately 260 kDa, is required for enzymatic activity. The C-terminal portion of the NOS protein closely resembles cytochrome P-450 reductase (Fig. 1.1), possessing many of the same cofactor binding sites.

Consequently, this portion is referred to as the reductase domain. At the extreme C-terminus is an nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) binding region, which is conserved in all NOS and aligns with that of cytochrome P-450 reductase. The NADPH binding site is followed, in turn, by flavin adenine dinucleotide and flavin mononucleotide consensus sequences (Bredt *et al.*, 1991; Djordjevic *et al.*, 1995). Unlike cytochrome P-450 reductase, NOS is a self-sufficient enzyme in that the oxygenation of its substrate, L-arginine, occurs at a heme-site in the N-terminal portion, termed the oxygenase domain of the protein. Stoichiometric amounts of heme are present in NOS and are required for catalytic activity (White & Marletta, 1992). Bridging the reductase and oxygenase domains is a calmodulin binding site, which seems to act as a switch to regulate electron flow from NADPH, through the flavins, to heme-iron within the oxygenase domain (Abu-Soud & Stuehr, 1993). This pattern is also observed in the inducible (Xie *et al.*, 1992) and endothelial isoforms (Sessa *et al.*, 1992; Figure 1.1).

1.3.1. Neuronal nitric oxide synthase

Neuronal NOS, is a soluble, constitutive enzyme which requires calcium/calmodulin for its activation. It has been cloned from rat cerebellum (Bredt *et al.*, 1991) and human brain (Nakane *et al.*, 1993), and has an open reading frame of 4.3 kb encoding a protein of 160 kDa. Immunocytochemical and histochemical studies have shown that nNOS is present in the epithelium of both human bronchi and rat trachea (Kobzik *et al.*, 1993) as well as in human skeletal muscle (Nakane *et al.*, 1993).

In the central nervous system, glutamate activates NMDA receptors leading to an influx of calcium which is followed by the activation of calcium/calmodulin-dependent NOS. The NO produced may participate in long-term depression or long-term potentiation, indicating that it may be associated with memory and learning functions (Garthwaite, 1991). Excessive release of excitatory amino acids is associated with convulsions and neurotoxicity. The link between stimulation of receptors by these amino acids and the activation of NOS, raises the possibility that overproduction of NO may be involved in cerebral ischemia and epilepsy (Lowenstein & Snyder, 1992).

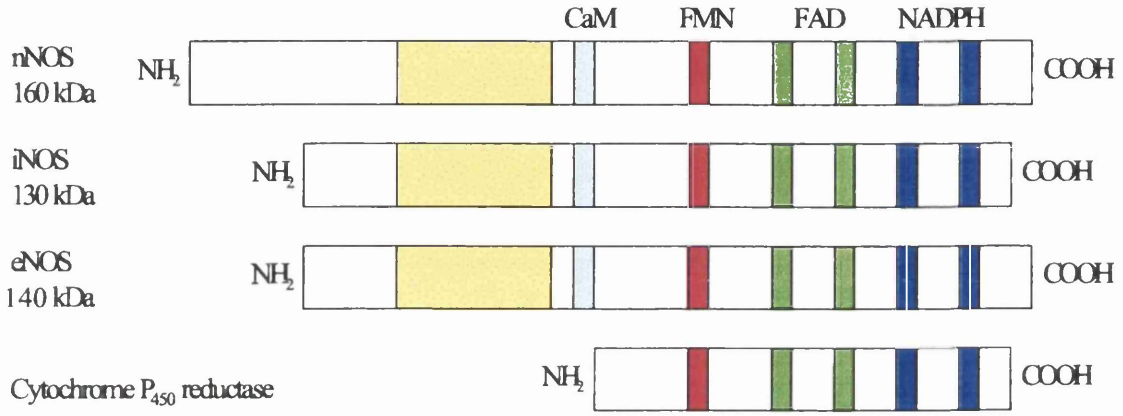


Figure 1.1. Subunit characteristics of NOS isoforms. Consensus sequences for the binding of the cofactors NADPH (adenine and ribose), FAD (isoalloxazine and pyrophosphate), FMN, and for calmodulin. The N-terminal region shows 65 % sequence identity between the three types of NOS. This region contains the putative L-arginine, tetrahydrobiopterin, and heme binding sites (Adapted from Griffith & Stuehr, 1995).

There is an extensive system of peripheral nerves, which are NANC in nature and of this population some that use NO as a neurotransmitter (Garthwaite, 1991). These are known as nitrergic nerves, which innervate organs such as the respiratory, gastrointestinal and urinogenital tract.

1.3.2. Inducible nitric oxide synthase

This enzyme is *de novo* synthesised by macrophages (Yui *et al.*, 1991a), leukocytes (Yui *et al.*, 1991b), liver (Evans *et al.*, 1992), and vascular smooth muscle cells (Busse & Mulsch, 1990) in response to stimulation by cytokines and/or LPS. The induced enzyme generates large amounts of NO and works in host defence and immunologic reactions through inhibition of deoxyribonucleic acid (DNA) synthesis, mitochondrial electron transport, and the tricarboxylic acid (TCA) cycle (Nathan & Hibbs, 1991). This isoform is believed to be involved in septic shock as a result of vasodilatation resulting from the production of excess NO (Ochoa *et al.*, 1991). In LPS-induced septic shock model in rats, iNOS is expressed in vascular endothelial and smooth muscle cells.

Inducible NOS was first cloned from murine macrophages (Lowenstein *et al.*, 1992; Lyons *et al.*, 1992; Xie *et al.*, 1992), and then from rat vascular smooth muscle cells (Nunokawa *et al.*, 1993), and hepatocytes (Wood *et al.*, 1993). It is the smallest of the three isoforms, with an open reading frame of 3.4 kb encoding a 130 kDa protein. This isoform also has a calmodulin-binding domain with higher affinity than that of the other types of NOS. As a result, calmodulin is already so tightly bound at physiological intracellular calcium concentrations that calcium is not required for enzyme activation (Cho *et al.*, 1992). It has been reported that leukocyte NOS requires calcium for full activation (Yui *et al.*, 1991b).

1.3.3. Endothelial nitric oxide synthase

Endothelial NOS has been cloned from bovine (Lamas *et al.*, 1992; Nishida *et al.*, 1992; Sessa *et al.*, 1992) and human (Janssens *et al.*, 1992; Marsden *et al.*, 1992) endothelial cells. It is a constitutive, calcium/calmodulin-dependent enzyme. This isoform is smaller than nNOS, and has an open reading frame of 3.6 kb encoding a 135 kDa protein. Studies using antibodies raised against eNOS have revealed that this enzyme is concentrated in a variety of neuronal populations in rat brain (Dinerman *et al.*, 1994). In mice in which the gene encoding nNOS is selectively deleted, as well as in wild-type mice, hippocampal neurons express eNOS (O'Dell *et al.*, 1994).

In endothelium, certain receptor agonists such as ACh, BK or histamine can stimulate NO synthesis by elevating the intracellular calcium level. Diffusing NO inhibits platelet adhesion and aggregation at the luminal surface and causes relaxation of smooth muscle in the media (Radomski *et al.*, 1987a).

Endothelial NOS is present in particulate form, but does not have any membrane-spanning region. It is bound to the membrane by N-myristoylation (Pollock *et al.*, 1992). The endothelial enzyme is translocated to the cytosolic fraction in association with phosphorylation of the enzyme following exposure to an agonist (Michel *et al.*, 1993a). Moreover, the membrane association of eNOS involving colocalisation with caveolin, a coat protein of caveolae plays an important role in the biosynthesis of NO in the vascular endothelium (Garcia-Cardena *et al.*, 1996).

1.4. Mechanisms of action of nitric oxide

Many of the physiological actions of NO are brought about by its activation of the soluble guanylate cyclase (Murad *et al.*, 1990; Ignarro, 1991; Hobbs, 1997). Binding of NO to the heme moiety of this enzyme causes a conformational change that increases enzyme activity and results in the formation of the intracellular second messenger, cGMP.

Prolonged exposure to NO inhibits the activity of a number of enzymes, such as aconitase and complexes I and II and cytochrome *c* oxidase (Nathan & Hibbs, 1991; Clementi *et al.*, 1998). In addition, DNA synthesis can be impaired by the inhibitory action of NO on ribonucleotide reductase. Such actions render NO cytotoxic or

cytostatic for invading microorganisms and sometimes for the generating cells. These actions explain, in part, the pathophysiological actions of NO. However, it is not clear to what extent these actions are due to NO itself or result from the combination of NO and other molecules, predominantly superoxide ($O_2^{\cdot -}$). Indeed the interaction between NO and $O_2^{\cdot -}$ leads to the generation of peroxynitrite ($ONOO^-$), which is a powerful oxidant (Beckman *et al.*, 1990; Radi *et al.*, 1991).

TABLE 1.1. ISOFORMS OF NITRIC OXIDE SYNTHASE

Characteristic	nNOS	iNOS	eNOS
Molecular weight (kDa)	160	130	135
Size of mRNA (kb)	8.5-9.5	4.2-4.5	4.3-4.8
Quaternary structure	Dimer	Dimer	Dimer
Calcium	Dependent	Independent	Dependent
Calmodulin	Dependent	Independent	Dependent
K_m L-arginine (μM)	2	20	3
K_m Tetrahydrobiopterin (μM)	0.6	0.03	0.1
Solubility	Soluble	Soluble	Particulate
Function	Cell signalling	Cytotoxicity	Cell signalling
Selective Inhibitors	7-nitroindazole	Aminoguanidine	N^{ω}-nitro-L-arginine

Characteristics of nitric oxide synthase isoforms. Adapted from Förstermann & Kleinert, 1995.

1.5. Nitric oxide synthases in the vessel wall

The production of NO from NO synthases in blood vessels contributes to the regulation of smooth muscle tone. Under resting conditions the activity of the constitutive form of the enzyme which is present in endothelial cells (Palmer & Moncada, 1989) is regulated by changes in cytosolic free calcium, calmodulin and NADPH. Increases in intracellular free calcium leads to an increase in NOS activity. In contrast to cyclo-oxygenase, where a threshold intracellular calcium (approximately 0.8 μM) is required for activity, eNOS is sensitive to calcium over a wide concentration range (Carter *et al.*, 1988) thus making it sensitive to moment by moment variations in intracellular calcium, e.g. by shear stress. However, under certain conditions in the presence of pro-inflammatory cytokines and/or bacterial endotoxin, large quantities of NO are generated by the inducible NO synthase. Apart from the anti-microbial role of NO under such conditions, NO may also interact with oxygen-derived radicals to produce other toxic substances such as peroxynitrite. Hence, NO plays multiple and diverse roles and is also involved in the pathogenesis of conditions such as septic shock and inflammation.

1.6. Role of co-factors in NOS enzymology

1.6.1. Calmodulin in the regulation of NOS

Calmodulin is a 17 kDa protein composed of an eight-turn central α-helix, flanked by globular N- and C-terminal calcium binding lobes. Upon calcium binding, these globular lobes compact, exposing sequestered hydrophobic and acidic amino acids that engage in binding to target sites (Babu *et al.*, 1988). Calmodulin regulates a wide range of cellular functions through its reversible calcium-dependent binding to target enzymes, including NOS (Abu-Soud & Stuehr, 1993). It is notable that each lobe of calmodulin has been shown to independently bind to nNOS (Persechini *et al.*, 1994), although the C-lobe of calmodulin binds calcium with approximately 10-fold greater affinity than the N-lobe (Linse *et al.*, 1991). nNOS and eNOS do not bind calmodulin under resting physiological calcium concentrations, however when intracellular calcium concentrations rise, the affinity of these enzymes for calmodulin increases resulting in calmodulin binding which triggers NO production (Schmidt *et al.*, 1991).

Based on kinetic studies of calmodulin binding and activation of purified nNOS and eNOS (Salerno *et al.*, 1997), the following sequential steps have been proposed: (1) calcium binds to calmodulin's C-lobe, exposing hydrophobic and acidic binding residues, (2) the C-lobe of calmodulin binds NOS, (3) calcium binds to the N-lobe of NOS-bound calmodulin, exposing N-lobe NOS-binding residues, and (4) the N-terminal lobe of calmodulin binds NOS, followed by NOS activation. Inducible NOS which is expressed in macrophages stimulated by LPS and/or cytokines, contains tightly bound calmodulin (Cho *et al.*, 1992). Binding of calmodulin enables electron flow from NADPH to the reductase domain (Abu-Soud *et al.*, 1994), and from the reductase to the heme group of the oxygenase domain with calmodulin acting as a molecular switch. It is thought that the close association of calmodulin with iNOS results in a tonically active enzyme, which produces NO in high concentrations over long time periods.

1.6.2. Role of tetrahydrobiopterin in the regulation of nitric oxide synthase

Prior to the discovery of NOS's heme cofactor, tetrahydrobiopterin (BH_4) was identified as an absolute catalytic requirement for NOS activity (Kwon *et al.*, 1989; Tayeh & Marletta, 1989). The precise function for BH_4 has been elusive and is one of the least understood aspects of NOS catalysis. One role for BH_4 in NOS appears to be as an allosteric effector. Radioligand-binding studies with [^{3}H] *N*-nitro-L-arginine have been used to probe the arginine site of rat nNOS (Michel *et al.*, 1993b; Klatt *et al.*, 1994b). Because BH_4 is largely retained in NOS after its purification, the importance of BH_4 for modulation of the arginine-binding site was not appreciated by these initial studies. However, studies of *N*-nitro-L-arginine binding to BH_4-depleted iNOS has revealed that BH_4 is an absolute requirement for specific *N*-nitro-L-arginine binding to occur (Liu & Gross, 1996). Similar results have been obtained using a truncated oxygenase domain comprising $nNOS_{220\text{-}721}$, expressed in *Escherichia coli*. This NOS fragment bound *N*-nitro-L-arginine with a similar high affinity to that observed with native holo-nNOS (50 nM) and, again, BH_4 was found to be essential for binding (Nishimura *et al.*, 1995). This confirms by direct measurement that binding sites for BH_4 and arginine reside within $nNOS_{220\text{-}721}$ and homologous sequences of other NOS isoforms.

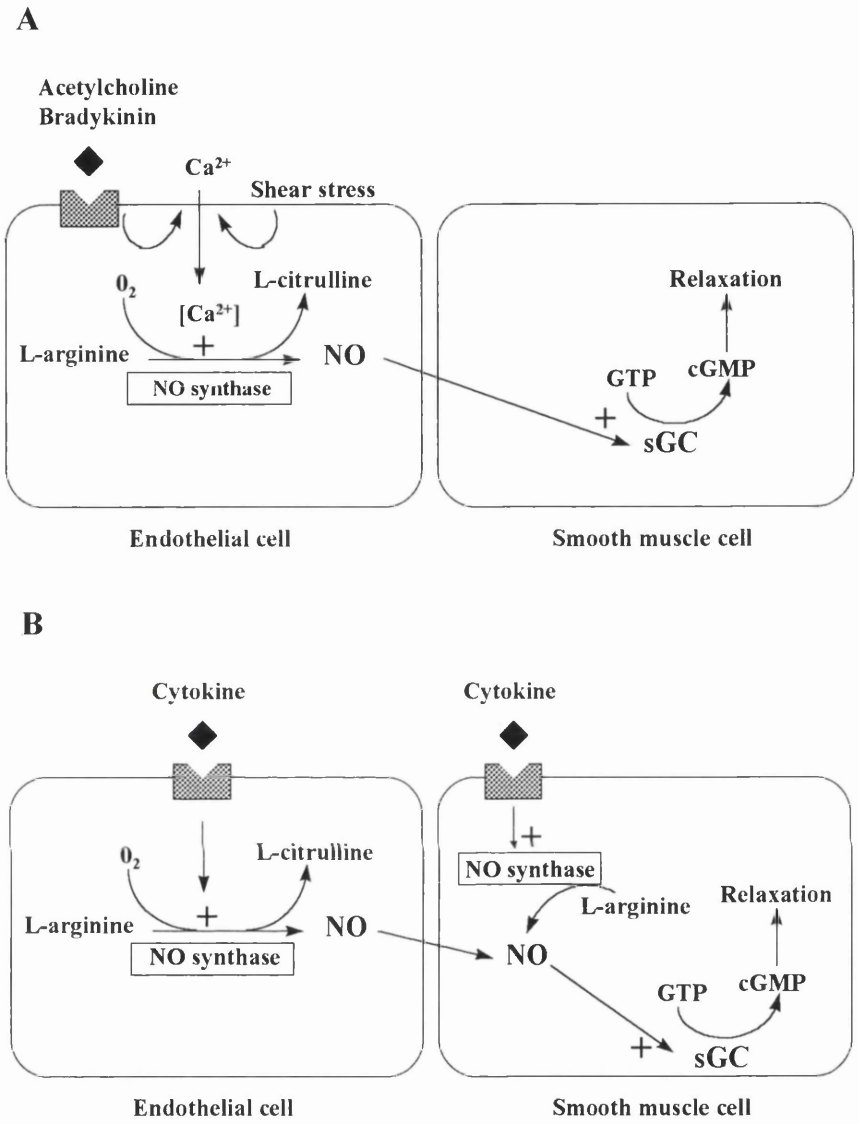


Figure 1.2. Vascular relaxation mediated by nitric oxide. In Panel A, shear stress or receptor activation of vascular endothelium stimulates the constitutive nitric oxide synthase via the release of intracellular calcium and predominantly calcium influx from the extracellular space, which generates nitric oxide that stimulates the soluble guanylate cyclase (sGC) in nearby smooth muscle cells. This leads to the enhanced synthesis of cyclic GMP, activation of protein kinase G resulting in vascular relaxation. In Panel B, the interaction of cytokines with their receptors on endothelial and smooth muscle cells results in the induction of a calcium independent nitric oxide synthase, which generates nitric oxide continuously resulting in sustained activation of soluble guanylate cyclase leading to prolonged smooth muscle relaxation, reduced responsiveness to vasoconstrictors, and possibly tissue damage. + indicates stimulation.

Induction of iNOS by cytokines and immunostimulants occurs concomitantly with induction of BH_4 synthesis in many cell types. Studies in fibroblasts (Werner-Felmayer *et al.*, 1990), endothelial cells (Gross *et al.*, 1991), vascular smooth muscle (Gross & Levi, 1992), and various other cell types have shown that induced BH_4 synthesis is essential for the activity of co-induced iNOS. This requirement for *de novo* BH_4 synthesis to support the high output NO pathway has similarly been observed *in vivo*. NO production by immunostimulant-activated cells can be further increased twofold to threefold upon supplementation with excess BH_4 (Gross & Levi, 1992). Induction of BH_4 synthesis by immunostimulants occurs, at least in part, by transcriptional upregulation of the gene encoding GTP cyclohydrolase-I (GTPCH-I), the first of three enzymes involved in conversion of GTP to BH_4.

1.6.3. Tetrahydrobiopterin synthesis

Pteridines are a class of compounds with a common heterocyclic ring structure, the pyrazino[2,3-*d*]pyrimidine. 2-Amino-4-hydroxypteridines are called pterins, whereas the 2,4-dihydroxy derivatives are called lumazines (Werner *et al.*, 1993). Pteridines include vitamins, such as folic acid and riboflavin, as well as several compounds endogenously synthesized in mammals, such as neopterin and biopterin derivatives.

The biosynthesis of tetrahydrobiopterin is from guanosine triphosphate (GTP), which is cleaved to the first intermediate 7,8-dihydroneopterin triphosphate (Fig. 1.3) by GTPCH-I (EC 3.5.4.16; Burg & Brown, 1968). The further steps from 7,8-dihydroneopterin triphosphate to tetrahydrobiopterin, the end product of the pathway and the biologically active metabolite, proceed via tetrahydroderivatives. At least two further enzymes are involved, i.e., 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase. GTPCH-I (Katzenmeier *et al.*, 1990; Hatakeyama *et al.*, 1991; Katzenmeier *et al.*, 1991), 6-pyruvoyl tetrahydropterin synthase (Inoue *et al.*, 1991; Hauer *et al.*, 1992), and sepiapterin reductase (Citron *et al.*, 1990; Oyama *et al.*, 1990; Ichinose *et al.*, 1991) have recently been sequenced and cloned. GTPCH-I does not require any additional cofactors, whereas the activity of 6-pyruvoyl tetrahydrobiopterin synthase depends on magnesium ions (Smith & Nichol, 1986; Takikawa *et al.*, 1986),

and NADPH is used as coenzyme for the reduction of the keto groups of 6-pyruvoyl tetrahydropterin (Smith & Nichol, 1986). It has also been demonstrated that the changes in GTPCH-I activity usually mediate or closely correlate with changes in tetrahydrobiopterin levels (Duch & Smith, 1991). GTPCH-I activity is controlled both by transcriptional regulation (Werner *et al.*, 1990) and by substrate levels (Hatakeyama *et al.*, 1992). For mammalian GTPCH-I several possible phosphorylation sites have been identified (Hatakeyama *et al.*, 1991), and it has been shown that the enzyme becomes phosphorylated *in vivo* (Imazumi *et al.*, 1994). Moreover, the rat liver enzyme is subject to end product feedback inhibition by tetrahydrobiopterin which, in turn, is mediated by the GTPCH-I feedback regulatory protein (Milstien *et al.*, 1996). In contrast, the two other enzymes implicated in tetrahydrobiopterin biosynthesis, 6-pyruvoyyl tetrahydropterin synthase and sepiapterin reductase, are both constitutively expressed (Werner *et al.*, 1990). GTPCH-I has been purified and characterised from different sources including *E. coli* (Yim & Brown, 1976), *Drosophila melanogaster* (Weisberg & O'Donnell, 1986), and human (Schoedon *et al.*, 1989), mouse (Cha *et al.*, 1991), and rat (Hatakeyama *et al.*, 1989) liver.

E.coli GTPCH-I has been studied in most detail by a combination of X-ray diffraction analysis and electron microscopy of freeze-etched specimens (Meining *et al.*, 1995). Its atomic structure has been determined by X-ray crystallography revealing a 250 kDa homodecameric protein complex consisting of two 5-fold symmetric pentameric rings associated face-to-face (Nar *et al.*, 1995). As yet, a detailed structural characterization of a mammalian GTP cyclohydrolase I homolog has remained elusive.

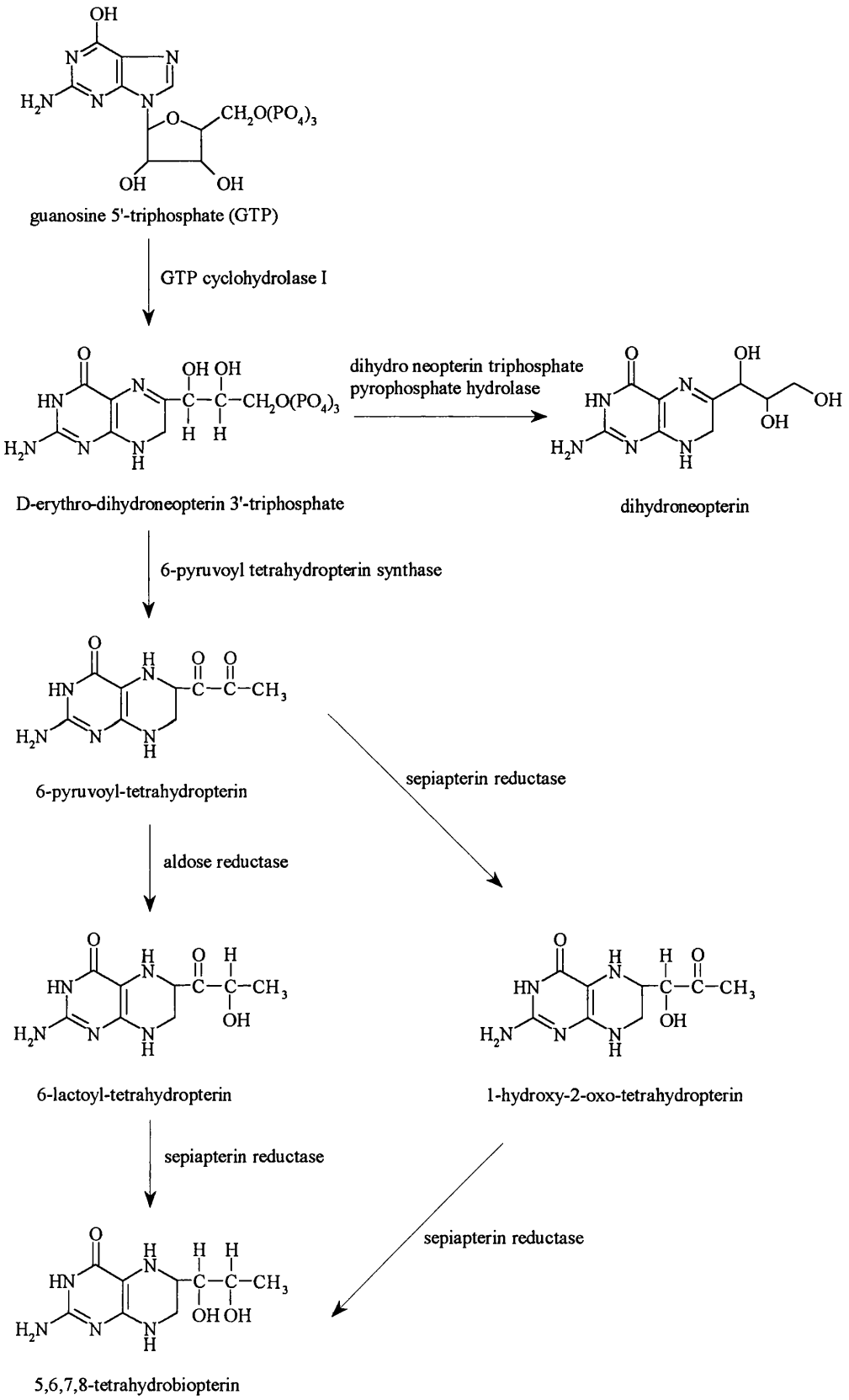


Figure 1.3. Biosynthesis of tetrahydrobiopterin from guanosine triphosphate. Adapted from Werner *et al.*, 1993.

1.7. Use of nitric oxide donors in pharmacological studies

A growing appreciation of the involvement of NO in numerous bioregulatory pathways has not only opened up new therapeutic avenues for organic nitrates and other NO donors but also led to an increased use of such compounds in pharmacological studies.

1.7.1. Biology and pharmacology of nitric oxide donors

NO donors produce NO-related activity when applied to biological systems and are therefore suited to either mimic an endogenous NO-related response or substitute for a NO deficiency. The pathways leading to enzymatic and/or non-enzymatic formation of NO differ among individual compound classes, as do their chemical reactivities and kinetics of NO release. The term "NO donor" implies that the compound releases the active mediator, NO. This may be true for many of the classes of NO donors, since the main NO-related species generated may be converted to NO. However, in a biological system, the redox form of nitrogen monoxide (NO^+, $NO^\cdot$ or NO^-) that is actually released or transferred may make a substantial difference to the NO donor's reactivity towards other biomolecules.

1.7.2. Organic nitrates

Organic nitrates are nitric acid esters of mono- and polyhydric alcohols. Clinically used representatives include glyceryl trinitrate (GTN), pentaerythrityl tetranitrate, isosorbide dinitrate, and isosorbide 5-mononitrate. After more than a century of therapeutic use the exact mode of action of organic nitrates remains unclear. For NO release to occur, organic nitrates require either enzymatic or non-enzymatic bioactivation. It is likely that multiple intracellular and extracellular pathways contribute to NO formation from these compounds *in vivo*, but the relative importance of individual metabolic systems is poorly understood (Ahlner *et al.*, 1991; Feelisch, 1993; Harrison & Bates, 1993; Bennett *et al.*, 1994).

Nonenzymatic formation of NO from nitrate esters requires interaction with sulfhydryl groups. In the course of this reaction, thiols are oxidised to their respective disulfides and nitrite is released as the major nitrogenous metabolite. Although virtually

all thiol compounds decompose organic nitrates to inorganic nitrite, only a select few (i.e. cysteine, N-acetyl-cysteine and thiosalicylic acid) promote concomitant NO generation as well (Feelisch *et al.*, 1988; Chong & Fung, 1991).

The site of enzymatic biotransformation of organic nitrates has not yet been identified. The biphasic profile of NO formation from GTN and the differential susceptibility of tissues that support bioactivation of GTN to heat inactivation (Feelisch & Kelm, 1991) suggest involvement of at least two distinct sites of biotransformation, one of which may be a membrane-bound enzyme (Seth & Fung, 1993). Two enzyme systems have been proposed to account for the bioactivation of organic nitrates-an NADPH-dependent cytochrome P450 pathway (Schröder, 1992; McDonald & Bennett, 1993; McGuire *et al.*, 1994) and certain isoenzymes of the glutathione S-transferase family (Kenkare *et al.*, 1994). In small coronary microvessels it has been demonstrated that administration of L-cysteine markedly enhances GTN-induced vasodilation. This effect may be the result of L-cysteine serving as a precursor for intracellular glutathione synthesis. Thus, glutathione is likely to participate in the intracellular enzymatic bioconversion of GTN to NO (Wheatley *et al.*, 1994), but results have not been consistent. Interestingly, glutathione also increases endogenous NO production (Vita *et al.*, 1998).

1.7.3. Nitrate Tolerance

A major therapeutic limitation to organic nitrates is the development of tolerance which occurs during chronic treatment with these agents (Elkayam, 1991). The mechanisms underlying nitrate tolerance remain poorly defined, and are likely multifactorial. One mechanism seems to be a diminished bioconversion of nitroglycerin to its active vasodilator metabolite (Chung & Fung, 1990). Other mechanisms likely include neurohumoral adaptations, e.g., increases in plasma volume (Dupuis *et al.*, 1990), activation of the renin angiotensin system (Packer *et al.*, 1987), and increases in plasma vasopressin and catecholamines (Parker *et al.*, 1991).

A phenomenon related to nitroglycerin tolerance is cross-tolerance to other nitrovasodilators and endothelium-dependent vasodilators. This has been observed most commonly in situations where nitroglycerin was administered chronically *in vivo*

(Molina *et al.*, 1987), and is usually not encountered in situations where nitroglycerin tolerance is produced by short-term exposure of vascular segments to nitroglycerin *in vitro* (Mulsch *et al.*, 1988).

1.7.4. S-Nitrosothiols

S-Nitrosothiols (RSNOs) are highly coloured solids or liquids which are obtained by *S*-nitrosation of primary and secondary or tertiary thiols. Both nitrosated low molecular weight thiols (such as *S*-nitrosoglutathione; GSNO) and protein thiols (in particular *S*-nitrosoalbumin) have been detected in biological fluids as a consequence of endogenous administration of NO, such as therapy with inhaled NO gas (Stamler *et al.*, 1992; Gaston *et al.*, 1993). It has been proposed that *S*-nitrosothiol groups in proteins serve in the metabolism of NO and in regulation of cellular functions: in transport and targeting of the NO group to specific, thiolregulatory effector sites, including enzymes, and signaling proteins (Stamler, 1995). RSNO intermediates have also been implicated in the smooth muscle relaxant effects of nitrovasodilators (Ignarro *et al.*, 1981).

It has recently been shown that homolytic decomposition of *S*-nitrosothiols, yielding thiyl radicals ($RS^{\cdot}$) and NO, is catalysed by trace amounts of cuprous (Cu^{+}) and ferrous ions, and that the biological activity of some RSNOs can be significantly reduced by chelation of copper ions (e.g. with bathocuproine sulphonate; Gordge *et al.*, 1995). Further studies have revealed that the effects of thiols and other reductants on the bioactivity of RSNOs can be explained by reduction of Cu^{2+} to Cu^{+} (Dicks *et al.*, 1996; Gorren *et al.*, 1996). In addition to homolytic cleavage, nitrosothiols can also decompose heterolytically with formation of NO^{+} or NO^{-} (Arnelle & Stamler, 1995), a pathway of decomposition that appears to predominate in biological systems.

1.7.5. Sydnonimines

Sydnonimines are mesoionic heterocycles which exhibit various biological activities (Ackermann, 1967). The most studied representative of this NO donor class is molsidomine which is in clinical use as an antianginal drug. Molsidomine is an *N*-acylprodrug which is converted by liver esterases to the active metabolite,

3-morpholinosydnonimine (SIN-1). Whereas molsidomine itself is poorly vasoactive *in vitro*, SIN-1 is a potent vasorelaxant and antiplatelet agent. These activities are thought to be mediated largely by spontaneous release of NO. Not all of the biological effects of sydnonimines can be attributed to NO. The molsidomine-related compound, CAS 936 has been shown to relax potassium-depolarised vascular tissue in a haemoglobin-insensitive manner (Bohn *et al.*, 1991) and SIN-1-induced suppression of neutrophil degranulation has been shown not to be mediated by cGMP, NO or $ONOO^-$, but by the NO-lacking metabolite, SIN-1C (Kankaanranta *et al.*, 1997).

At physiological and alkaline pH, SIN-1 undergoes rapid non-enzymatic hydrolysis to the ring-open form SIN-1A. Traces of oxygen promote oxidative conversion to a cation radical intermediate which, upon NO release and deprotonation, undergoes cleavage to the corresponding N-morpholino-aminoacetonitrile, SIN-1C. In the course of this reaction stoichiometric amounts of $O_2^{\cdot-}$ are formed as a result of oxygen reduction (Feelisch *et al.*, 1989). As NO is known to react with $O_2^{\cdot-}$ at almost diffusion-controlled rate (Huie & Padmaja, 1993), $ONOO^-$ production is likely. This and other observations have led to the use of SIN-1 as a $ONOO^-$ donor.

1.7.6. NONOates

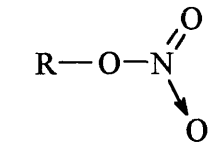
NONOates are adducts of NO with nucleophiles in which a NO dimer is bound to the nucleophilic residue via a nitrogen atom, thus forming a functional group of the structure $X\text{-}[N(O)NO]^-$. NONOates have been shown to relax vascular tissue in an endothelium-independent manner, to increase tissue cGMP and to inhibit platelet aggregation and cell proliferation *in vitro* (Maragos *et al.*, 1993; Morley *et al.*, 1993; Mooradian *et al.*, 1995). A selected candidate, the NO adduct with diethylamine NONOate, has also been shown to produce pulmonary and systemic vasodilation upon intravenous infusion *in vivo* (Vanderford *et al.*, 1994). It is thought that NONOates generate NO spontaneously (the rate of release being unaffected by biological reactants). However, high concentrations of thiol may decrease NO release, and enzymatic metabolism *in vivo* remains a distinct possibility. NO liberation properties of individual compounds are determined by the structure of the nucleophile, the pH and the temperature of the incubation solution (Morley & Keefer, 1993). Reported half-lives at

pH 7.4 and 37°C range from 2 min for DEA/NO to 20 h for diethylene triamine NONOate (DETA/NO; Maragos *et al.*, 1991; Mooradian *et al.*, 1995).

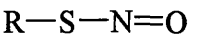
1.7.7. Sodium Nitroprusside

The most commonly studied of the iron nitrosyls is sodium nitroprusside (SNP), an inorganic complex where iron is in the ferrous state and NO is formally bound as NO^+. The compound is used clinically to reduce blood pressure, e.g. in hypertensive emergencies. The nitroprusside anion undergoes addition reactions with a variety of sulphur-, oxygen-, nitrogen- and carbon-centered nucleophiles. Despite intensive studies of its chemical reactivity with thiols (Leeuwenkamp *et al.*, 1984), the mechanism of NO release remains incompletely understood. It is clear, however, that SNP requires either irradiation with light or one-electron reduction to release NO (Singh *et al.*, 1995).

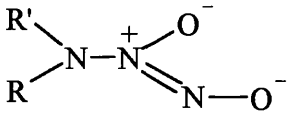
In biological systems, both non-enzymatic and enzymatic NO release from SNP may occur. Attack of thiolate anions may lead to decomposition of the complex with formation of disulfide, NO and cyanide. A membrane-bound enzyme may be involved in the generation of NO from SNP in biological tissues, and either nicotinamide adenine dinucleotide, reduced form (NADH) or NADPH appears to be required as a cofactor (Bates *et al.*, 1991; Rao *et al.*, 1991; Kowaluk *et al.*, 1992).



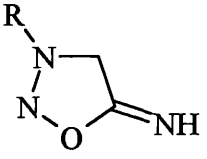
Organic nitrates



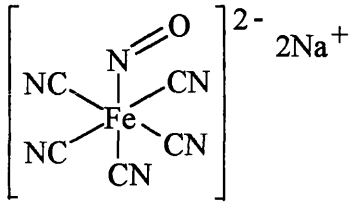
S-Nitrosothiols



NONOates



Sydnonimines



Sodium nitroprusside

Figure 1.4. Chemical structures of NO donors.

1.8. Superoxide generating systems

In recent years, reactive oxygen species (ROS) such as $O_2^{\cdot-}$ have been shown to play a number of roles in the body. Phagocytic cells produce ROS as a primary host defence mechanism (Miller & Brittigan, 1995), whereas other cells utilise ROS as intracellular second messengers for a wide range of cellular functions. For example, ROS participate in Ras-mediated mitogenic signaling in fibroblasts as well as in leukocyte apoptosis (Irani *et al.*, 1997).

The phagocyte NADPH oxidase or respiratory burst oxidase is the best characterised ROS-generating system and is a multicomponent enzyme complex that catalyses the one-electron reduction of oxygen to $O_2^{\cdot-}$. Its components include the two membrane-spanning polypeptides, $p22^{phox}$ and $gp91^{phox}$, which comprise flavocytochrome b_{558}, and three cytoplasmic polypeptides, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ (DeLeo & Quinn, 1996; Zhan *et al.*, 1996).

1.8.1. Superoxide generation in blood vessels

In addition to the tonic release of NO from the vessel wall, there is also a basal production of $O_2^{\cdot-}$. In contrast to the well characterised biology of NO in blood vessels and cell signalling, the biosynthetic pathways and function of $O_2^{\cdot-}$ remains unclear. The enzymatic source of $O_2^{\cdot-}$ varies between different cell types and potential generating systems include NADH/NADPH oxidases (Pagano *et al.*, 1995; Jones *et al.*, 1996), NOS (in the absence of L-arginine; Xia *et al.*, 1996), xanthine oxidase (Miyamoto *et al.*, 1996) and respiratory chain enzymes (Mohazzab *et al.*, 1994).

It has been demonstrated that $O_2^{\cdot-}$ is released from blood vessels by endothelial cells (Jones *et al.*, 1996) and vascular smooth muscle cells (Ushio-Fukai *et al.*, 1996) by a membrane bound NADH/NADPH oxidase. The NADPH oxidase systems in these cells have not yet been well characterised, and even the substrate specificity of these oxidase(s) with regard to NADH and NADPH is still not clear (Mohazzab & Wolin, 1994; Griendling *et al.*, 1994; Mohazzab *et al.*, 1994; Pagano *et al.*, 1995). Diphenyleneiodonium, a commonly used inhibitor of NADPH oxidase, reduces $O_2^{\cdot-}$ generation from blood vessels suggesting the presence of a functional NADPH oxidase in vascular cells (Pagano *et al.*, 1995). However, this agent has also been demonstrated to

inhibit endothelium-dependent vasodilatations *in vitro* (Wang *et al.*, 1993) possibly acting as an NO synthase inhibitor. Other evidence indicating the presence of an NADPH oxidase in endothelial cells has been obtained by identification of four (p22, gp91, p47 and p67) of the five recognised components, by reverse transcriptase polymerase chain reaction (PCR) using primers derived from known phagocyte sequences (Jones *et al.*, 1996). Sequence analysis has shown a high sequence homology between the human endothelial and neutrophil NADH oxidase. Expression of various components have also been detected by immunofluorescence and Western blotting analysis.

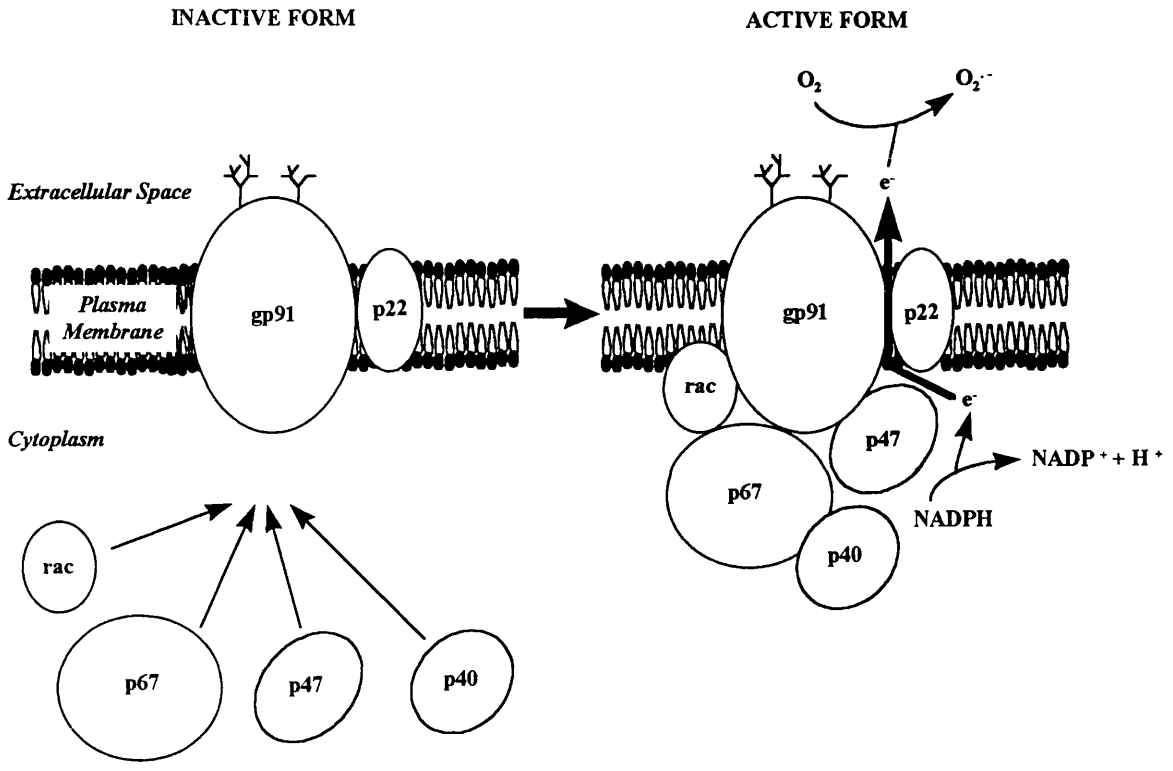


Figure 1.5. Schematic representation of the neutrophil NADPH oxidase. The two proteins comprising cytochrome b_{558}, p22 and gp91, are shown in the plasma membrane. Cytosolic components p40, p47 and p67 have translocated to the membrane where they are shown associated with the cytochrome *b*. The NADPH binding site, though illustrated in relation to the cytosolic components, is not known. Electrons are shuttled from NADPH to oxygen.

1.8.2. Antioxidant defences

Removal of excess $O_2^{\cdot-}$ by intracellular superoxide dismutase (SOD) enzymes is an important physiological antioxidant defence mechanism (Fridovich, 1989). The walls of blood vessels additionally contain an "extracellular SOD" enzyme, which could conceivably serve to modulate the $O_2^{\cdot-}$/NO interaction (Abrahamsson *et al.*, 1992). Mice lacking a functional extracellular SOD gene appear normal, but are more sensitive to hyperoxia (Carlsson *et al.*, 1995). The action of all types of SOD enzymes generates hydrogen peroxide (H_2O_2). Vascular endothelial cells can generate additional H_2O_2 by the action of several oxidase enzymes, including xanthine oxidase (Zweier *et al.*, 1994), which makes both $O_2^{\cdot-}$ and H_2O_2 during its catalytic cycle. In vascular endothelial and smooth muscle cells, both catalase and glutathione peroxidase enzymes are involved in removal of H_2O_2 (Verkerk *et al.*, 1992).

1.9. Interaction between nitric oxide and superoxide

Under certain conditions $O_2^{\cdot-}$ reacts with NO resulting in the formation of a powerful oxidant species, $ONOO^-$ (Blough & Zafiriou, 1985; Figure 1.6). The reaction rate for the formation of $ONOO^-$ has been determined as 6.7×10^9 M/s (Huie & Padmaja, 1993) and is faster than the dismutation of $O_2^{\cdot-}$ by SOD (2×10^9 M/s) at physiological ionic strength, pH 7.4 (Cudd & Fridovich, 1982). In effect, $ONOO^-$ is formed mainly when cells generate large amounts of NO and $O_2^{\cdot-}$. As eukaryotic cells contain large amounts of SOD, the vital determinant of $ONOO^-$ formation may be whether the concentration of NO rises to levels to high enough to overcome the dismutation of $O_2^{\cdot-}$ by NO.

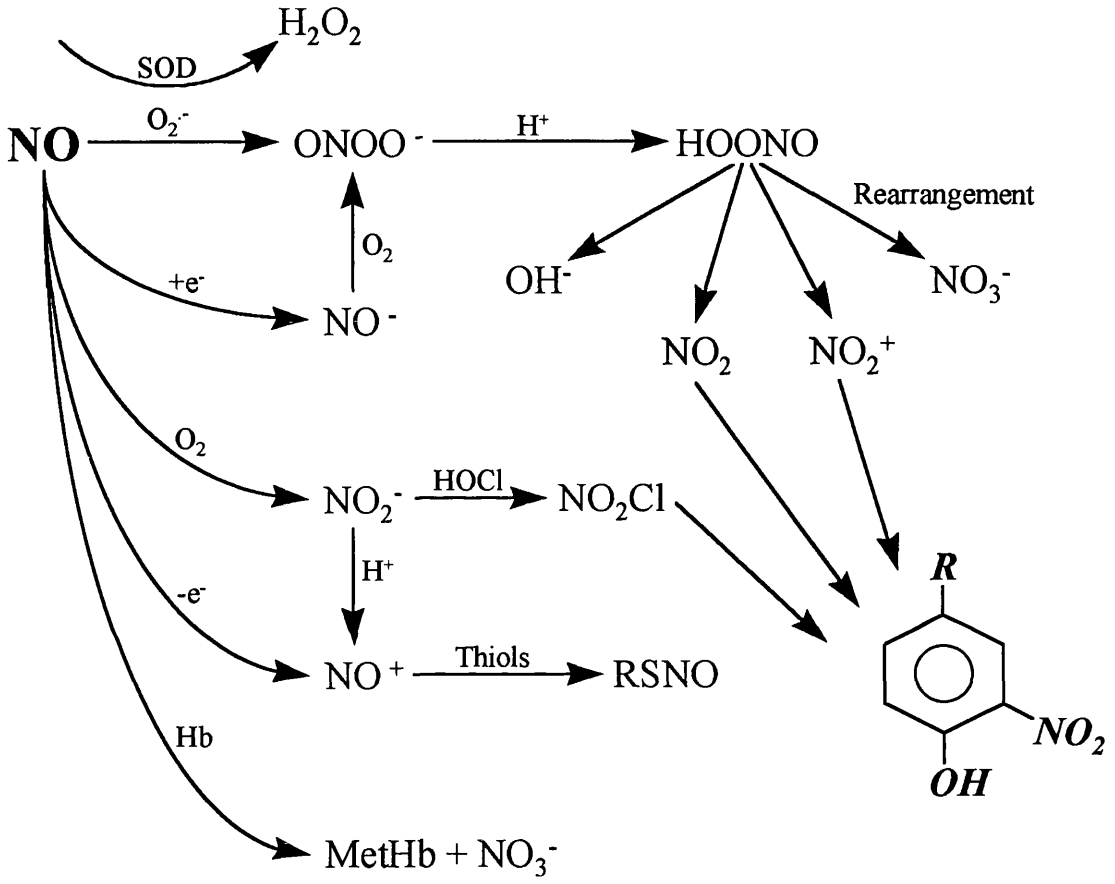


Figure 1.6. Reactions of nitric oxide, superoxide and peroxynitrite.

Under normal conditions superoxide is dismutated to hydrogen peroxide and oxygen by SOD. However, under certain conditions, when concentrations of NO rise to high levels, it can effectively compete with SOD because of its rapid rate constant. Abbreviations: H_2O_2, hydrogen peroxide; $ONOO^-$, peroxynitrite; HOONO, peroxynitrous acid; NO^-, nitroxyl anion; NO_2^-, nitrite; NO^+, nitrosyl cation; RSNO, nitrosothiol; MetHb, methaemoglobin; NO_3^-, nitrate; NO_2, nitrogen dioxide; NO_2^+, nitronium cation; OH^-, hydroxyl anion.

1.9.1. Biological effects of peroxynitrite

Increasing evidence suggests that $ONOO^-$ is a contributor to tissue injury. Furthermore, the formation of $ONOO^-$ results in lipid peroxidation and modification of enzymes and other proteins (Ischiropoulos & Al-Mehdi, 1995). An important reaction of $ONOO^-$ is its heterolytic cleavage by transition metals to form hydroxyl anion and nitronium ion. The nitronium ion is well known to attack phenolics to produce nitrophenolics. Furthermore, the nitration of protein tyrosine residues to give 3-nitrotyrosine is a chemical footprint left by $ONOO^-$ (Oury *et al.*, 1995). Tyrosine nitration is a convenient marker of reactive nitrogen-centred oxidants being formed. It is not entirely due to the formation of $ONOO^-$ but is the most likely candidate *in vivo* (Beckman & Koppenol, 1996). Tyrosine nitration has been found in human lung biopsy and autopsy samples from patients with sepsis or adult respiratory distress syndrome (Haddad *et al.*, 1994) and in atherosclerotic vessels (Beckman *et al.*, 1994). At present the functional consequences of tyrosine nitration are unknown, but potentially this chemical substitution could have important biological effects. Interestingly, it has been suggested that nitration of neurofilament L, which is greatly catalysed by SOD may have a significant role in the pathogenesis of amyotrophic lateral sclerosis (Crow *et al.*, 1997).

1.10. Nitrogen dioxide

Exposure of tyrosine to nitrogen dioxide (NO_2) in aqueous solution generates both nitrotyrosine and bityrosine (Prutz *et al.*, 1985; Kikugawa *et al.*, 1994; Van der Vliet *et al.*, 1995) and NO_2 can also nitrate tyrosine residues in proteins (Prutz *et al.*, 1985; Kikugawa *et al.*, 1994; Van der Vliet *et al.*, 1995). Indeed, NO_2 production may be one mechanism accounting for tyrosine nitration on addition of $ONOO^-$ at physiological pH. However, it seems that high levels of NO_2 are needed to achieve tyrosine nitration, even *in vitro* (Kikugawa *et al.*, 1994). NO_2 can arise from NO oxidation, by oxidation of nitrite and perhaps from $ONOO^-$ at physiological pH.

1.11. Nitryl (nitronium) chloride

It has been demonstrated that the reaction of nitrite with hypochlorous acid, generates a product that can nitrate tyrosine and other aromatic compounds (Eiserich *et al.*, 1996). Detailed chemical characterization (Eiserich *et al.*, 1996) has identified this compound as nitryl (nitronium) chloride. Hypochlorous acid is well known to be produced at sites of inflammation by activated neutrophils where nitrite can be present in μM amounts (Weiss, 1989). Addition of hypochlorous acid (Domigan *et al.*, 1995; Kettle, 1996) or of nitryl chloride (Eiserich *et al.*, 1996) can lead to chlorination of tyrosine, generating 3-chlorotyrosine. Although there is no direct evidence for nitryl chloride production *in vivo* (hypochlorous acid generated *in vivo* can react with many molecular targets other than nitrite or tyrosine), however, it has been suggested that caution should be taken in attributing nitration to the effects of $ONOO^-$ (Eiserich *et al.*, 1996). Of course, $ONOO^-$ cannot chlorinate aromatic rings, therefore, observations of nitration in the absence of chlorination would suggest that nitryl chloride is not involved.

1.12. Myeloperoxidase

Hypochlorous acid is generated by the H_2O_2-dependent oxidation of chloride ions by the haem-containing enzyme myeloperoxidase (Weiss, 1989). Myeloperoxidase is a 'non-specific'peroxidase, capable of using H_2O_2 to oxidize a wide range of substrates. It has been shown that myeloperoxidase can oxidize nitrite in the presence of H_2O_2 into a species able to nitrate tyrosine (Van der Vliet *et al.*, 1997). Myeloperoxidase/H_2O_2 is capable of oxidising tyrosine to tyrosyl radicals (Heinecke *et al.*, 1993; Marquez & Dunford, 1995). Such radicals could combine with H_2O_2 to form nitrotyrosine (Van der Vliet *et al.*, 1997). Indeed, any oxidizing species capable of oxidizing both tyrosine to tyrosyl radicals and nitrite to H_2O_2 should lead to nitrotyrosine formation: $ONOO^-$-derived species may generate nitrotyrosine in this way (Van der Vliet *et al.*, 1995).

1.13. Aims of the thesis

The overall aim of this thesis was to study the mechanisms underlying the regulation of NO production by co-factors and the consequent interaction between NO and $O_2^{\cdot-}$ in the vasculature.

The specific aims addressed by this thesis were to investigate:

1. the regulation of NO and $O_2^{\cdot-}$ production.
2. the interactions between $O_2^{\cdot-}$ and NO *in vitro* and *in vivo*.
3. whether $ONOO^-$ can be generated in the absence of iNOS and what mechanism(s) might be responsible for this.

To address these aims, a wide range of methodological approaches were used in cultured cells, isolated blood vessels and whole animals.

CHAPTER 2

METHODOLOGY

2.1. Cell culture techniques

2.1.1. Culture and maintenance of J774 murine macrophage cell line

The murine macrophage cell line, J774, was obtained from the European Collection of Animal Cell Cultures (Porton Down, Wilts.) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with foetal calf serum (10 % v/v), L-glutamine (4 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were kept in continuous culture in stirrer flasks (Techne), stirred at a rate of 30 revolutions/min in a humidified incubator at 37 °C in an atmosphere of 95 % O_2 / 5 % CO_2.

2.1.2. Sub-culture of cells into 96-well plates

For use, an aliquot of cells was obtained from the stirrer flask and counted using a haemocytometer. Cells were diluted in DMEM to a final concentration of 5×10^5 cells/ml and 200 μl of cell suspension were inoculated to the inner 60 wells of a 96-well plate (final cell density = 10^5 cells/well). Cells were allowed to adhere for 2-4 h in a humidified incubator at 37 °C in an atmosphere of 95 % O_2 / 5 % CO_2.

2.1.3. Release of nitric oxide from cultured J774 cells

Production of NO by J774 cells was determined by measurement of nitrite accumulation in the culture medium. NO is released from cells and broken down spontaneously to nitrite and nitrate in culture medium. Following plating, J774 cells were activated by addition of medium containing 1 μg/ml lipopolysaccharide (LPS; *Eschericia coli,* serotype 055:B5). Twenty four hours later 100 μl medium were removed for measurement of nitrite.

2.2. In vivo techniques

2.2.1. Preparation of rabbit aortic rings

Male New Zealand white rabbits (3-4 kg) were killed by injection of sodium pentobarbitone (125 mg/kg) via the lateral ear vein. Thoracic aorta was removed and placed in ice-cold Krebs bicarbonate buffer (composition mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 0.6, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, Na_2-EDTA 0.026 and glucose 5.5). Vessels were cleaned of surrounding fat and adventitia, sectioned into 5 mm rings, rinsed with ice-cold bicarbonate buffer and stored at 4 °C, until time of assay.

2.2.2. Induction of endotoxin shock in mice

Two group of mice were used in these studies. The first group consisted of male MF1 wild-type (+/+) and homozygous (-/-) iNOS-deficient mice (obtained from Professor Liew, Department of Immunology, University of Glasgow). Mice deficient in the gene for iNOS were generated by disruption of the iNOS gene in embryonic stem cells which were then used to produce germline chimeras. F_1 mice heterozygous for the mutation were bred to obtain homozygotes for the disrupted iNOS gene (Wei *et al.*, 1995). The second group consisted of male C57BL/6J wild-type (+/+) and homozygous (-/-) iNOS-deficient mice (purchased from The Jackson Laboratory, USA). To generate chimeric mice, C57BL/6 blastocytes were injected with the recombinant embryonic stem cells and implanted into pseudopregnant females. Chimeric males were mated with females, and the resulting F_1 heterozygotes were mated to generate F_2 mice homozygous for the iNOS disruption (Laubach *et al.*, 1995). Genotyping of mice was performed by Southern analysis. All mice were aged 8 to 12-weeks and were maintained on ordinary laboratory chow and tap water *ad libitum*, in a temperature-controlled room. All studies were performed in accordance with the United Kingdom Home Office regulations for the care and use of animals (Animals (Scientific Procedures) Act, 1986). Mice were injected with either LPS (*Escherichia coli* serotype 0126:B6; 12.5 mg/kg; i.v.) or vehicle (saline) as a bolus via the tail vein. All animals were killed 12 h later by cervical dislocation following gaseous anaesthesia with isoflurane (2 %). A 12 h time point was selected, since it has been established in a model of endotoxin shock in the conscious mouse that LPS (12.5 mg/kg, i.v.) induced the expression of iNOS in the

mouse heart and elevated the concentrations of nitrite and nitrate in the plasma, reaching a maximum at 12 h. This was accompanied by a progressive fall in blood pressure over the same period (Rees *et al.*, 1998). Blood samples were collected immediately by cardiac puncture with a syringe containing 0.1 ml of 250 U/ml heparinised saline (0.9 %). Samples of aorta, heart, and liver were frozen in liquid nitrogen, and stored at -80 °C.

2.2.3. 2,4-Diamino-6-hydroxypyrimidine administration

DAHP (75 mg/ml) was dissolved in 2 % carboxymethylcellulose (CMC) immediately prior to use, and administered orally as a suspension (15 mg in 0.2 ml), via a stomach catheter, at 12 h intervals for 2 days. Control animals received 2 % CMC. On day 3 animals received either LPS (12.5 mg/kg; i.v.) or saline as a bolus dose, as described by Bune *et al.* (1996).

2.3. Biochemical techniques

2.3.1. Measurement of nitrite production from cells in culture (Griess Reaction)

Production of nitrite was used as an indicator of NO synthesis. J774 cells were incubated in culture medium and after specified times nitrite production was measured using the Griess reaction (Green *et al.*, 1981). Griess reagent was prepared by mixing 1 % w/v sulphanilamide and 0.1 % w/v napthylethylenediamine dihydrochloride in 2.5 % v/v orthophosphoric acid. 100 µl of Griess reagent were added to an equal volume of culture medium and the optical density at 540-620 nm ($OD_{540\text{-}620}$) measured using a Molecular Devices Spectramax 250 microplate reader. Nitrite concentrations were determined by comparison to values obtained using standard solutions of sodium nitrite prepared in culture medium (Figure 2.1).

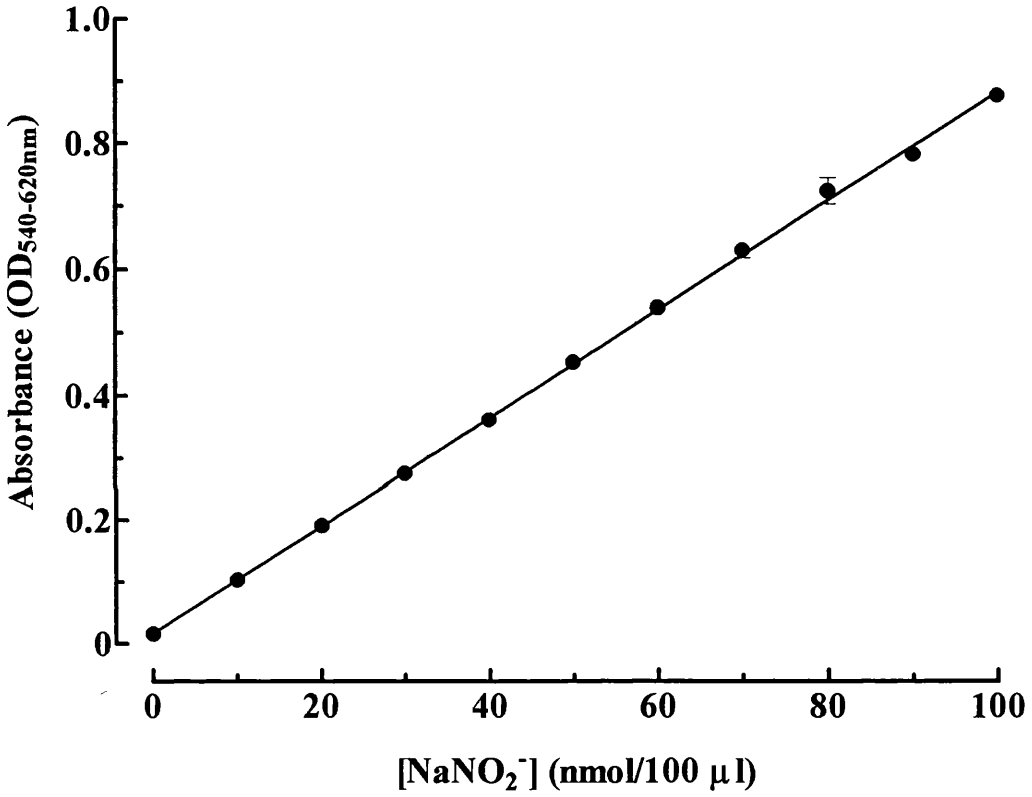


Figure 2.1. Typical standard curve for sodium nitrite in DMEM. Figure shows standard curve in a representative experiment. Griess reagent (100 µl) was mixed with $NaNO_2$ in serum containing DMEM at room temperature for 10 min. Absorbance at 540-620 nm was measured as described previously. Results are expressed as mean ± s.e.m of 3 observations. Correlation coefficient $r^2 = 0.999$.

2.3.2. Measurement of blood plasma nitrite/nitrate (Griess Reaction)

Blood samples were centrifuged (14,000 × g, for 5 min) to produce plasma. Plasma was ultra-filtered by centrifugation (14,000 × g, for 10 min) using a 10,000 kDa molecular weight filter. If the filtrate was not clear and colourless, due to defects in the filters, the samples were refiltered. The nitrate in the deprotinised filtrate was reduced to nitrite with nitrate reductase (aspergillus species) as described previously (Verdon *et al.*, 1995) and nitrite measured by the Griess reaction. Typically, 10 µl of sample, standards ($NaNO_2^-$ or $NaNO_3^-$ varying in concentration from 10 to 100 µM), or 50 µl of blank were placed in wells of a 96-well microassay plate. Following this, 40 µl of phosphate buffered saline were added to all assay wells except blanks. Next, 10 µl of NADPH (1 µM) were added followed by 40 µl of a freshly prepared mixture containing glucose-6-phosphate (500 µM), glucose-6-phosphate dehydrogenase (160 U/ml), and nitrate reductase (80 U/ml) in phosphate buffered saline (pH 7.4) and the microplate was incubated for 60 min at 37 °C. It has been reported that NADPH can interfere with the Griess reaction at higher concentrations than that used in the above assay (Verdon *et al.*, 1995). The resultant nitrite was measured by addition of the components of the Griess reagent: 100 µl of 1 % w/v sulphanilamide in 5 % orthophosphoric acid immediately followed by 100 µl of 0.1 % w/v napthylethylenediamine dihydrochloride. After incubation for 15 min at room temperature, the absorbance was read at 540-620 nm ($OD_{540-620}$) using a Molecular Devices Spectramax 250 microplate reader. The efficiency of nitrate conversion was 98.9 ± 0.4 % (n=6).

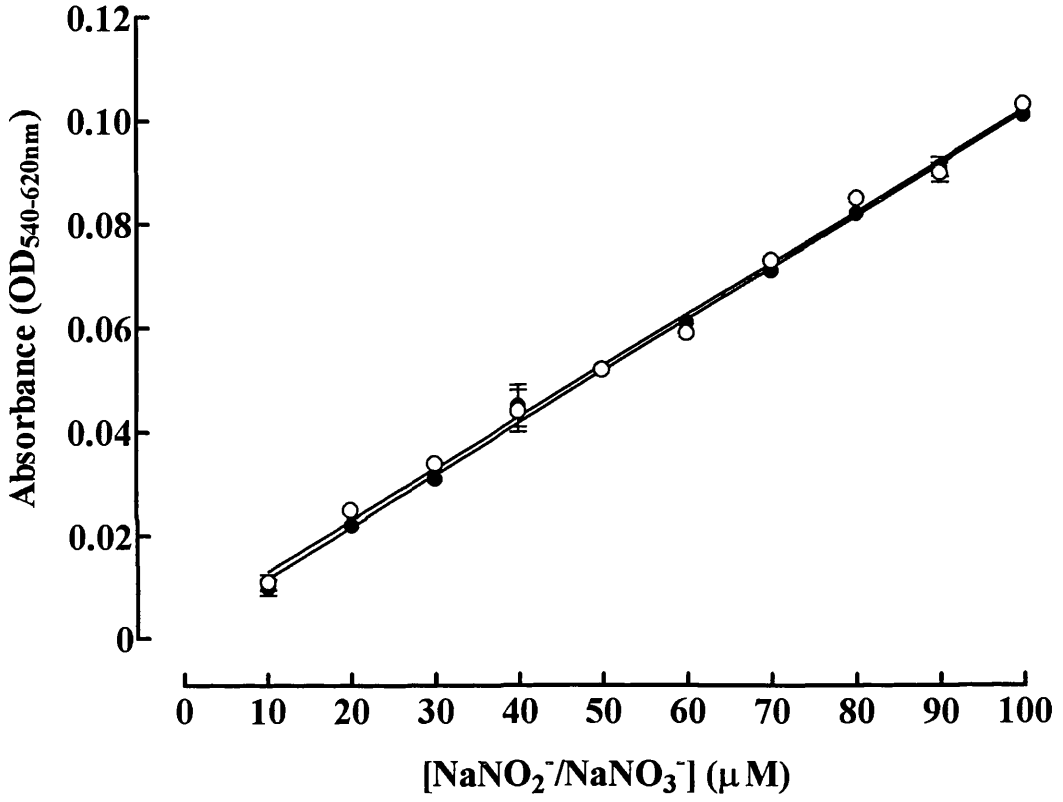


Figure 2.2. Typical standard curve for nitrite and nitrate in plasma. Figure shows standard curve in a representative experiment. 100 μl of 1 % w/v sulphanilamide in 5 % orthophosphoric acid immediately followed by 100 μl of 0.1 % w/v napthylethylenediamine dihydrochloride was added to sodium nitrite (open circles) and sodium nitrate (closed circles) standards treated with nitrate reductase. Absorbance at 540-620 nm was measured as described previously. Results are expressed as mean ± s.e.m of 3 observations. Nitrite and nitrate correlation coefficient $r^2 = 0.995$ and 0.998, respectively.

2.3.3. *Isolation of nitric oxide synthase from J774 cells*

J774 cells, maintained in stirrer flasks, were incubated in L-arginine free medium containing LPS (10 μg/ml) and murine interferon-γ (IFN-γ; 50 U/ml) for 20 h. Following incubation, cells were lysed by sonication (on ice) in homogenization buffer (composition 0.1 M HEPES, 0.1 mM dithiothreitol, pH 7.4) using a Janke & Kunkel Ultra-Turrax T25 (5 sec bursts with a 20 sec delay; repeated 3 times) and centrifuged at 100,000 × g for 30 min at 4 °C. Endogenous L-arginine was removed by adding crude homogenates to Dowex-50 (200-400; 8 % cross linked, sodium form) 1:1 w/v and incubating for 5 min at room temperature. After centrifugation (100,000 × g) to collect the resin, supernatants were removed and stored on ice until use. This homogenate was then used as a crude source of inducible NO synthase.

2.3.4. *Preparation of reduced haemoglobin*

Commercially available human haemoglobin contains both a small percentage (approximately 1 %) of reduced or ferrous haemoglobin and, in the vast majority, its oxidised derivative, methaemoglobin. Thus, as supplied, the haemoglobin must be chemically reduced before use. This was achieved according to the methods of Feelisch & Noack (1987). A 2-fold molar excess of sodium dithionite was added to 10mM solution of haemoglobin and stirred for 30 min at room temperature to ensure complete reduction of haemoglobin. Sodium dithionite was removed by passing the resulting oxyhaemoglobin solution through a Sephadex G-25 column equilibrated and eluted with phosphate buffered saline (10 mM, pH 7.4). The resulting solution was stored frozen in 200 μl aliquots at -20 °C. The purity of solutions was checked spectrophotometrically, reduced haemoglobin exhibiting characteristic absorption maxima at 414, 542 and 577 nm (Figure 2.3). The concentration of oxyhaemoglobin was calculated according to the following equation:

$$[oxyhaemoglobin] = \Delta\, Absorbance_{(542\text{-}510\ nm)} \times \Delta\varepsilon_{(542\text{-}510\ nm)} \times l$$

where ε = extinction coefficient for oxyhaemoglobin at 542-510 nm (9.61 mM/cm)

l = path length (0.183 cm)

2.3.5. Measurement of nitric oxide synthase activity

NOS enzyme activity was assessed by monitoring the conversion of oxyhaemoglobin to methaemoglobin in a spectrophotometer as described previously (Feelisch & Noack, 1987). For the assay, 30 µl of assay buffer (composition: Tris-HCl 200mM, $CaCl_2$ 0.24 mM, pH 7.4) were incubated with NADPH (1 mM), L-arginine (100 µM), dithiothreitol (1 mM), tetrahydrobiopterin (10 µM), oxyhaemoglobin (50 µM) and 10 µl of cell homogenate for 20 min at 37 °C in a 96-well plate. Production of NO was initiated by rapid addition of 10 µl of NADPH to the reaction mixture. The rate of change in $A_{401\text{-}411}$, was measured at 15 s intervals for 15 min at 37 °C in a Molecular Devices Spectramax 250 microplate reader. Protein content of the cytosol was determined according to the methods of Bradford (1976), as described in section 2.3.8. Activity was expressed as pmol of NO/mg protein/min.

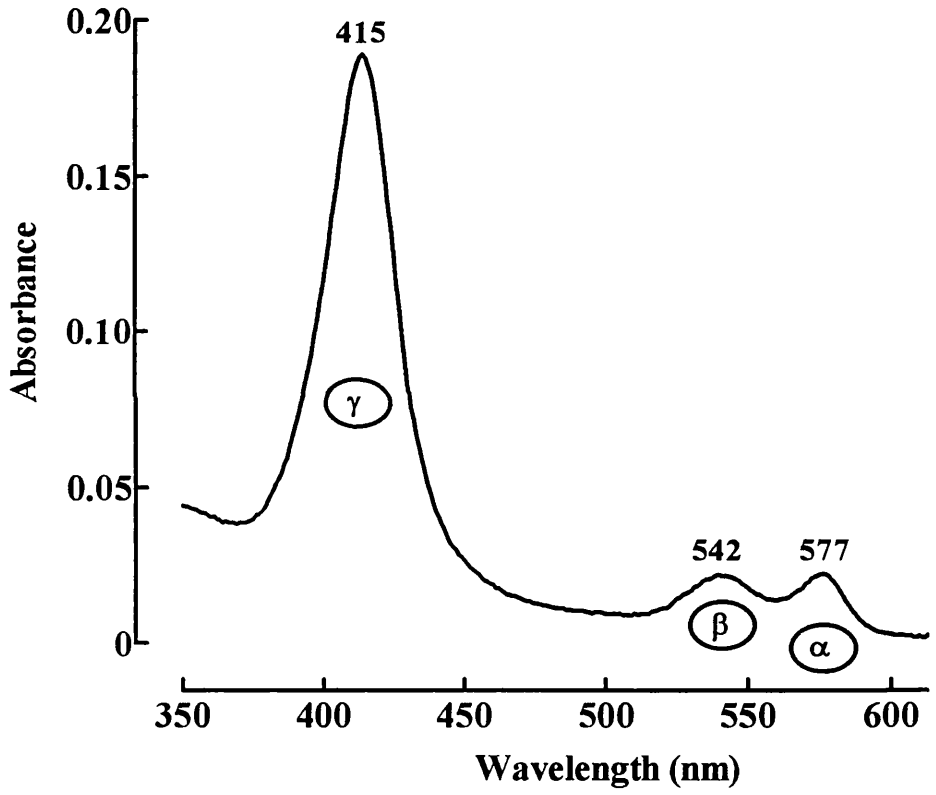


Figure 2.3. Typical absorption spectrum of oxyhaemoglobin at neutral pH. Spectrum was recorded in 200mM Tris buffer (pH 7.4) at room temperature.

2.3.6. Measurement of nitric oxide production from nitric oxide donors

Release of NO by GSNO and SNP was measured by monitoring the conversion of oxyhaemoglobin to methaemoglobin in aqueous solution according to the method of Feelisch & Noack (1987). Briefly, oxyhaemoglobin (5 μM) was incubated in 1 ml HEPES-containing buffer in a cuvette in the presence of NO donor at 37 °C. The differential absorbance at 401 nm and 411 nm was monitored using a dual-wavelength Shimadzu UV-3000 spectrophotometer. NO production was calculated from the following equation:

$$[NO] = \frac{\Delta Absorbance_{(401\text{-}411nm)}}{\Delta \varepsilon_{(401\text{-}411nm)}}$$

where ε = extinction coefficient for oxyhaemoglobin at 401 - 411 nm (19.7 mM/cm)

2.3.7. Measurement of cell viability

Cell viability was assessed by measuring the ability of the J774 cells to exclude the vital stain trypan blue (0.4 % w/v in DMEM) as determined by light microscopy. Additionally, cell respiration was assessed by measuring the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide (MTT; 0.5 mg/ml) to formazan according to the method of Mosmann (1983). At the end of each experiment, cells in 96-well plates were incubated (37 °C; 1 h) with 200 μl of MTT (0.2 mg/ml) dissolved in culture medium, after which time the medium was removed by aspiration and cells were solubilised in DMSO (200 μl). The extent of reduction of MTT to formazan within cells was quantified by measurement of optical density at 650 nm (OD_{650}) using a Molecular Devices Spectramax 250 microplate reader.

2.3.8. Measurement of protein in cells and organs

Homogenates from cells grown *in vitro* or organs were assayed for protein content using a Coomassie blue protein reagent (Bradford, 1976). Two methods were used:

(1) 80 µl of supernatants from homogenates of J774 cells were mixed with 20 µl of Bradford reagent (Biorad) used undiluted and optical densities determined at 595 nm using a Molecular Devices Spectramax 250 microplate reader. Regression analysis of the optical densities from a standard curve constructed with bovine serum albumin dissolved in distilled water was used to interpolate the concentrations of the unknown protein concentrations (Figure 2.4A).

(2) 5 µl of supernatants from tissue homogenates were mixed with 250 µl of Bradford reagent used diluted 1:5 in distilled water. Optical densities were determined as above and protein concentrations determined by interpolation from a bovine serum albumin standard curve (Figure 2.4B).

2.3.9. Measurement of superoxide by lucigenin chemiluminescence

Measurements of $O_2^{\cdot-}$ chemiluminescence were made according to the methods of Pagano *et al.* (1995). Aortic rings (5 mm) were equilibrated in bicarbonate buffer (95 % O_2/5 % CO_2, 37 °C) for 30 min. Rings were rinsed with HEPES-buffered Krebs (composition mM: NaCl 119.0, HEPES 20.0, KCl 4.6, $MgSO_4$ 1.0, Na_2HPO_4 0.15, KH_2PO_4 0.4, $NaHCO_3$ 5.0, $CaCl_2$ 1.2, Na_2-EDTA 1.0 and glucose 5.5) and incubated with 1 ml volumes of Krebs solution containing lucigenin (250 µM) in clear polystyrene cuvettes. These were positioned in a LKB-Wallac 1250 luminometer maintained at 37 °C and chemiluminescence signal (measured in mV) monitored continuously both by digital readout and also in analogue form, by means of an attached pen-recorder. Certain rings were pre-treated with the SOD inhibitor, diethyldithiocarbamate (DDC, 10 mM) to reveal basal superoxide release. To increase superoxide production, rings were incubated with NADH or NADPH. The ability of NO donors, including *S*-nitrosoglutathione (GSNO) and sodium nitroprusside (SNP) to quench $O_2^{\cdot-}$ chemiluminescence was also investigated.

2.3.10. Calibration of lucigenin assay

The lucigenin assay was calibrated by using xanthine/xanthine oxidase to produce superoxide anion in both the lucigenin and ferricytochrome *c* assay. Xanthine (100 μM) and xanthine oxidase (0.001-0.008 U/ml) were incubated with lucigenin (250 μM) in a quartz cuvette and production of superoxide measured by chemiluminescence. Signals were calibrated by measurement of the reduction of cytochrome *c* (5 μM) in the prescence or abscence of superoxide dismutase (1000 U/ml) spectrophotometrically and determining the amount of superoxide anion produced from xanthine/xanthine oxidase using the formula:

$$[O_2^{\cdot}] = \frac{Absorbance_{(550\ nm)}}{\varepsilon}$$

where ε = extinction coefficient for cytochrome *c* at 550 nm (21.1 mM/cm)

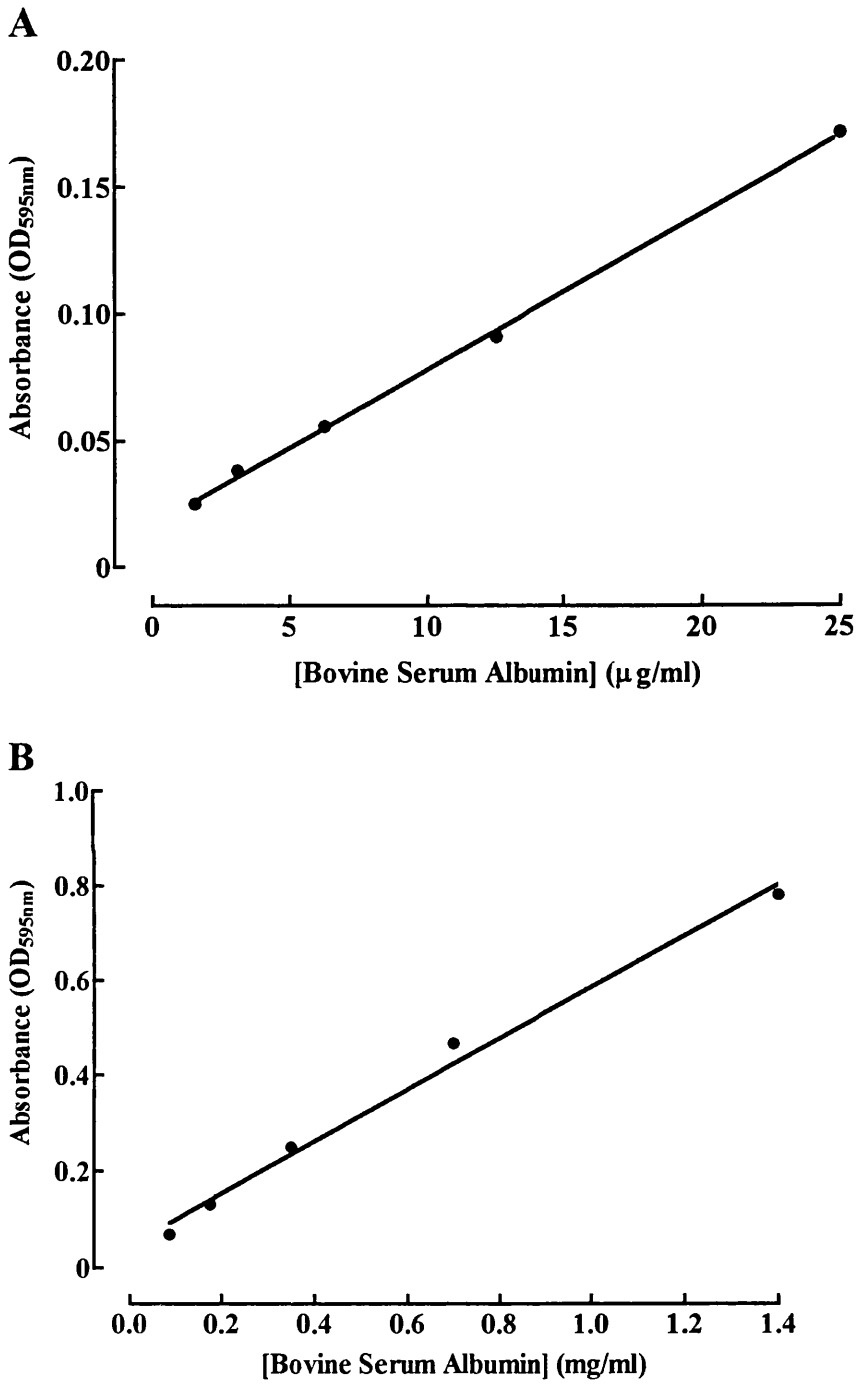


Figure 2.4. Typical standard curves for protein assay. Absorbance at 595 nm was measured as described previously. Results are expressed as mean ± s.e.m of 3 observations. The limits of the s.e.m lie within the margins of the data points. Correlation coefficients for standard curves A and B were 0.999 and 0.991, respectively.

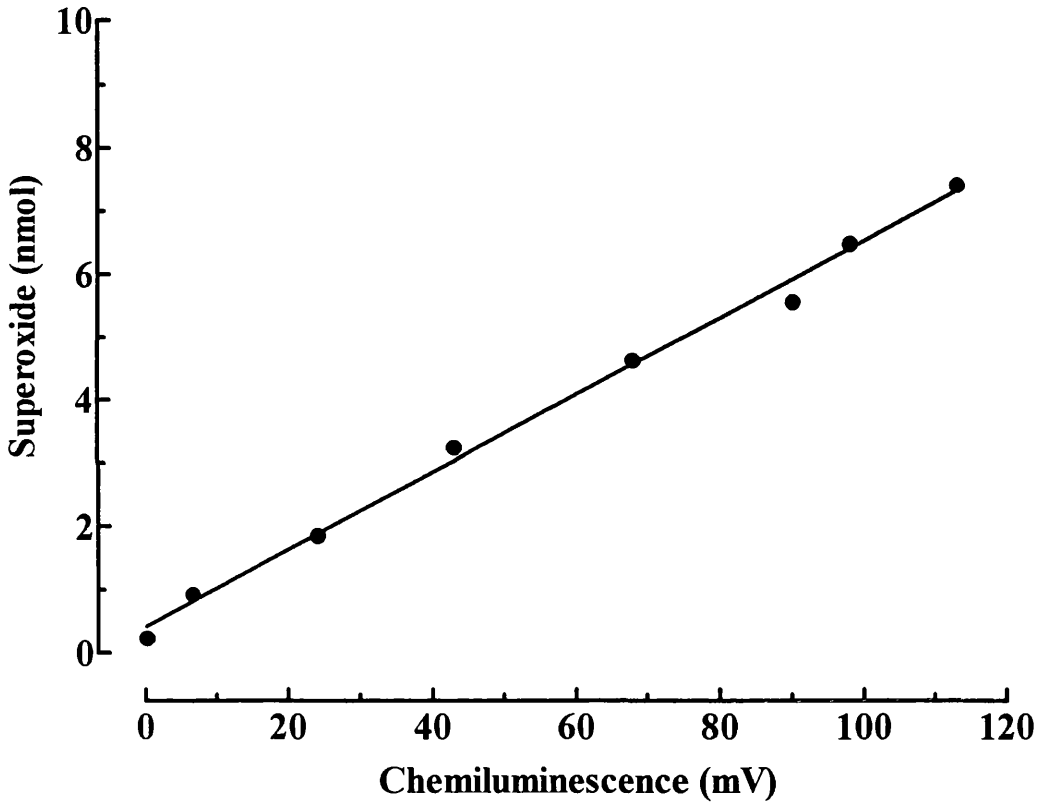


Figure 2.5. Calibration curve for the lucigenin assay. Results are expressed as mean ± s.e.m of 3 observations. The limits of the s.e.m lie within the margins of the data points. Correlation coefficient $r^2 = 0.995$.

2.3.11. Western blotting

Nitrotyrosine, iNOS, eNOS and GTPCH-1 in tissue homogenates from either rabbit aorta, heart or liver were detected by immunoblotting. Tissues were homogenised under liquid nitrogen with a pestle and mortar and resuspended in phosphate buffered saline (pH 7.4) containing Triton X-100 (1 % v/v), phenylmethylsulfonyl fluoride (1 mM), pepstatin A (50 μM) and leupeptin (200 μM). Tissue homogenates were then centrifuged at 14,000 × g for 10 min, supernatant removed and the protein content of each sample determined using Bradford reagent (Bradford, 1976), as described in section 2.3.8. Equivalent amounts of each sample (25 μg protein) were mixed with gel loading buffer (composition: Tris 50 mM, 10 % w/v SDS, 10 % v/v glycerol, 10 % v/v 2-mercaptoethanol and 2 mg/ml bromophenol blue) and boiled for 3 min. Samples were loaded onto a SDS-PAGE gel (10 % w/v SDS) and separated (150 V, 30 mA, 60 min). The separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon) for 55 min at 80 V. After transfer, membranes were incubated in blocking solution (5 % w/v non-fat dried milk) for 60 min to block non-specific protein binding and then with either a polyclonal rabbit anti-nitrotyrosine, or an anti-iNOS or anti-eNOS antibody overnight at 4 °C. Subsequently, membranes were washed and then incubated with a goat anti-rabbit antibody (peroxidase-linked; 1:3000) for 120 min. Secondary antibody binding was detected using either diaminobenzidine (0.05 % w/v) or ECL™ reagent. Densitometric analysis (using a Seescan densitometer) on each blot was performed to allow quantification of any stained bands.

2.3.12. Detection of nitrotyrosine by ELISA

The assay is a competitive inhibition of binding ELISA that utilises peroxynitrite-treated bovine serum albumin (containing nitrotyrosine) and a commercial affinity purified polyclonal rabbit antibody with a stated specificity for nitrotyrosine. Briefly, the assay was performed in 96-well plates coated with 5 μg/ml nitro-BSA/well (immobilized antigen). Each well was subsequently blocked with ovalbumin to prevent non-specific binding. A standard curve was constructed by incubating in the wells serial dilutions of nitro-BSA in PBS with immunoaffinity purified polyclonal anti-nitrotyrosine rabbit IgG (1:30000) for 2 h at 37 °C, followed by washing the plate with

PBS/Tween20. Sequential incubations with biotinylated donkey anti-rabbit IgG and then avidin-biotinylated horseradish peroxidase complex were performed with washing steps in between. Colour development was stopped by the addition of 4 M sulphuric acid. Antibody binding was determined from the absorbance at 490 nm. The competitive inhibition of antibody binding by plasma was performed in triplicate by substituting them for nitro-BSA in the incubation. The concentrations of nitrated proteins that inhibit anti-nitrotyrosine antibody binding were interpolated from the standard curve and are expressed as nitro-BSA equivalents (an equivalent concentration of 3-nitrotyrosine in nitro-BSA that produces the equivalent inhibition as the nitrated proteins; 0.00582 to 100 μg/ml).

2.3.13. GTP cyclohydrolase-I assay

The activity of GTPCH-I in heart tissue was determined according to the methods of Kerler *et al.* (1990). Briefly, tissues were homogenized in Tris buffer (50 mM, pH 8.0; containing EDTA 2.5 mM) using an Ultraturrax and cleared by centrifugation (10 min at 14 000 × g). Aliquots of the supernatant were incubated with GTP (2 mM) at 37 °C for 90 min in the dark. The reaction product was oxidized to neopterin triphosphate by acidic iodine solution. After reduction of excess iodine by ascorbic acid, the sample was immediately separated by ion pair reversed phase HPLC with the liquid phase consisting of 1 % triethylamine and 0.4 % 2-propanol (pH 7.0) and detected by its fluorescence. For further verification, aliquots of the oxidized sample were neutralized and dephosphorylated by alkaline phosphatase (0.005 U/ml). Neopterin was then determined by reversed phase HPLC as described above but with the liquid phase consisiting of 3 % methanol and 1 % acetonitrile. Protein concentrations were estimated by the Coomassie dye binding reagent as detailed in section 2.3.8.

2.3.14.Determination of tissue biopterin

The levels of tissue biopterin was measured according to the methods of Ziegler (1985). Briefly, dihydroneopterin and tetrahydrobiopterin were determined after acidic iodine oxidation of the reduced forms. Aliquots of the cell extracts were deproteinized by trichloroacetic acid and subsequently prepurified by centrifugation (10 min at 14,000 × g) and cation exchange chromatography, using DOWEX 50 WX 8-400 resin. After lyophilization the samples were separated by reversed-phase HPLC and detected fluorometrically as described in section 2.3.13.

2.3.15. Determination of plasma biopterin

Plasma total biopterins were determined according to the methods of Fukushima & Nixon (1980) after deproteination with 60 % perchloric acid and oxidised with 3 % iodine, 6 % potassium iodide solution for 1 h in the dark at room temperature. Oxidation was terminated by addition of excess ascorbate, and total biopterins were measured by HPLC.

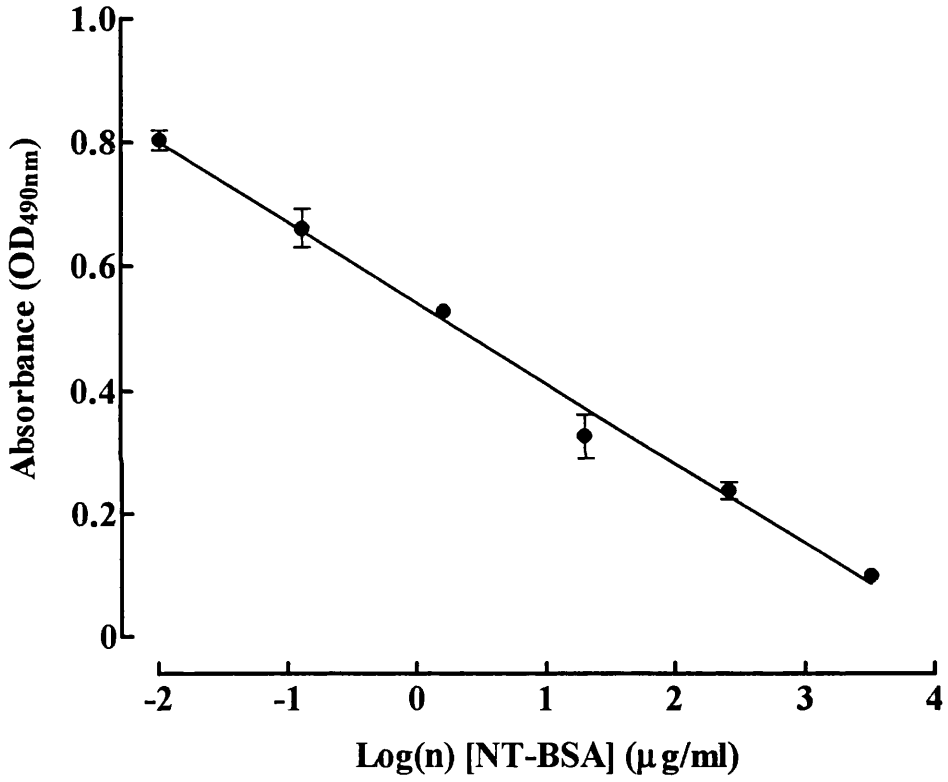


Figure 2.6. Typical standard curve for nitrated bovine serum albumin. Results are expressed as mean ± s.e.m of 3 observations. Correlation coefficient $r^2 = 0.993$.

2.4. Molecular techniques

2.4.1. Isolation of mRNA

Poly(A)$^+$-selected mRNA was isolated from mouse liver and heart using a Micro FastTrack mRNA isolation kit according to the manufacturer's instructions. Briefly, Frozen mouse liver and heart were homogenised using a Janke & Kunkel Ultra-Turrax T25 homogeniser in 1 ml lysis buffer, preheated to 45 °C. Following an initial incubation of the tissue lysate at 45 °C for 20 min, the sodium chloride concentration was adjusted to 0.5 M by adding 5 M sodium chloride. The DNA was then sheared by passing the lysate through an 18 gauge needle. Oligo (dT) cellulose was added to the lysate to bind to Poly(A)$^+$-selected mRNA and was then washed in binding buffer. Non-polyadenylated RNA was removed by washing with a low salt buffer and Poly(A)$^+$ RNA was eluted off using an elution buffer. RNA was allowed to precipitate (in 2 mg/ml glycogen, 2 M sodium acetate and 100 % ethanol) overnight at -70 °C before centrifugation at 14,000 × g for 30 min. The resulting pellet was resuspended in 10 µl nuclease-free water. The differential absorbance at 260 nm and 280 nm was monitored using a Cecil CE 2020 (Cecil Instruments Ltd, Cambridgeshire, U.K.) spectrophotometer. The concentration of mRNA was determined using the following formula:

$$[\text{mRNA}] = (A_{260}) \times (0.04\ \mu g/\mu l) \times D$$

where D = dilution factor

2.4.2. Production of cDNA probe for GTP cyclohydrolase-I

Poly(A)$^+$-selected mRNA was isolated from mouse liver using a Micro FastTrack mRNA isolation kit according to the manufacturer's instructions, as described in section 2.4.1. The mRNA obtained was used for the synthesis of first strand cDNA primed with random hexanucleotides (random primed cDNA labelling kit, Roche Diagnostics Ltd), according to manufacturer's instructions (conditions: extension 42 °C, 50 min; inactivation 72 °C, 10 min). The cDNA product was then used as a template in a polymerase chain reaction (PCR; conditions: 35 cycles of denaturing, 96 °C, 35 sec;

annealing 58 °C, 1 min and polymerization 72 °C, 2 min; final extension 72 °C, 10 min), utilizing oligonucleotide primers designed from the published sequence of murine GTPCH-1 (Primer 1: 5'-gctgcttactcgtccattct-3' (sense), Primer 2: 5'-cgcattaccatgcacatgtg-3' (antisense); Nomura *et al.*, 1993). The PCR product was resolved on a 1 % agarose gel and the molecular weight was determined by comparison of its migration with that of molecular weight standards. A product of expected size (430 base pairs) was used as a probe in Northern blotting. The PCR product was sequenced on forward and reverse strands using a dRhodamine terminator cycle sequencing kit, ready reaction with AmpliTaq DNA polymerase, FS (Applied Biosystems), according to manufacturer's instructions. The nucleotide sequence determination (conducted by Miss Lisa Jones) of the probe revealed that the cDNA product corresponded to the published sequence (Nomura *et al.*, 1993).

2.4.3. Production of cDNA probe for β-actin

Poly(A)$^+$-selected mRNA was isolated from mouse liver using a Micro FastTrack mRNA isolation kit according to the manufacturer's instructions, as described in section 2.4.1. The mRNA obtained was used for the synthesis of first strand cDNA primed with random hexanucleotides, as used in section 2.4.2 (conditions: extension 42 °C, 50 min; inactivation 72 °C, 10 min). The cDNA product was then used as a template in a polymerase chain reaction (conditions: 35 cycles of denaturing, 96 °C, 35 sec; annealing 56 °C, 1 min and polymerization 72 °C, 2 min; final extension 72 °C, 10 min), utilizing oligonucleotide primers designed from the published sequence of murine β-actin (Primers were a kind gift from Dr. Vinod Achan, Department of Clinical Pharmacology, UCL). The PCR product was used as a probe in Northern blotting.

2.4.4. Production of cDNA probe for Hypoxanthine phosphoribosyl transferase

Poly(A)$^+$-selected mRNA was isolated from mouse liver using a Micro FastTrack mRNA isolation kit according to the manufacturer's instructions, as described in section 2.4.1. The mRNA obtained was used for the synthesis of first strand cDNA primed with random hexanucleotides, as used in section 2.4.2 (conditions: extension 42 °C, 50 min; inactivation 72 °C, 10 min). The cDNA product was then used as a template in a

polymerase chain reaction (conditions: 30 cycles of denaturing, 95 °C, 30 sec; annealing 55 °C, 30 sec and polymerization 72 °C, 1 min; final extension 72 °C, 10 min), utilizing oligonucleotide primers designed from the published sequence of murine HPRT (Primer 1: 5'-cctgctggattacattaaagcactg-3' (sense), Primer 2: 5'-cctgaagtactcattatagtcaagg-3' (antisense); Primers were a kind gift from Dr. Monira Hussain, Department of Pharmacology, UCL). The PCR product was resolved on a 1 % agarose gel and the molecular weight determined by comparison of its migration with that of molecular weight standards. A product of expected size (370 base pairs) fragment was used as a probe in Northern blotting.

2.4.5. Northern Blotting

The mRNA samples (3 μg quantified from the $OD_{260\text{-}280\ nm}$) were electophoresed on a 1 % agarose/formaldehyde gel containing 20 mM morpholinosulphonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA (pH 7.0). Blotting of mRNA was achieved by capillary action using standard sodium citrate (20× SSC; pH 8.0) as a buffer, containing sodium chloride 3.6 M, sodium phosphate 0.2 M, pH 7.7; Na_2-EDTA 0.002 M onto a nylon membrane (Hybond N+) and fixed by exposure to ultraviolet irradiation using a UV Stratalinker-1800 (Stratagene, U.K.) for 12 sec. The cDNA probe for GTPCH-1, β-actin or HPRT was labelled with [α^{32}P]-dCTP (3000 Ci/mmol) using a multiprime DNA labelling system according to the manufacturer's instructions. Pre-hybridization was performed as follows, nylon membranes were incubated at 68 °C for 30 min with ExpressHyb solution supplemented with 100 μg/ml denatured salmon sperm DNA. Following this, membranes were incubated with the ^{32}P-labelled probe for 60 min at 68 °C in the above buffer in the absence of salmon sperm DNA. Following hybridisation, the blots were washed to remove non-specific binding of the cDNA probe. Washes were carried out for 15 min intervals in increasing temperature and reduced salt in the presence of 0.05 % SDS to a final stringency of 2× SSC at 50 °C. The membranes were then sealed in Saranwrap before being placed in a cassette for autoradiography, for 4-96 h at -80 °C. Equal mRNA loading was verified by stripping the hybridised probe from the membrane (0.5 % SDS for 20 min at 100 °C) and hybridising the membrane with β-actin or HPRT cDNA probe.

2.5. Analysis of results

Graphs were plotted using GraphPad Prism v.2.01 and statistical analysis of results carried out with GraphPad InStat v.2.04a computer software. Densitometry on western and northern blots was conducted by using ImageMaster v.1.20 computer software. The statistical analysis of the result was carried out by a paired comparison (paired Student's *t* test), which determines whether the differences between the mean values analysed are significant. Differences were considered significant when $P<0.05$. Values of $P>0.05$ indicated that the differences between the mean values analysed were not significant.

CHAPTER 3

ROLE OF CALMODULIN IN THE INDUCTION OF INDUCIBLE NITRIC OXIDE SYNTHASE

3.1. Introduction

Vascular smooth muscle of arteries is inflamed in diseases such as atherosclerosis and in septicaemia. Strategies designed to inhibit inflammation in blood vessels may lead to useful treatments for these conditions. NO which is produced by blood vessels by NOS requires protein co-factors such as calmodulin. Selective inhibition of iNOS may be brought about by inhibitors of the enzyme or of these co-factors. An understanding of how calmodulin interacts with NOS may be important for the development of novel therapy for the prevention of inflammatory conditions.

Inducible NOS is dependent on the dimerisation and binding of certain co-factors which are essential for full enzyme activity (Baek *et al.*, 1993; Cho *et al.*, 1995). Whilst eNOS and nNOS are tightly controlled by intracellular calcium concentrations, iNOS is characterised by its functional insensitivity to calcium/calmodulin and apparent lack of requirement for calmodulin in enzyme assays (Hevel *et al.*, 1991; Stuehr *et al.*, 1991). However, there is accumulating evidence to suggest that agents which block the action of calmodulin may have anti-inflammatory properties in the cardiovascular system. Although iNOS activity appears functionally independent of calmodulin, in fact calmodulin is bound tightly to iNOS and is essential for enzyme activity. Consistent with this, it has recently been reported that anti-fungal imidazoles such as econazole inhibit the induction, but not activity of iNOS in the J774 murine macrophage cell line (Bogle *et al.*, 1994, Bogle & Vallance, 1996). Evidence from this study suggests that econazole acted by blocking the activation and/or binding of calmodulin to the enzyme during its induction phase. Based on previous findings, my hypothesis was that calmodulin-binding to iNOS is an essential step leading to the expression of active enzyme and hence NO production and that agents that interfere with this process will lead to the expression of a functionally inactive iNOS which can then be activated by calcium-calmodulin similar to iNOS and eNOS. This might be of physiological and pathophysiological relevance and might (i) explain the occurrence of an apparently calcium/calmodulin-dependent iNOS in certain tissues (Evans *et al.*, 1992; Palmer *et al.*, 1992, 1993) and (ii) have therapeutic implications for the design of novel cardiovascular anti-inflammatory drugs for the treatment of diseases as diverse as septic shock and atherosclerosis in which expression of iNOS has been implicated. Therefore, the aim of

this study was to develop this idea focusing in particular on the regulation of NOS induction in an established model of iNOS expression in macrophages by examining the effects of calmodulin antagonists on the induction of iNOS.

3.2. Methods

3.2.1. Effects of calmodulin antagonists on nitric oxide production

J774 cells were activated with LPS as described in chapter 2, sections 2.1.2. and 2.1.3, in the presence of a series of structurally unrelated calmodulin antagonists or their respective vehicles for 24h following which production of nitrite was measured as described later. In some experiments the antagonists were added to J774 cells following induction of NO synthase (24h after addition of LPS) and incubated for a further 24h prior to nitrite measurements.

3.2.2. Measurement of cell viability

At the end of experiments in which antagonists were incubated with J774 cells for 24 h following induction of NOS, cellular viability was measured by assessment of the ability of the cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide (MTT; 0.5 mg/ml) to formazan according to the method of Mosmann (1983), as described in chapter 2, section 2.3.7.

3.2.3. Measurement of nitric oxide synthase activity

NOS activity was determined in crude homogenates isolated from J774 cells activated with LPS (10 μg/ml) and IFN-γ (50 U/ml) in DMEM in the absence of L-arginine. Cell extracts were incubated with oxyhaemoglobin and either econazole, miconazole, clotrimazole (10 μM.), ophiobolin A (1 μM.) or aminoguanidine (1 mM), as described in chapter 2, section 2.3.5.

3.3. Results

3.3.1. Induction of nitric oxide synthase in cultured J774 cells

Under basal conditions production of nitrite by cultured J774 macrophage cells was below the level of detection (<0.1 nmol/10^5 cells/24 h) using the Griess reagent (n=4). Stimulation of cells with LPS (1 μg/ml, 24 h) resulted in increased nitrite production which reached 4.4 ± 0.3 nmol/10^5 cells at 24 h (n=4).

3.3.2. Effects of calmodulin antagonists on nitrite production

The effects of structurally unrelated calmodulin antagonists on the induction of NOS in cells was assessed. J774 cells were stimulated with LPS (1 μg/ml) in the presence of increasing concentrations of calmodulin antagonists. 24 h later nitrite production was measured. Incubation with calmodulin antagonists resulted in a concentration-dependent inhibition of nitrite production (Fig. 3.1A, 3.2A, 3.3A and 3.4A). IC_{50} values for each dose-response curve are shown in Table 3.1. All respective vehicles had no significant (P>0.05) effect on nitrite production.

Further experiments were performed to determine whether calmodulin antagonists inhibited the induction or activity of NOS. Cells were induced to express NOS by stimulation with LPS (1 μg/ml), 24 h later, cells were incubated for a further 24 h with medium containing a calmodulin antagonist. At the end of the incubation, medium was removed and assayed for nitrite. Incubation with all calmodulin antagonists resulted in a significant (P<0.05) inhibition of nitrite production. The rank order of potency of the antagonists to inhibit nitrite production are as follows: ophiobolin A > calmidazolium > clotrimazole > econazole > miconazole > trifluoperazine > chlorpromazine > W13 > W7 > W5. As shown in Fig. 3.1B, 3.2B, 3.3B and 3.4B the degree of inhibition by calmodulin antagonists in cells pre-induced to express iNOS was less than that occurring in cells treated with the antagonists at the time of induction.

3.3.3. Effects of calmodulin antagonists on cell viability

Cells were incubated with LPS (1 μg/ml) in the presence of various calmodulin antagonists for 24 h. Cell viability was measured by determining the ability of J774 cells to convert MTT to formazan. This allowed us to discriminate between cytotoxic agents and those directly influencing nitric oxide production.

3.3.4. Effects of calmodulin antagonists on isolated nitric oxide synthase

In order to investigate the mechanism of inhibition of nitrite production the effects of calmodulin antagonists were assessed on a crude preparation of NOS isolated from J774 cells. Cells were activated with LPS (10 μg/ml) and IFN-γ (50 U/ml) in the absence of L-arginine for 24 h. Crude extracts were obtained as described in chapter 2, section 2.3.3. Enzyme activity was determined by measurement of the conversion of oxyhaemoglobin to methaemoglobin in a microplate spectrophotometer. In control cells NOS activity was 0.022 ± 0.017 which increased to 1.3 ± 0.1 pmol NO/min/mg protein in activated cells. NOS activity was significantly ($P<0.01$) inhibited in the presence of aminoguanidine (1 mM; a selective iNOS inhibitor (Griffiths *et al.*, 1993); Fig. 3.1D and 3.2D), confirming the specificity of the assay. Incubation of cell extracts with imidazoles (Fig. 3.1D) or ophiobolin A (Fig. 3.2D) did not affect NOS activity.

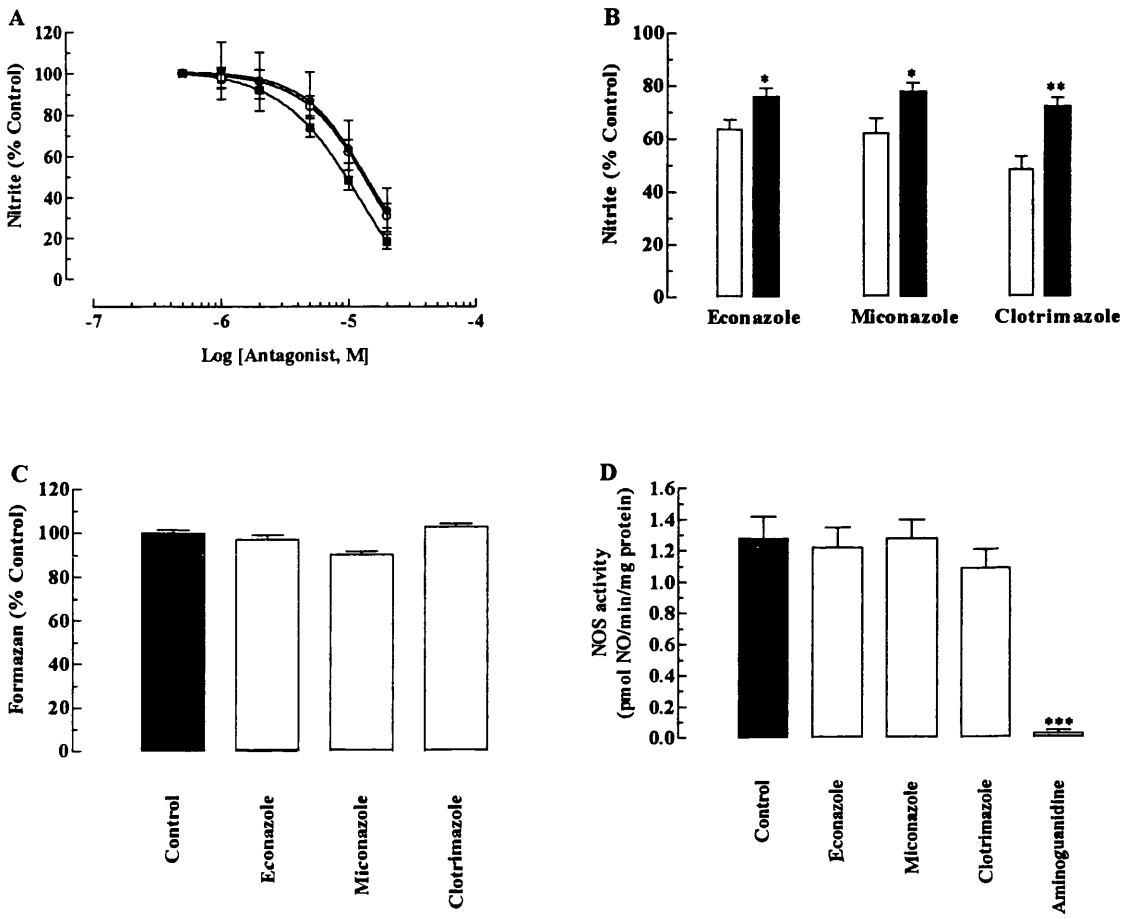


Figure 3.1. Effects of anti-fungal imidazoles on J774 cells. (A) LPS-stimulated J774 cells were treated with increasing concentrations of either econazole (closed circle), miconazole (open circle), or clotrimazole (closed square). (B) J774 cells were either stimulated with LPS (1 μg/ml) for 24 h in the presence (open bars) of econazole (10 μM), miconazole (10 μM) or clotrimazole (10 μM) or were pre-stimulated for 24 h with LPS after which anti-fungal imidazoles were added for a further 24 h (closed bars). (C) Cell respiration was assessed by measuring cell conversion of MTT to formazan in J774 cells treated with anti-fungal imidazoles (concentrations as in panel B) 24 h after LPS stimulation. (D) Crude extracts prepared from LPS/IFN-γ-stimulated J774 cells were incubated with oxyhaemoglobin and econazole (10 μM), miconazole (10 μM), clotrimazole (10 μM) or aminoguanidine (1 mM) and the conversion to methaemoglobin was measured by spectroscopy. Results are shown as the mean ± s.e.m of data obtained in 4 separate experiments. $^{*}P<0.05$ and $^{**}P<0.01$, pre-treated vs post-treated; $^{***}P<0.0001$, control vs treatment.

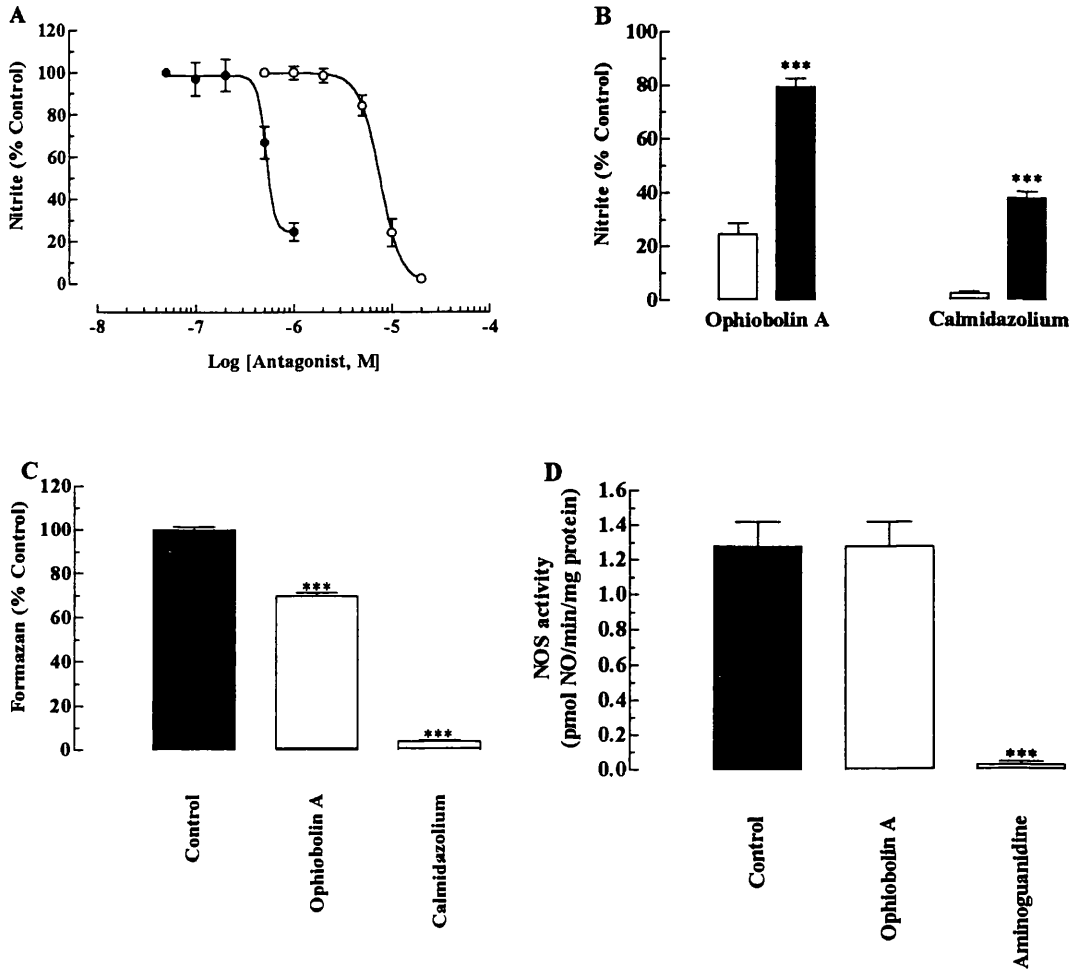


Figure 3.2. Effects of calmodulin antagonists on J774 cells. (A) LPS-stimulated J774 cells were treated with increasing concentrations of either ophiobolin A (closed circle) or calmidazolium (open circle). (B) J774 cells were either stimulated with LPS (1 μg/ml) for 24 h in the presence (open bars) of ophiobolin A (1 μM) or calmidazolium (20 μM) or were pre-stimulated for 24 h with LPS after which antagonists were added for a further 24 h (closed bars). (C) Cell respiration was assessed by measuring cell conversion of MTT to formazan in J774 cells treated with antagonists (concentrations as in panel B) 24 h after LPS stimulation. (D) Crude extracts prepared from LPS/IFN-γ-stimulated J774 cells were incubated with oxyhaemoglobin and ophiobolin A (1 μM) or aminoguanidine (1 mM) and the conversion to methaemoglobin was measured by spectroscopy. Results are shown as the mean ± s.e.m of data obtained in 4 separate experiments. $^{***}P<0.0001$, control vs treatment.

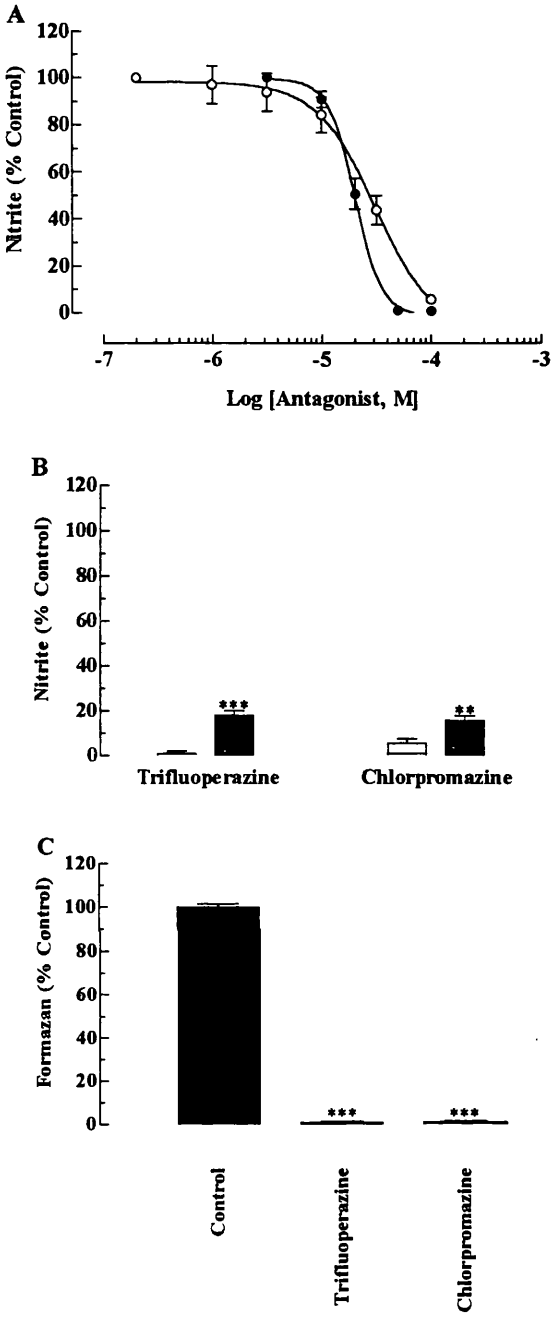


Figure 3.3. Effects of phenothiazines on J774 cells. (A) LPS-stimulated J774 cells were treated with increasing concentrations of either trifluoperazine (closed circle) or chlorpromazine (open circle). (B) J774 cells were either stimulated with LPS (1 μg/ml) for 24 h in the presence (open bars) of trifluoperazine (50 μM) or chlorpromazine (100 μM) or were pre-stimulated for 24 h with LPS after which antagonists were added for a further 24 h (closed bars). (C) Cell respiration was assessed by measuring cell conversion of MTT to formazan in J774 cells treated with antagonists (concentrations as in panel B) 24 h after LPS stimulation. Results are shown as the mean ± s.e.m of data obtained in 4 separate experiments. $^{**}P<0.01$, $^{***}P<0.0001$, control vs treatment.

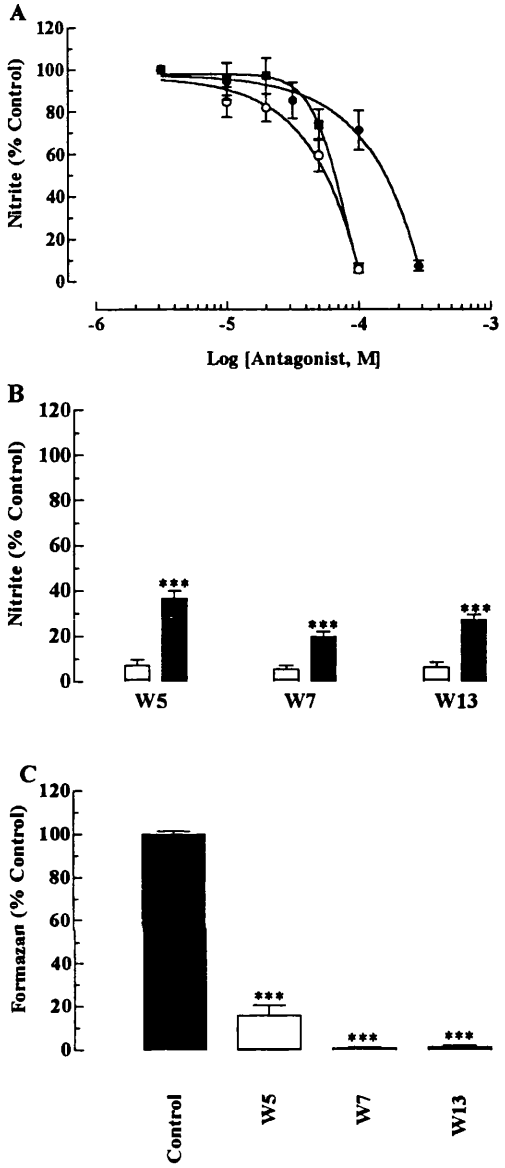


Figure 3.4. Effects of naphthalene sulfonamides on J774 cells. (A) LPS-stimulated J774 cells were treated with increasing concentrations of either W5 (closed circle), W7 (open circle) or W13 (closed square). (B) J774 cells were either stimulated with LPS (1 μg/ml) for 24 h in the presence (open bars) of W5 (300 μM), W7 (100 μM) or W13 (100 μM) or were pre-stimulated for 24 h with LPS after which antagonists were added for a further 24 h (closed bars). (C) Cell respiration was assessed by measuring cell conversion of MTT to formazan in J774 cells treated with antagonists (concentrations as in panel B) 24 h after LPS stimulation. Results are shown as the mean ± s.e.m of data obtained in 4 separate experiments. $^{***}P<0.0001$, control vs treatment.

TABLE 3.1. EFFECTS OF CALMODULIN ANTAGONISTS ON INHIBITION OF NITRITE ACCUMULATION FROM ACTIVATED J774 CELLS *IN VITRO*

Compound	Calculated IC_{50} (μM)
Ophiobolin A	0.7 ± 0.1
Calmidazolium	7.7 ± 1.5
Clotrimazole	9.6 ± 2
Econazole	13 ± 4
Miconazole	15 ± 4
Trifluoperazine	18 ± 2
Chlorpromazine	27 ± 4
W-13	59 ± 6
W-7	59 ± 8
W-5	128 ± 27

J774 cells were incubated with LPS (1μg/ml) in the presence of increasing concentrations of calmodulin antagonists. Twenty four hours later medium was removed and nitrite production measured using the Greiss reagent. IC_{50} values were calculated following fitting of data using a computer program (GraphPad Prism). Results are shown as the mean ± s.e.m of data obtained in 4 separate experiments.

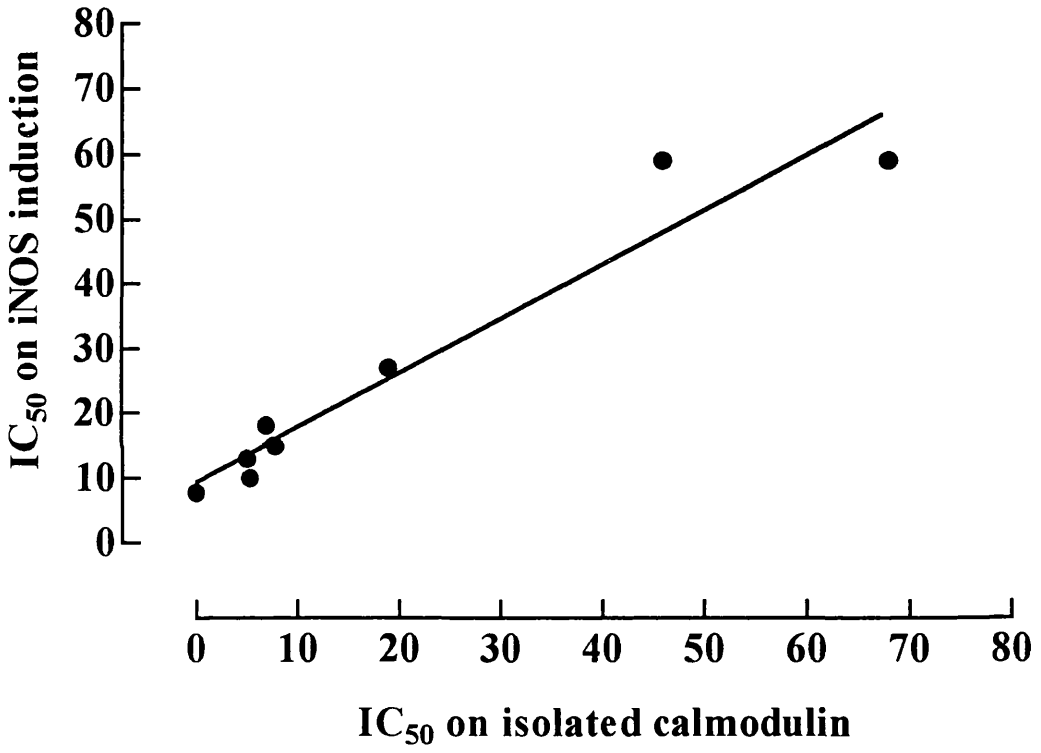


Figure 3.5. Correlation of inhibitory effects of calmodulin antagonists on induction of iNOS in J774 cells and inhibition of isolated calmodulin. Results are shown as the mean ± s.e.m of data obtained in 4 separate experiments. Correlation coefficient $r^2 = 0.94$.

3.4. Discussion

Calmodulin regulates a wide range of cellular functions through its reversible calcium-dependent binding to target enzymes, including NOS. Inducible NOS, which is expressed in a wide variety of cells following stimulation by LPS and/or cytokines, contains tightly bound calmodulin (Cho *et al.*, 1992). Binding of calmodulin enables electron flow from NADPH to the reductase domain (Abu-Soud *et al.*, 1994), and from the reductase to the heme group of the oxygenase domain with calmodulin acting as a molecular switch. It is thought that the close association of calmodulin with iNOS results in a tonically active enzyme which produces NO in high concentrations over long time periods.

The expression of iNOS is likely to be of importance in the excess generation of NO that occurs during inflammation. To explore the regulation of iNOS by calmodulin a range of structurally unrelated compounds was used in an established model of iNOS induction, namely the LPS-stimulated J774 cells. In this system NO generation was induced and an attempt was made to identify inhibitor(s) that were selective in the inhibitory effects on induction of nitrite production. A range of compounds were used which are structurally unrelated but have anti-calmodulin activity in isolated preparations of calcium/calmodulin kinase II (Fig. 3.5; Tanaka & Hidaka, 1980; Hidaka *et al.*, 1981; Gietzen *et al.*, 1982; Gietzen *et al.*, 1983; Hidaka & Tanaka, 1983; Itoh & Hidaka, 1984; Leung *et al.*, 1984; Marshak *et al.*, 1985; Leung *et al.*, 1988) or on isolated crude enzyme preparation (Bogle & Vallance, 1996).

Results from this study demonstrate that all the calmodulin antagonists inhibit nitrite synthesis in J774 cells in a concentration-dependent manner. Furthermore, the rank order of potency of these agents on inhibition of NO production in J774 cells correlates closely ($r^2 = 0.94$) with the reported inhibitory potency of these compounds on either isolated calcium/calmodulin kinase II or crude iNOS activity (Fig. 3.5). From the panel of antagonists tested in the model of iNOS induction, ophiobolin A appears to be the most potent ($IC_{50} = 0.7 \pm 0.1$ μM), consistent with other findings (Leung *et al.*, 1984; Lueng *et al.*, 1988).

Recently it has been shown that certain anti-fungal imidazoles (econazole, miconazole and clotrimazole) inhibit the induction, but not the activity of iNOS in J774

cells (Bogle *et al.*, 1994). This study suggested that anti-fungal imidazole agents could be acting as calmodulin antagonists. In agreement with these findings, it has been shown that anti-fungal imidazoles do not inhibit activity of expressed iNOS obtained from LPS-treated murine macrophage RAW264 cells (Wolff & Gribin, 1994). The inhibition of NOS induction by anti-fungal imidazoles appeared to occur post-transcriptionally because whilst LPS-stimulated nitrite production was inhibited by co-incubation with LPS and econazole, levels of NOS mRNA were similar under both conditions (Bogle *et al.*, 1994). Others have confirmed this finding and shown that clotrimazole inhibits the induction of NOS activity in rat hepatocytes stimulated with LPS (Kuo & Abe, 1995). In this study the level of iNOS protein in cells treated with LPS and clotrimazole was similar to that observed in cells treated with LPS alone, but the expressed protein was inactive. However, other studies have suggested that the anti-fungal imidazoles inhibit NOS through interaction with heme (Sennequier *et al.*, 1999) rather than through calmodulin. To determine whether inhibition of calmodulin does block iNOS, a range of compounds was studied, which would not be expected to interact with heme.

Experiments were designed in which calmodulin antagonists were added at the same time as the LPS or 24 h after LPS treatment. In agreement with previous findings (Bogle *et al.*, 1994) when the calmodulin antagonists were added to cells pre-stimulated with LPS, they were less effective although for most compounds the difference was quite small. In concentrations similar to those achieved therapeutically (e.g. 2 μM for miconazole; Benson & Nahata, 1988), clotrimazole, econazole and miconazole inhibited nitrite production by J774 cells if added to the cells at the same time as the LPS but were ineffective in cells pre-stimulated with LPS. This suggests that the anti-fungal imidazoles inhibit NOS induction by interfering with calmodulin binding to NOS. Moreover, the observation that these compounds inhibit calmodulin activity at low micromolar concentrations (e.g. 12.8 μM. for clotrimazole), similar to those concentrations required to inhibit expression of NO synthesis in J774 cells (IC_{50} = 5.0 μM. for econazole) suggests that binding to calmodulin may be the predominant mode of action of these agents. Moreover the rank order of potency of the anti-fungal imidazoles in inhibiting NO production (Table 3.1) or calmodulin

(Hegemann *et al.*, 1993) was similar, consistent with blocking of the induction of iNOS. The possibility that calmodulin antagonists might have a direct inhibitory effect on iNOS was examined further by directly measuring iNOS activity *in vitro*. In these experiments none of the calmodulin antagonists significantly inhibited activity of the enzyme. This is consistent with previous reports on the sensitivity of murine macrophage NOS to calmodulin antagonists (Palacios *et al.*, 1993).

Despite the apparent relationship between the ability of the antagonists to block calmodulin and inhibit nitrite production, it was noted that, with the exception of the anti-fungal imidazoles and ophiobolin A, the degree of inhibition of nitrite production also correlated with the adverse effects of these compounds on cell viability as assessed by MTT formation. This suggests that certain calmodulin antagonists may have a direct cytotoxic effect, but it is unclear whether this is due to calmodulin binding or other non-specific effects of the compounds. In this respect, trifluoperazine has been shown to inhibit growth of J774 cells at concentrations greater than 20μM (Horwitz *et al.*, 1981). The finding that many of the compounds caused cell damage makes interpretation of the results difficult. However, one compound, ophiobolin A significantly depressed iNOS without causing a major effect on cell viability. The results with this compound support an effect of calmodulin inhibition on iNOS function. Specific effects of ophiobolin A on calmodulin inhibition appear to be through its interaction with lysine residues in calmodulin (Kong & Chow, 1998). Interestingly, several compounds with inhibitory effects on calmodulin have been reported previously to inhibit induction of NOS or have therapeutic effects that might be accounted for by such an action. These include chlorpromazine (Palacios *et al.*, 1993) and trifluoperazine (Fig. 3.3A). However, these authors did not assess cell viability. Results in this chapter demonstrate the importance of assessment of cell viability when assessing the potency of antagonists. Studies have also provided evidence that direct interaction of azole derivatives, such as ketoconazole with calmodulin might contribute to their therapeutic activity (Yu & Thomasa, 1993), particularly to their efficacy in the treatment of inflammatory skin disorders (Hegemann *et al.*, 1993). Ketoconazole inhibits procoagulant activity of activated macrophages (Williams & Maier, 1992) and significantly reduces mortality and the incidence of acute lung failure in septic patients (Yu & Tomasa, 1993).

Chlorpromazine has a protective action in both glucocorticoid-sensitive and resitant models of endotoxic shock (Gadina *et al.*, 1991).

In conclusion, the data suggest that the selected antagonists appear to inhibit the induction of NO synthase. With the exception of the anti-fungal imidazoles and ophiobolin A, the inhibitory effects do not appear to be significantly selective or non-toxic and make most of the calmodulin antagonists inappropriate tools for the study of the regulation of NOS induction in macrophages. However, ophiobolin A is a particularly selective and relatively non-toxic calmodulin antagonist, which can potentially be used as a tool for future studies on the regulation of NOS induction.

CHAPTER 4

INTERACTION BETWEEN NITRIC OXIDE & SUPEROXIDE IN BLOOD VESSELS

4.1. Introduction

Blood vessels generate free radical species including NO and $O_2^{\cdot-}$. In the vasculature, the enzymatic sources and biological effects of NO have been well characterised. NO is released in small amounts to regulate local blood flow and inhibit interactions between circulating platelets, white cells and the vessel wall (Moncada *et al.*, 1991). In healthy blood vessels NO is synthesised by a calcium/calmodulin-dependent NO synthase present in endothelium (eNOS; Palmer & Moncada, 1989; Mayer *et al.*, 1989). However, during inflammatory episodes, a cytokine-inducible NO synthase is expressed throughout the vessel wall which results in production of larger quantities of NO (Bogle & Vallance, 1996).

In contrast to the extensively characterised biology of NO, the biosynthetic pathways and roles of $O_2^{\cdot-}$ in blood vessels remain unclear. The source of $O_2^{\cdot-}$ (endothelial/smooth muscle cells) may vary and potential generating systems include NADH/NADPH oxidases (Pagano *et al.*, 1993; Jones *et al.*, 1996), NO synthases in the absence of L-arginine (Xia *et al.*, 1996), xanthine oxidase (Miyamoto *et al.*, 1996) or arachidonic acid-metabolising enzymes (Cross & Jones 1991).

NO and $O_2^{\cdot-}$ react rapidly at almost diffusion-limited rate (Huie & Padmaja, 1993) to form $ONOO^-$. This product may isomerise to form nitrate, which has little biological activity, and this may provide a mechanism to remove and inactivate both NO and $O_2^{\cdot-}$. However, $ONOO^-$ is also a powerful oxidant, which can lead to the generation of other reactive radical species and result in cell damage (Beckman & Koppenol, 1996). In addition, $ONOO^-$ reacts with proteins resulting in nitration of tyrosine residues and formation of 3-nitrotyrosine (Beckman *et al.*, 1994; Oury *et al.*, 1995).

Nitration of tyrosine may alter protein function and initiate cellular damage, and the presence of 3-nitrotyrosine has been used as a marker for $ONOO^-$-mediated tissue damage (Martin *et al.*, 1990; Ohshima *et al.*, 1990; Liu *et al.*, 1994; Van-der-Vliet *et al.*, 1996). Nitrotyrosine has been detected in human atherosclerotic plaques (Beckman *et al.*, 1994; White *et al.*, 1994), in acute lung (Haddad *et al.*, 1994; Kooy *et al.*, 1995) and myocardial injury (Kooy *et al.*, 1997). Its presence is regarded as an indication of the induction of both NO and $O_2^{\cdot-}$ generation. However, in some situations only one of the radical species may be up-regulated e.g. $O_2^{\cdot-}$ in diabetes

(Giugliano *et al.*, 1996), or concentrations of NO or $O_2^{\cdot-}$ may be altered independently by drugs. For example, levels of NO may increase due to exogenous administration of NO donors. Therefore, the aim of this study was (i) to estimate $O_2^{\cdot-}$ production from isolated rabbit aortic rings *in vitro* and identify its possible enzymatic sources and (ii) to test the hypothesis that an increase in NO generated from an NO donor would be sufficient to combine with endogenous $O_2^{\cdot-}$ to form $ONOO^-$.

4.2. Methods

4.2.1. Measurement of superoxide chemiluminescence

Rabbit aortic rings were prepared as described in chapter 2, section 2.2.1. In some experiments endogenous SOD was inhibited by pre-treatment of rings with diethyldithiocarbamate (DDC; 10 mM; an irreversible inhibitor of copper zinc SOD) for 30 min. Specificity of lucigenin chemiluminescence was assessed by conducting experiments in the presence of exogenous SOD (0.01-10000 U/ml) or 4,5-dihydroxy-1,3-benzene disulfonic acid (tiron; 10 mM), a non-enzymatic $O_2^{\cdot-}$ scavenger. In some experiments, substrates for potential $O_2^{\cdot-}$ generating enzymes were added to the rings NADH (0.01-10 mM), NADPH (0.01-30 mM), xanthine (1 mM), arachidonic acid (100 μM) or succinate (5 mM). The ability of NO donors to scavenge superoxide was investigated by incubation of rings with NADH (300 μM) and adding increasing concentrations of GSNO (0.01-3 mM) or SNP (0.01 μM - 1 mM).

4.2.2. Calibration of the lucigenin assay

The lucigenin assay was calibrated by using xanthine/xanthine oxidase to produce superoxide anion in both the lucigenin and ferricytochrome *c* assay, as described in chapter 2, section 2.3.10.

4.2.3. Measurement of nitric oxide production from GSNO

The release of NO by GSNO (0.001-1 mM) was determined by the measurement of the reduction of oxyhaemoglobin to methaemoglobin as described in chapter 2, section 2.3.6.

4.2.4. Nitration of bovine serum albumin

Solutions of bovine serum albumin were prepared (0.7 mg/ml) in HEPES buffer (pH 7.4). Aliquots (1 ml) were incubated with 3-morpholinosydnonimine (SIN-1; 1 mM), GSNO (1 mM), or combinations of SIN-1 and oxyhaemoglobin (50 μM) or SOD (10-1000 U/ml) for 10 min at 37°C. In additional experiments bovine serum albumin (dissolved in HEPES buffer, pH 10.8) was incubated with GSNO (1 mM), pyrogallol (200 μM), or GSNO and pyrogallol. Samples (10 μg protein) were loaded

onto SDS/PAGE gels, transferred to nylon membranes and blotted for nitrotyrosine as described in chapter 2, section 2.3.11.

4.2.5. Nitrotyrosine detection by Western blotting

Treated rings of rabbit aorta were homogenised, loaded onto a SDS-PAGE gel, and probed for nitrotyrosine using a polyclonal rabbit anti-nitrotyrosine antibody as described in chapter 2, section 2.3.11. Control experiments were conducted to confirm the presence of nitrotyrosine. Prior to the addition of the primary antibody to the nitrocellulose membranes, the antibody was incubated with 10 mM 3-nitro-L-tyrosine in potassium phosphate buffer, pH 7.4.

4.3. Results

4.3.1. Characterisation of superoxide production in rabbit aorta

Under basal conditions $O_2^{\cdot-}$ production was not detected from rabbit isolated aortic rings. Addition of DDC (10 mM), an inhibitor of copper-zinc SOD unmasked basal $O_2^{\cdot-}$ production which reached $0.9 \pm 0.01 \times 10^{-12}$ mol/min/mg tissue (n=4). Basal $O_2^{\cdot-}$ production was completely abolished in the presence of SOD (150 U/ml). All subsequent $O_2^{\cdot-}$ measurements were performed in the presence of DDC (10 mM).

Addition of NADH (0.01-10 mM) or NADPH (0.01-30 mM) resulted in a concentration-dependent increase in $O_2^{\cdot-}$ production (Fig. 4.1, Table 4.1). Calculated EC_{50} values for NADH and NADPH were 0.234 ± 0.118 and 6.21 ± 1.79 mM respectively (n=4). NADH (0.3 mM)-stimulated $O_2^{\cdot-}$ chemiluminescence was reduced in the presence of SOD (IC_{50} = 8.8 U/ml Fig. 4.2, n=4). In the absence of tissue, neither NADH nor NADPH increased lucigenin chemiluminescence.

Addition of substrates for xanthine oxidase (xanthine, 0.1 mM), cyclooxygenase/lipoxygenase (arachidonic acid, 0.1 mM) or mitochondrial complex II (succinate, 5 mM in the presence of the complex III inhibitor antimycin A (0.03 mM)) did not alter vascular $O_2^{\cdot-}$ production from basal levels (Table 4.1, n=3).

TABLE 4.1. SUBSTRATE DEPENDENCE OF SUPEROXIDE CHEMILUMINESCENCE

Substrate	Superoxide production (pmol/min/mg tissue)
Basal	0.9 ± 0.01
NADH	9.8 ± 1.00*
NADPH	1.5 ± 0.10*
Xanthine	1.0 ± 0.07
Arachidonic acid	0.9 ± 0.02
Succinate + Antimycin A	1.0 ± 0.10

Chemiluminesence was measured in aortic rings in response to NADH (0.1 mM), NADPH (0.1 mM), xanthine (1 mM), arachidonic acid (0.1 mM) or succinate (5 mM) and antimycin A (0.03 mM). Results are mean ± s.e.m of data obtained in 3-4 experiments, *$P<0.05$, unpaired t-test.

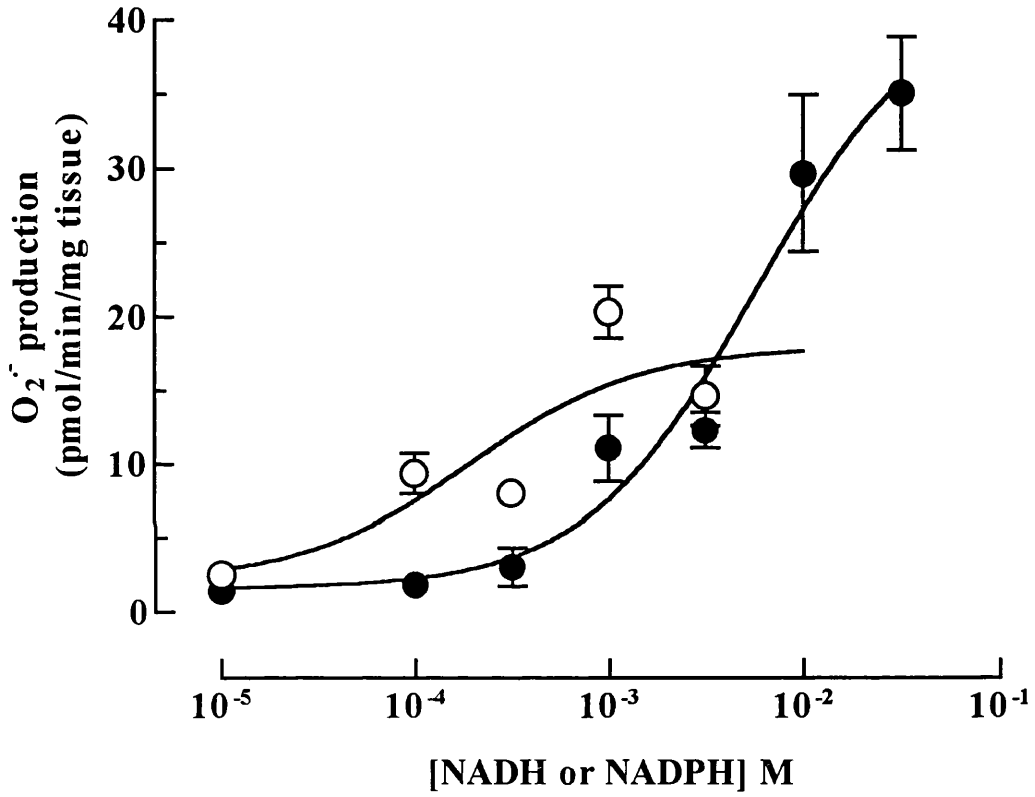


Figure 4.1. Generation of $O_2^{\cdot-}$ by rabbit aortic rings *in vitro*. Rings of rabbit aorta were placed in a cuvette containing bicarbonate buffer solution and lucigenin. Chemiluminescence was measured in response to increasing concentrations of NADH (open circle) or NADPH (closed circle). Results are mean ± s.e.m of data obtained in 4 experiments.

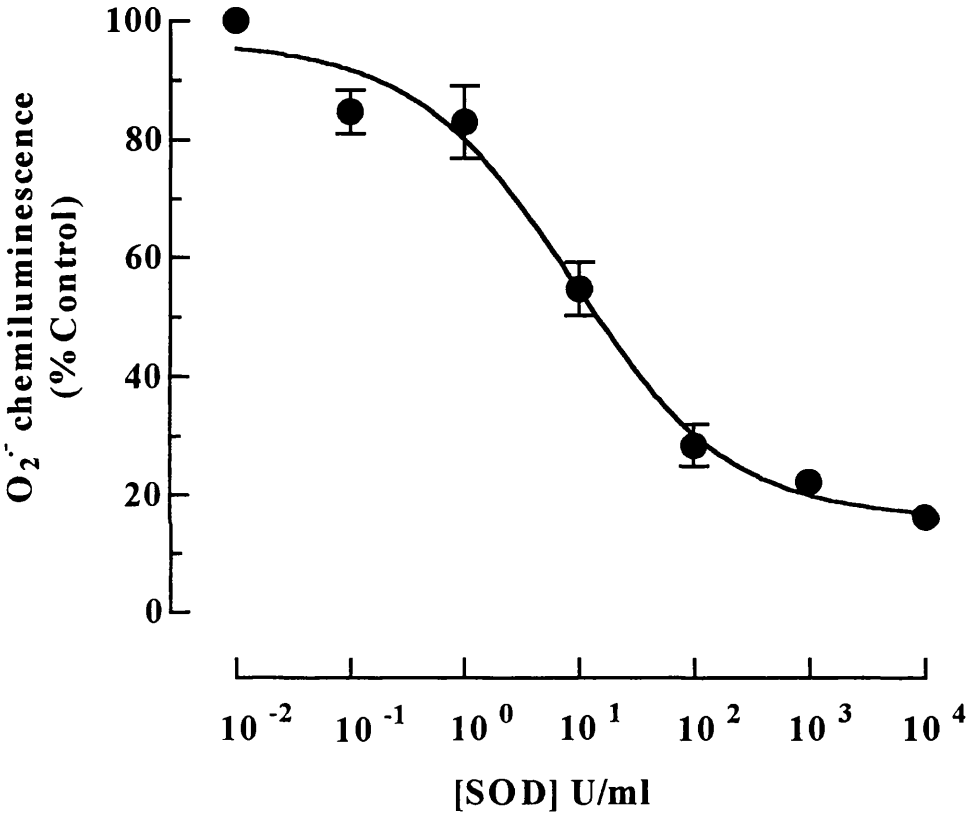


Figure 4.2. Inhibition of $O_2^{\cdot-}$ chemiluminescence signal by SOD. Rings of rabbit aorta were incubated with NADH and increasing concentrations of SOD. $O_2^{\cdot-}$ was measured by lucigenin chemiluminescence. Results are mean ± s.e.m of data obtained in 4 experiments.

4.3.2. Scavenging of $O_2^{\cdot-}$ by NO donors

The ability of NO donors to scavenge $O_2^{\cdot-}$ was investigated in rabbit aortic rings. Incubation of rings with NADH (0.3 mM) and increasing concentrations of GSNO (0.01-3 mM) or SNP (0.01-1000 μM) resulted in a significant ($P<0.01$) reduction of detected $O_2^{\cdot-}$ chemiluminescence (Fig. 4.3, n=4). Calculated IC_{50} values for GSNO and SNP were 360 ± 10 and 2.3 ± 0.1 μM respectively.

The effects of GSNO on the $O_2^{\cdot-}$ chemiluminescence produced by reaction of xanthine and xanthine oxidase were assessed and compared with the release of NO from GSNO. Release of NO by GSNO occurred over a similar concentration range to that which resulted in the quenching of $O_2^{\cdot-}$ chemiluminescence (Fig. 4.4). Incubation with reduced glutathione (up to 1 mM), did not affect $O_2^{\cdot-}$ detection.

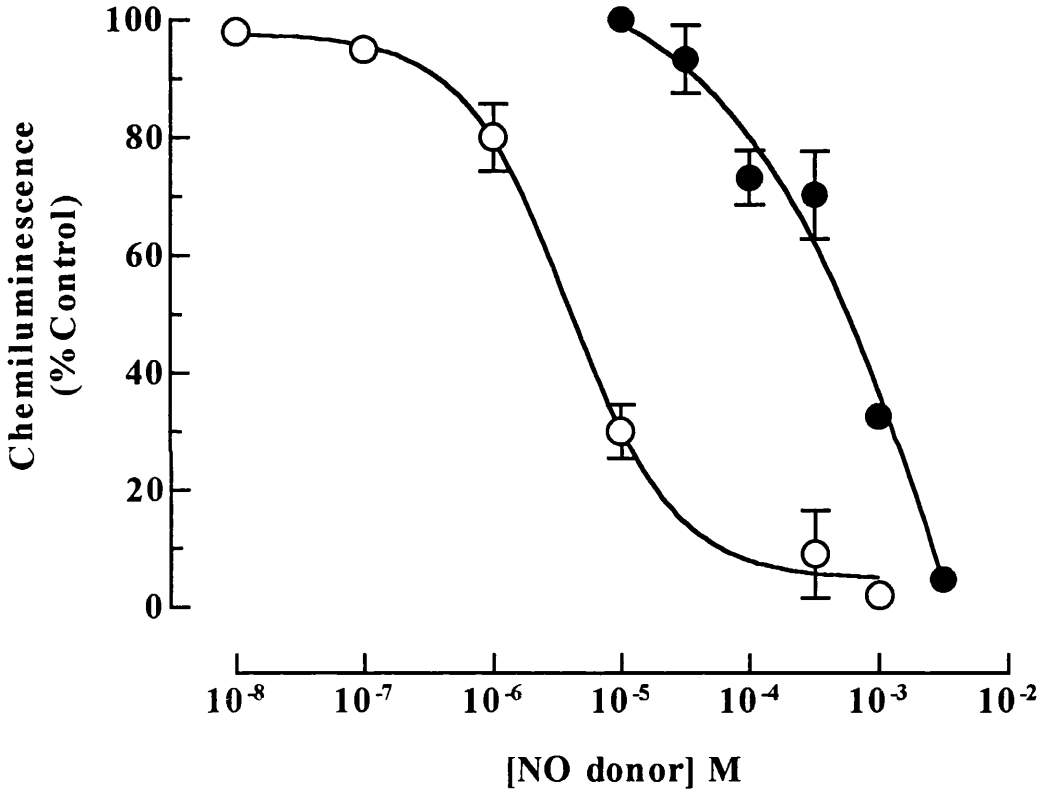


Figure 4.3. Interaction between NO and $O_2^{\cdot-}$ in rabbit aortic rings. Rings of rabbit aorta were incubated with NADH (300 μM) in the presence of lucigenin and increasing concentrations of GSNO (closed circles) or SNP (open circles). $O_2^{\cdot-}$ production was measured by chemiluminescence. Results are the mean ± s.e.m of 4 experiments.

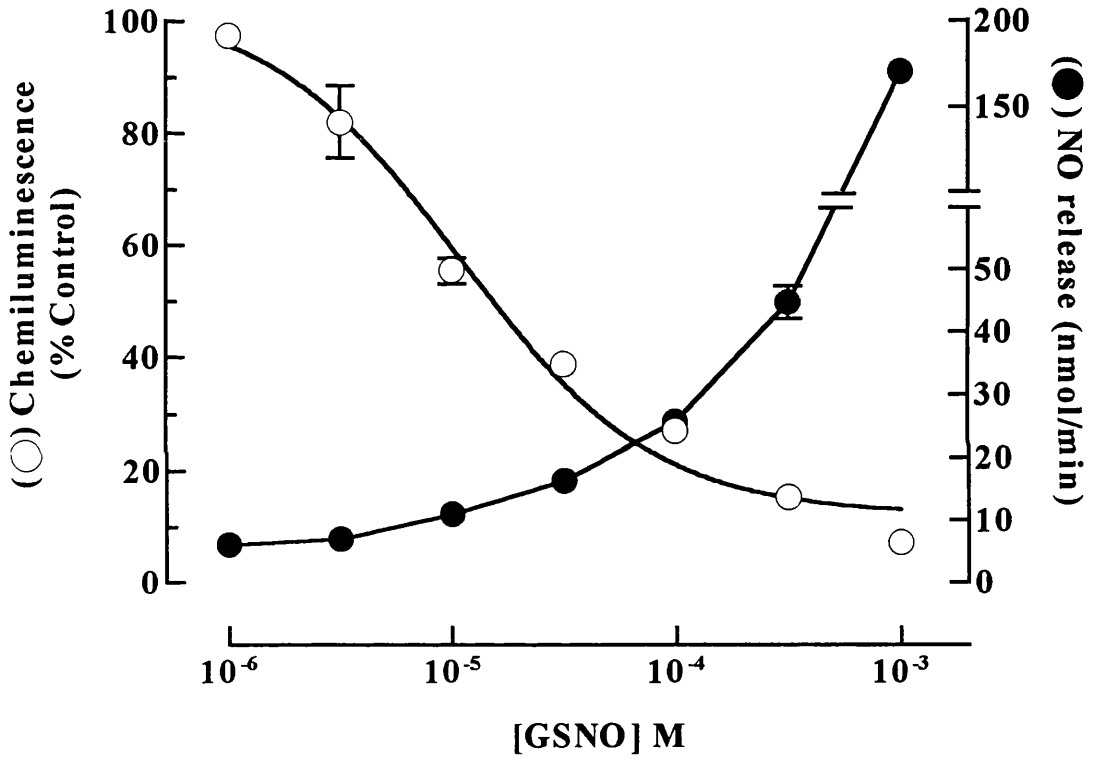


Figure 4.4. Release of NO and quenching of $O_2^{\cdot-}$ chemiluminescence by GSNO. $O_2^{\cdot-}$ was generated by incubation of xanthine oxidase (0.1 U/ml) and xanthine (100 μM). The effects of increasing concentrations of GSNO on $O_2^{\cdot-}$ chemiluminescence and release of NO measured by reduction of oxyhaemoglobin are shown. Results are the mean ± s.e.m of 4 experiments.

4.3.3. Formation of ONOO⁻ assessed by nitrotyrosine formation

Under basal conditions or in the presence of NADH (300 μM) a faint band of nitrotyrosine (corresponding to a molecular weight of ~38 kDa) was detected in protein extracts from rabbit aortic rings. This may represent a nitrated protein present constitutively. Incubation of rings with GSNO (1 mM, 10 min) alone or GSNO in combination with NADH (300 μM) resulted in the appearance of additional nitrotyrosine bands of which the most prominent had an apparent molecular weight of approximately 30 kDa (Fig. 4.5). Nitrotyrosine staining was similar whether the tissues were prepared in the presence or absence of DDC. Formation of nitrotyrosine was not inhibited in the presence of SOD (1×10^4 U/ml; not shown) or tiron (10 mM, Fig. 4.5). Specific nitrotyrosine immunoreactivity was not observed when the blot was incubated in the presence of primary antibody and excess 3-nitro-L-tyrosine (10 mM). The conditions required for tyrosine nitration were examined further using bovine serum albumin as a substrate for nitration. Nitrotyrosine residues were not detected on serum albumin incubated (10 min) with HEPES buffer solution or GSNO alone. Incubation of albumin with SIN-1 (1 mM), a compound which co-generates $O_2^{\cdot-}$ and NO (Feelisch *et al.*, 1989), resulted in nitration of albumin (Fig. 4.6). Treatment of albumin with SIN-1 (1 mM) in the presence of oxyhaemoglobin (50 μM) or a high concentration of SOD (1×10^3 U/ml) reduced albumin nitration. The effects of SOD to inhibit SIN-1 induced nitrotyrosine formation were concentration-dependent and even at very high concentrations only partial inhibition was possible (Fig. 4.7). The inhibitory effects of high concentrations of SOD on nitrotyrosine formation were partially reversed in the presence of GSNO (1 mM; Fig. 4.6).

Further experiments were conducted to investigate whether nitrotyrosine formation was dependent on the presence of both NO and $O_2^{\cdot-}$. Incubation of albumin with HEPES buffer and pyrogallol (1 mM; a potent donor of $O_2^{\cdot-}$) alone (10 min) did not result in nitrotyrosine formation (Fig. 4.8) whereas in the presence of GSNO and pyrogallol nitration of albumin occurred (Fig. 4.8).

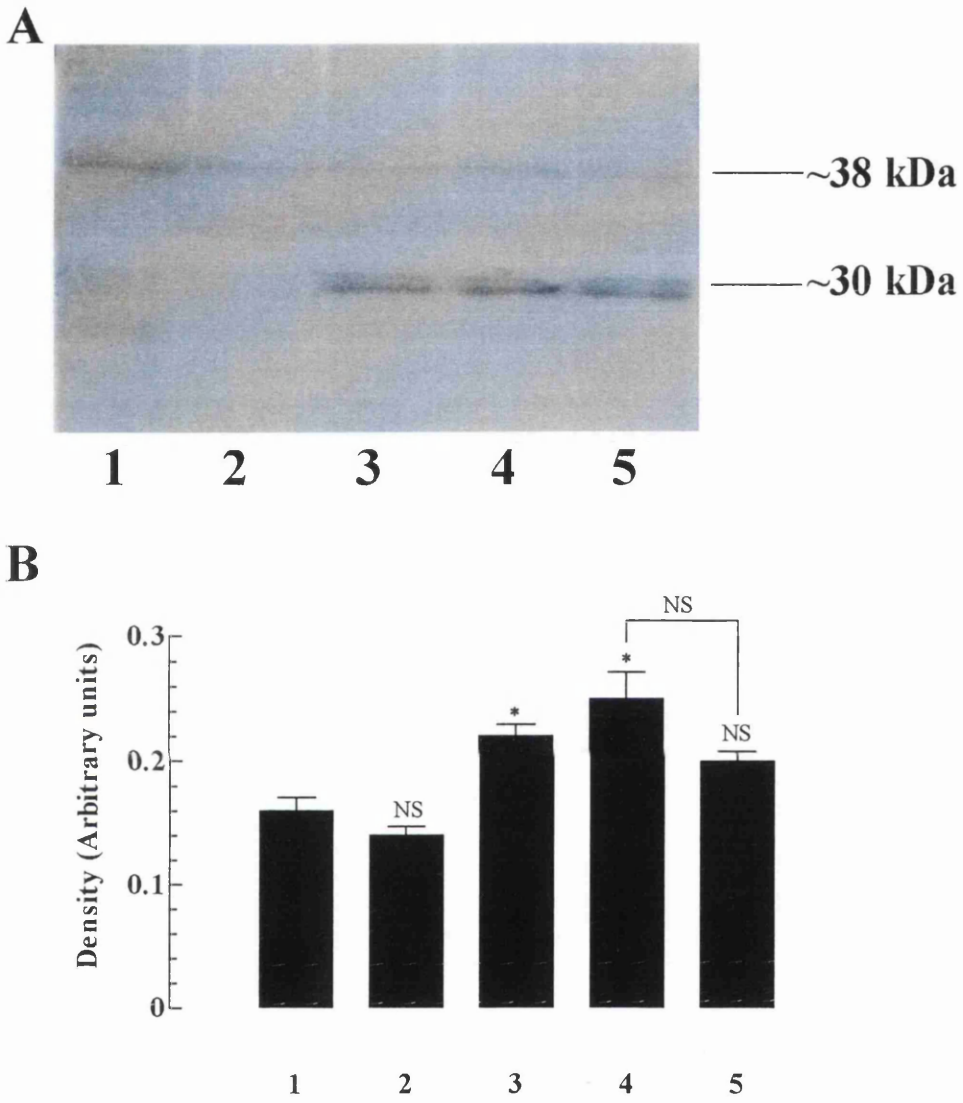


Figure 4.5. Nitrotyrosine formation by GSNO in rabbit aorta. $ONOO^-$ generation was assessed by monitoring nitrotyrosine formation in rabbit aorta. Panel A: SDS-PAGE analysis of tissue protein probed with polyclonal anti-nitrotyrosine antibody; lane 1, control; 2, NADH (300 μM); 3, NADH and GSNO (1 mM); 4, GSNO, (1 mM); 5, GSNO and tiron (1×10^{-2} M). Panel B: densitometric analysis of n=3 experiments. This gel is representative of those obtained in 3 separate experiments. NS denotes no significance and $*P<0.05$, control vs treatment.

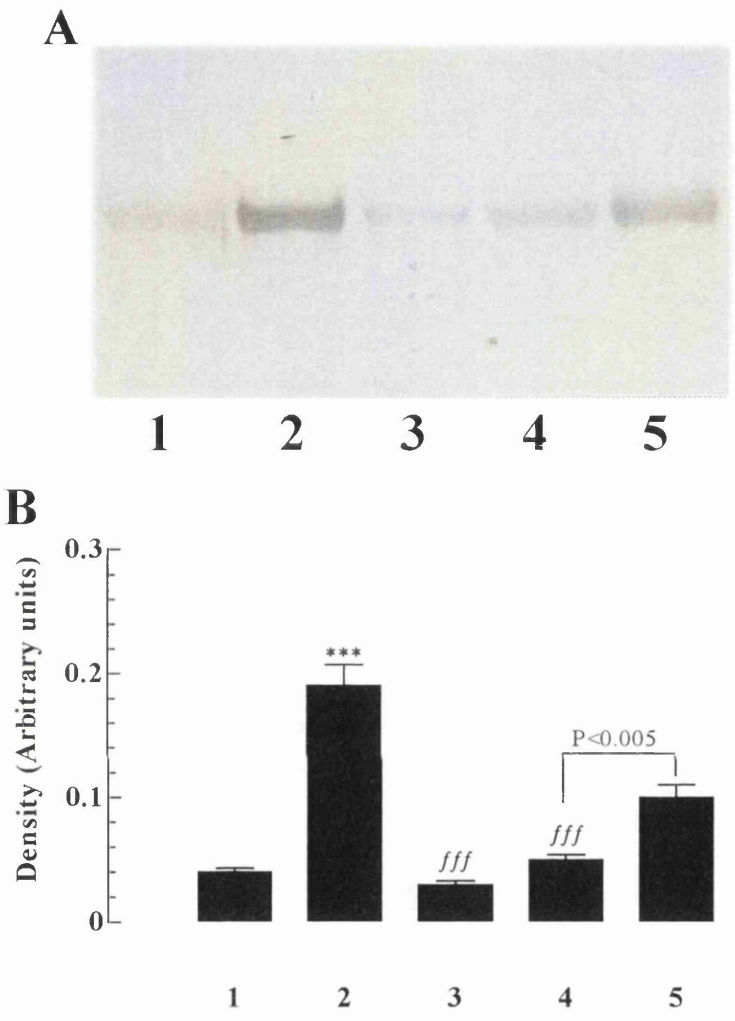


Figure 4.6. Nitrotyrosine formation by SIN-1 in bovine serum albumin. $ONOO^-$ generation was assessed by monitoring nitrotyrosine formation in bovine serum albumin. Panel A: SDS-PAGE analysis of treated bovine serum albumin probed with polyclonal anti-nitrotyrosine antibody; bovine serum albumin was incubated for 10 min with 1, HEPES buffer (control); 2, SIN-1 (1 mM); 3, SIN-1 and oxyhaemoglobin (50 μM); 4, SIN-1 and SOD (1000 U/ml); 5, SIN-1, SOD + GSNO (1 mM). Panel B: densitometric analysis of n=3 experiments. This gel is representative of those obtained in 3 separate experiments. ***$P<0.0005$, control vs SIN-1; $^{fff}P<0.0005$, control vs treatment.

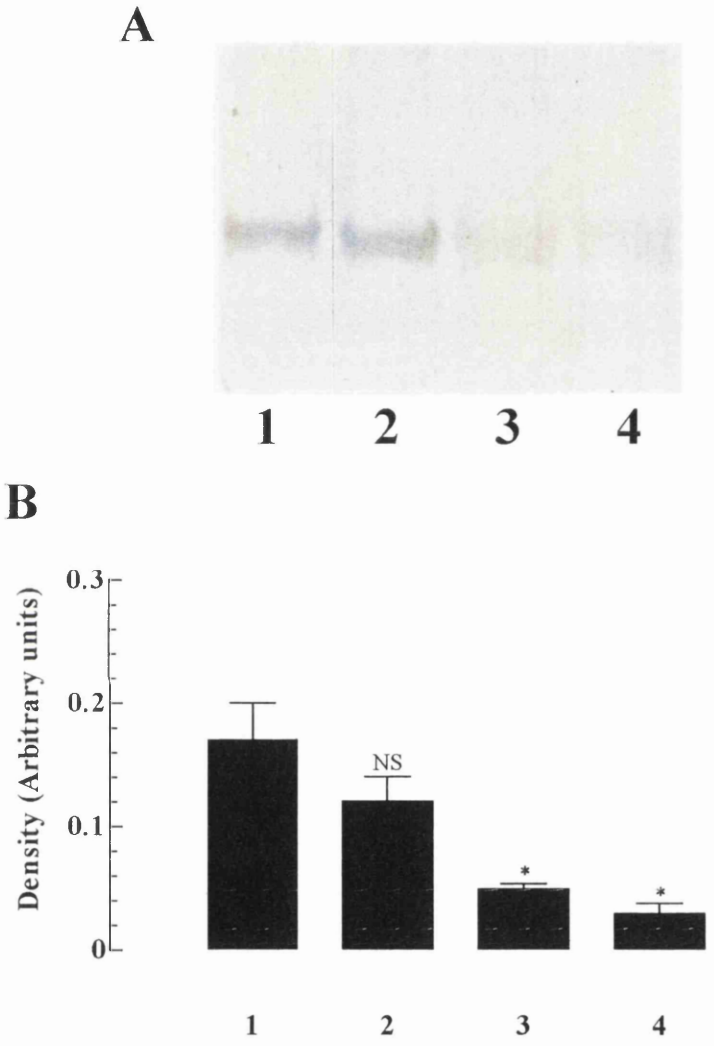


Figure 4.7. Inhibition of nitrotyrosine formation by SOD. $ONOO^-$ generation was assessed by monitoring nitrotyrosine formation in bovine serum albumin. Panel A: SDS-PAGE analysis of treated bovine serum albumin probed with polyclonal anti-nitrotyrosine antibody; bovine serum albumin and SIN-1 (1 mM) was incubated for 10 min with 1, SIN-1 (1 mM; control); 2, SOD (10 U/ml); 3, SOD (100 U/ml); 4, SOD (1000 U/ml). Panel B: densitometric analysis of n=3 experiments. This gel is representative of those obtained in 3 separate experiments. *P<0.05 control vs SOD.

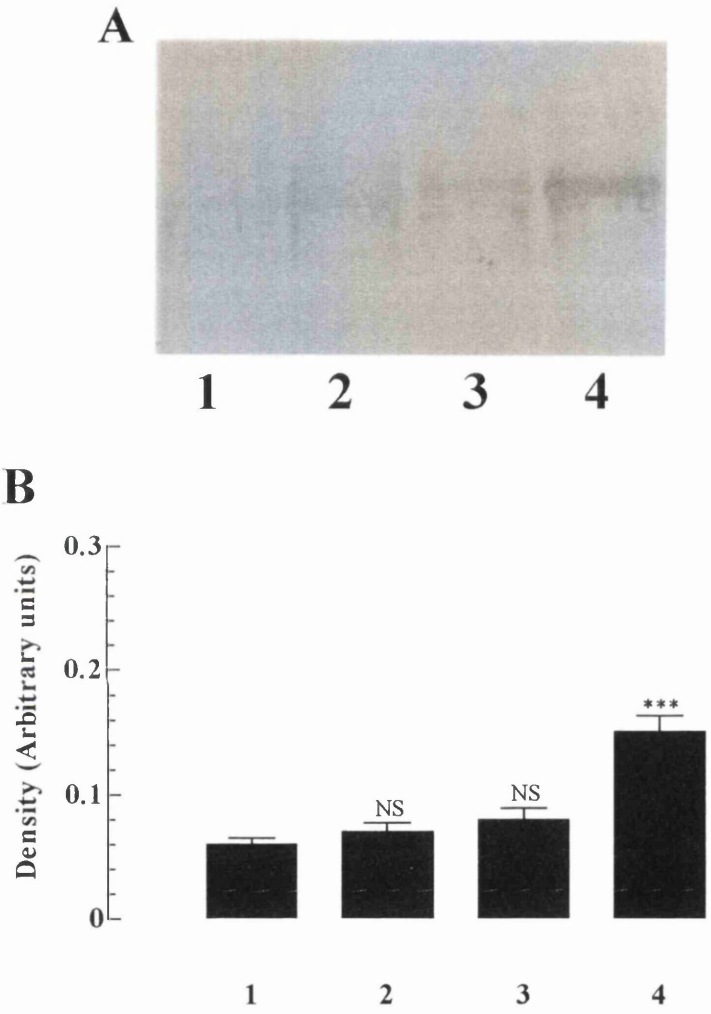


Figure 4.8. Nitrotyrosine formation by NO and $O_2^{\cdot-}$. $ONOO^-$ generation was assessed by monitoring nitrotyrosine formation in bovine serum albumin. Panel A: SDS-PAGE analysis of treated bovine serum albumin probed with polyclonal anti-nitrotyrosine antibody; bovine serum albumin was incubated for 10 min with 1, HEPES buffer (control); 2, GSNO (1 mM); 3, Pyrogallol (1 mM); 4, GSNO and Pyrogallol. Panel B: densitometric analysis of n=3 experiments. This gel is representative of those obtained in 3 separate experiments. NS denotes no significance and ***P<0.005 control vs treatment.

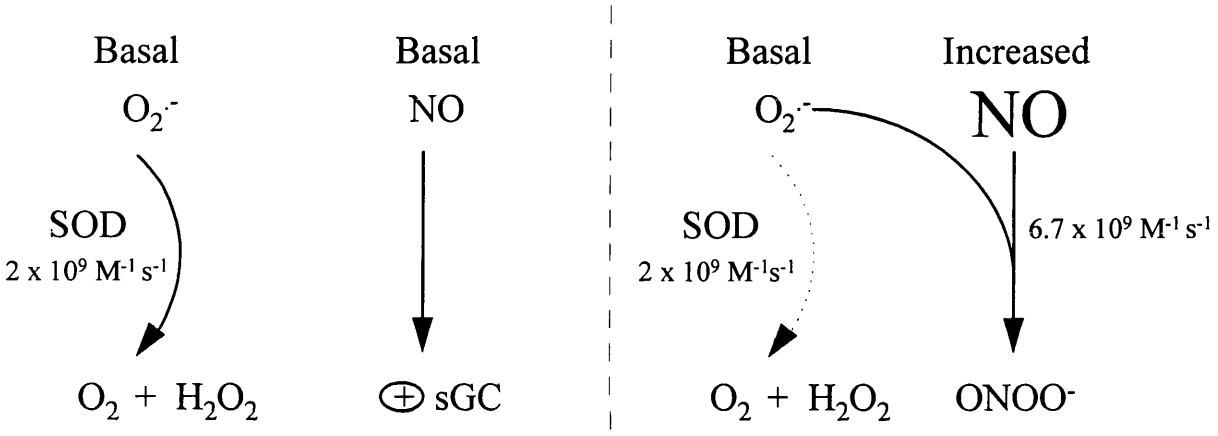


Figure 4.9. Interactions of NO, $O_2^{\cdot-}$ and SOD. Formation of $ONOO^-$ is dependent on the concentration of $O_2^{\cdot-}$ and NO. When levels of NO rise above a critical level, the reaction between NO and $O_2^{\cdot-}$ predominates and SOD is unable to compete.

4.4. Discussion

This study investigated the interaction between $O_2^{\cdot-}$ and NO in rabbit aorta. Under normal conditions intracellular $O_2^{\cdot-}$ concentrations are kept at low levels because eukaryotic cells contain large amounts of SOD (4-10 μM; Fridovich, 1978). In the present study endogenous generation of $O_2^{\cdot-}$ by aortic rings was not evident unless intrinsic SOD activity was inhibited. Once SOD was inhibited, the amount of $O_2^{\cdot-}$ generated in rabbit aorta was in the order of 1 pmol/min/mg tissue. Under certain conditions autooxidation of the lucigenin cation radical may result in redox cycling and $O_2^{\cdot-}$ generation from lucigenin itself (Li *et al.*, 1998b). Thus the figure of 1 pmol/min/mg tissue $O_2^{\cdot-}$ generation should be considered an estimate rather than precise value. The results of this and other studies (Pagano *et al.*, 1993, Jones *et al.*, 1996) are compatible with significant vascular generation of $O_2^{\cdot-}$ which may greatly exceed the capacity of the endothelium to generate NO (Guo *et al.*, 1996; Kelm *et al.*, 1997).

Potential sources of $O_2^{\cdot-}$ in blood vessels include xanthine oxidase, mitochondrial enzymes, cyclooxygenases, NO synthases and enzymes similar to the leukocyte-NADPH oxidase. Addition of NADH or NADPH, but not substrates selective for the other enzyme systems, resulted in increased $O_2^{\cdot-}$ production from aortic rings and this was inhibited in the presence of SOD. In leukocytes, NADPH oxidase is a multi-component enzyme system capable of generating rapidly large amounts of $O_2^{\cdot-}$ (nmol/min) following cellular activation (Cross *et al.*, 1984). In blood vessels and cultured endothelial cells (Jones *et al.*, 1996) NADH/NADPH-oxidase like systems have been identified (Pagano *et al.*, 1993; Mohazzab *et al.*, 1994) and the present experiments clearly demonstrate that $O_2^{\cdot-}$ generation by aortic rings can be stimulated by NADH or NADPH. Endothelium-intact aortic rings were used throughout this study and thus the contribution of the endothelium to the measured $O_2^{\cdot-}$ release has not been assessed but previous studies suggest that most vascular $O_2^{\cdot-}$ may be released from the adventitia (Wang *et al.*, 1998).

The NO donors GSNO and SNP quenched $O_2^{\cdot-}$ chemiluminesence originating from either aortic rings stimulated with NADH or from xanthine/xanthine oxidase used as an artificial cell-free $O_2^{\cdot-}$ generating system. These effects occurred over the concentration range 0.01-3 mM for GSNO and 0.01 μM-1 mM for SNP.

The relationship between NO release and quenching of $O_2^{\cdot-}$ was determined and found that the quantity of NO released from GSNO in this acellular system was proportional to the quenching of $O_2^{\cdot-}$ chemiluminesence (Fig. 4.4), suggesting that the quenching of the signal occurred as a result of interaction between the two radicals. Quenching of $O_2^{\cdot-}$ occurred at relatively low concentrations of SNP and would be expected to occur at therapeutic doses. The apparent greater potency of SNP may be related to its ability to release NO spontaneously without metabolism, whereas GSNO is more stable in solution (Jourd'heuil *et al.*, 1998).

In aortic rings no $O_2^{\cdot-}$ was detected in the absence of DDC suggesting that metabolism of $O_2^{\cdot-}$ by SOD is an important regulator of basal $O_2^{\cdot-}$ levels. Addition of GSNO resulted in nitrotyrosine formation and this was not increased by inhibition of SOD. The rapid reaction between NO and $O_2^{\cdot-}$ (Fig. 4.6) would result in a relatively small contribution of SOD to the disposal of $O_2^{\cdot-}$ when concentrations of NO are high. Thus in the presence of a NO donor, inhibition of SOD would not be expected to alter peroxynitrite formation and thus tyrosine nitration. Incubation of aortic rings with NADH and GSNO did not increased tyrosine nitration compared to that observed in the presence of GSNO alone. This may reflect the relatively small increase in superoxide production with NADH and the semi-quantitative nature of the Western blotting technique for detection of nitrotyrosine.

The reaction between NO and $O_2^{\cdot-}$ results in the formation of $ONOO^-$ which is capable of nitrating tyrosine (Beckman *et al.*, 1994) and addition of GSNO to aortic rings resulted in tyrosine nitration, suggesting that $ONOO^-$ formation was occurring. However, attempts to scavenge endogenous $O_2^{\cdot-}$ with SOD did not prevent the appearance of nitrotyrosine in aortic rings incubated with GSNO. SOD does not cross cell membranes and this may explain why it was unable to reduce nitrotyrosine formation. Tiron, a relatively weak scavenger of $O_2^{\cdot-}$ (0.1 - 0.05 $\mu M^{-1} s^{-1}$; Greenstock & Miller, 1975; Bors *et al.*, 1979; Mok *et al.*, 1998), but one which is cell permeable, also did not inhibit nitrotyrosine formation. One hypothesis is that GSNO can nitrate tyrosine residues directly, independent of its reaction with $O_2^{\cdot-}$. Alternatively, the reaction between NO and $O_2^{\cdot-}$ might be particularly resistant to SOD under certain conditions. To test these hypotheses a cell-free system was used.

Incubation of serum albumin with GSNO did not result in nitrotyrosine formation suggesting that a direct reaction of NO does not occur at least on tyrosine residues on albumin. We examined the dependence of nitrotyrosine formation on $O_2^{\cdot-}$ and NO using a $O_2^{\cdot-}$ donor, pyrogallol. This agent releases $O_2^{\cdot-}$ at alkaline pH in a reproducible manner and using serum albumin as a substrate for nitration we found that GSNO and pyrogallol in combination but not alone resulted in nitrotyrosine formation. These results are consistent with the hypothesis that that nitration of albumin is dependent on the presence of both NO and $O_2^{\cdot-}$. Experiments were conducted with SIN-1, a co-donor NO and $O_2^{\cdot-}$. GSNO and SIN-1 at a concentration of 1 mM release similar amounts of NO (1.02 and 1.23 μM respectively; Kelm *et al.*, 1997) but SIN-1 will also liberate $O_2^{\cdot-}$ (Feelisch *et al.*, 1989). Oxyhaemoglobin (a scavenger of NO) or SOD (a scavenger of $O_2^{\cdot-}$) inhibited nitrotyrosine formation by SIN-1, suggesting that both NO and $O_2^{\cdot-}$ are required for tyrosine nitration. Whereas oxyhaemoglobin readily inhibited nitrotyrosine formation by SIN-1, high concentrations of SOD (>1000 U/ml) were required to suppress SIN-1-induced nitrotyrosine formation on serum albumin and its IC_{50} in this system lies somewhere between 100-1000 U/ml. This is in marked contrast to the calculated IC_{50} (8.8 U/ml) for SOD inhibition of NADH-stimulated lucigenin chemiluminescence measured in aortic rings (Fig. 4.2). Furthermore, even in the presence of 1000 U/ml of SOD, addition of GSNO to the SIN-1 solution increased nitrotyrosine formation.

The reaction between $O_2^{\cdot-}$ and NO is effectively diffusion limited ($6.7 \times 10^9\ M^{-1}\ s^{-1}$) and this rate constant is three times that for the reaction of $O_2^{\cdot-}$ and SOD ($2 \times 10^9\ M^{-1}\ s^{-1}$; Huie & Padmaja, 1993). Thus, in a system in which NO, $O_2^{\cdot-}$ and SOD are all present, the dominant reaction will be critically dependent on the concentration of each reagent (Fig. 4.6). At low NO concentrations the dismutation reaction between SOD and $O_2^{\cdot-}$ would be expected to predominate, but when the NO concentration rises the reaction between NO and $O_2^{\cdot-}$ will predominate and SOD will become progressively less able to compete (Fig. 4.6). Thus, the failure of SOD or tiron to prevent GSNO-induced nitrotyrosine formation in the rabbit aorta is most likely to be due to the inability of these $O_2^{\cdot-}$ scavengers to compete for $O_2^{\cdot-}$ in the presence of the large amounts of $O_2^{\cdot-}$ generated basally and the large amounts of NO liberated by the

NO-donor. However, we cannot exclude the possibility that GSNO might directly nitrate proteins present in aortic rings, even though it was unable to do so to albumin.

The results of this study show that GSNO, a NO donor, leads to nitrotyrosine formation in the vessel wall. This effect occurs because as the concentration of NO rises, $O_2^{\cdot-}$ reacts preferentially with NO and SOD cannot compete. Alternatively, it is possible that the SOD is inactivated by $ONOO^-$. Whichever mechanism occurs, these results are consistent with the hypothesis that as the concentration of NO rises, the endogenous SOD system is unable to keep oxidant stress under control and this might have implications for pathophysiology. However, these studies were performed *in vitro* in a haemoglobin-free environment and it remains to be determined whether NO donors cause nitrotyrosine formation *in vivo,* especially under conditions where oxyhaemoglobin may scavenge NO.

The biological significance of protein tyrosine nitration remains unclear. Several reports suggest that nitration may alter the function of important proteins. In murine macrophages, peroxynitrite nitrates the regulatory subunit of phosphatidylinositol 3-kinase. This protein is involved in the signal transduction cascade initiated by many agonists including growth factors (Hellberg *et al.*, 1998). Similarly, in human neuroblastoma cells nitration of cytosolic proteins inhibits phosphoinositide hydrolysis in response to carbachol (Li *et al.*, 1998a). Modification of enzymes such as glutamine synthetase by nitration results in loss of enzyme activity and may alter cellular metabolism (Berlett *et al.*, 1996). Protein nitration may well have marked biological effects on regulatory enzymes and signal transduction pathways. Furthermore, the finding of nitrotyrosine staining in the aorta of patients with atheroma (Beckman *et al.*, 1994) might be due to nitrovasodilator therapy *per se* rather than inflammatory induction of endogenous NO and $O_2^{\cdot-}$ in the vessel wall. Whilst NO donors have been used extensively in the management of ischaemic heart disease evidence suggests that long term treatment with these agents might paradoxically increase cardiac events, especially in patients with healed myocardial infarction (Ishikawa *et al.*, 1996).

CHAPTER 5

CONTRIBUTION OF NITRIC OXIDE SYNTHASES TO THE GENERATION OF PEROXYNITRITE

5.1. Introduction

NO is a potentially cytotoxic mediator implicated in the pathogenesis of endotoxin-induced tissue injury and septic shock (Thiemermann & Vane, 1990). Administration of bacterially derived LPS results in the induction of iNOS, leading to the production of large quantities of NO. This occurs in many cells however, the largest contribution to NO generation is thought to come from macrophages and vascular smooth muscle (Busse & Mülsch, 1990, Förstermann *et al.*, 1993). The interaction of NO with $O_2^{\cdot-}$ forms $ONOO^-$, a powerful oxidant capable of causing pathological damage in conditions such as endotoxemia or sepsis, inflammation, atherosclerosis, and ischemia-reperfusion (Haddad *et al.*, 1994; Kaur & Halliwell, 1994; Van der Vliet *et al.*, 1994; Szabo *et al.*, 1995; Wada *et al.*, 1995). The production of $ONOO^-$ has been extensively studied *in vitro*. $ONOO^-$ decomposes to give various products, including NO_2 or NO_2^+ that can nitrate aromatic amino acids (e.g. tyrosine) existing as free forms or components of proteins. Nitration on the *ortho* position of tyrosine is a major reaction. Plasma concentrations of nitrotyrosine may therefore reflect $ONOO^-$ production.

Although it has been demonstrated that injection of endotoxin into rats leads to the expression of iNOS and increased immunoreactivity of nitrotyrosine in the thoracic aorta (Szabo *et al.*, 1995), no evidence exists in order to understand the sources of NO for $ONOO^-$ formation under condition were elevated nitrotyrosine is evident. The major aim of this study was to determine whether $ONOO^-$ production is related to production of NO from the constitutive and/or inducible NOS pathway using iNOS mutant mice as a tool to explore these two potential NO generating pathways. Moreover, as recent studies have demonstrated phenotypic differences between iNOS mutant mice, studies in this chapter have used two different iNOS mutants.

5.2. Methods

5.2.1. Induction of endotoxin shock in mice

Experiments were conducted in male MF1 and C57BL/6 wild-type (+/+) and homozygous (-/-) iNOS-deficient mice, obtained from Professor Liew and The Jackson Laboratory (USA), respectively. Mice were injected with saline (3 ml/kg) or LPS (*E. coli*, 026:B6, 12.5 mg kg^{-1}, bolus dose; i.v.) as described in chapter 2, section 2.2.2. Twelve hours later blood samples were collected and plasma prepared as described in chapter 2, section 2.2.2.

5.2.2. Measurement of plasma nitrite/nitrate

Collected blood samples from both group of animals were assayed for plasma nitrite/nitrate as described in chapter 2, section 2.3.2.

5.2.3. Nitrotyrosine, iNOS & eNOS detection by Western blotting

Collected aorta and heart tissues from treated wild-type and iNOS mutant mice were homogenised as described in chapter 2, section 2.3.11. Homogenates from tissue samples were assayed for protein content using a Coomassie blue protein reagent as described previously (section 2.3.8). Equivalent amounts of each sample (25 μg protein) were loaded onto a SDS-PAGE gel, and probed for nitrotyrosine, iNOS or eNOS using a polyclonal rabbit anti-nitrotyrosine, iNOS or eNOS antibody, respectively, as described in chapter 2, section 2.3.11. Control experiments were conducted to confirm the presence of nitrotyrosine. Prior to the addition of the primary antibody to the nitrocellulose membranes, the antibody was incubated with 10 mM 3-nitro-L-tyrosine in potassium phosphate buffer, pH 7.4.

5.2.4. Measurement of plasma urea and creatinine levels

Plasma was prepared from collected blood samples from treated wild-type and iNOS mutant mice, as described in chapter 2, section 2.3.2. Plasma samples were sent to Vet Diagnostics Ltd for analysis of urea and creatinine levels.

5.2.5. Nitrotyrosine detection by ELISA

Aortic and heart homogenates and plasma samples collected from treated wild-type and iNOS mutant mice were assayed for 3-nitrotyrosine by ELISA as described in chapter 2, section 2.3.12. Limit of detection was 0.1327 μg/ml.

5.3. Results

5.3.1. Induction of endotoxin shock

Levels of nitrite/nitrate in the plasma of wild-type and iNOS mutant mice (obtained from Professor Liew) treated with saline were 37 ± 7 and 41 ± 9 μM (P>0.05; n=8), respectively. Administration of LPS (12.5 mg/kg; i.v; bolus dose) significantly (P<0.0005) increased plasma nitrite/nitrate by 9.9 and 4.7 fold at 12h (Fig. 5.1, n=8), in wild-type and iNOS mutant mice respectively. Levels of nitrite/nitrate in the plasma of wild-type and iNOS mutant mice (obtained from Jackson Labs) treated with saline were 32 ± 7 and 26 ± 3 μM (P>0.05; n=8), respectively. Administration of LPS (12.5 mg/kg; i.v; bolus dose) significantly (P<0.0005) increased plasma nitrite/nitrate by 18.4 and 2.3 fold at 12h (Fig. 5.3, n=8), in wild-type and iNOS mutant mice respectively. No deaths occurred in the wild-type and iNOS mutant mice treated with saline or LPS.

5.3.2. Expression of nitrotyrosine, iNOS and eNOS in LPS-treated mice

In iNOS mutant mice immunoreactive iNOS protein was detected in mice obtained from Professor Liew (Fig. 5.2) but not from Jackson Laboratory, USA (Figs. 5.4A & B). Wild-type mice treated with endotoxin displayed a band at relative molecular mass 130kDa in aortae corresponding to iNOS (Fig. 5.4B). A band at molecular mass 140kDa was present in saline and LPS-treated wild-type and iNOS mutant mice consistent with constitutive eNOS expression (Figs. 5.5A & B).

Nitrotyrosine staining was observed in aorta and heart of control (saline treated) wild-type or iNOS mutant mice, as assessed by western blotting (Fig. 5.6A & 5.6B). Specific nitrotyrosine immunoreactivity was not observed when the blot was incubated in the presence of primary antibody and excess 3-nitro-L-tyrosine (10 mM). Treatment of wild-type or iNOS mutant mice with LPS appeared to increase nitrotyrosine formation (Fig. 5.6).

5.3.3. Levels of tissue and plasma nitrotyrosine

An attempt was made to quantify nitrotyrosine formation using an ELISA method. The limit of detection for the assay was 0.133 μg/ml. As shown in Table 5.1, nitrotyrosine levels were below the limit of detection in aorta under all

conditions. However in heart and plasma there was detectable nitrotyrosine which was not further increased in wild-type or iNOS mutant mice following endotoxin treatment, as measured by western blotting.

5.3.4. Levels of plasma urea and creatinine

Since plasma nitrite/nitrate levels increased in iNOS mutant mice after LPS treatment, an attempt was made to determine whether nitrite/nitrate clearance was altered in endotoxaemia. Levels of urea in the plasma of wild-type and iNOS mutant mice treated with saline were 6.7 ± 0.3 and 7.4 ± 1.1 mmol/l (P>0.05; n=8), respectively. Administration of LPS (12.5 mg/kg; i.v; bolus dose) significantly (P<0.0005) increased plasma urea by 2.9 and 2.7-fold at 12h (Figure 5.7, n=8), in wild-type and iNOS mutant mice respectively. Levels of creatinine in the plasma of wild-type and iNOS mutant mice treated with saline were 44.7 ± 7 and 69 ± 20.7 μmol/l (P>0.05; n=8), respectively. Administration of LPS (12.5 mg/kg; i.v; bolus dose) increased (P>0.05) plasma creatinine by 1.4 and 1.9-fold at 12h (Figure 5.7, n=8), in wild-type and iNOS mutant mice respectively.

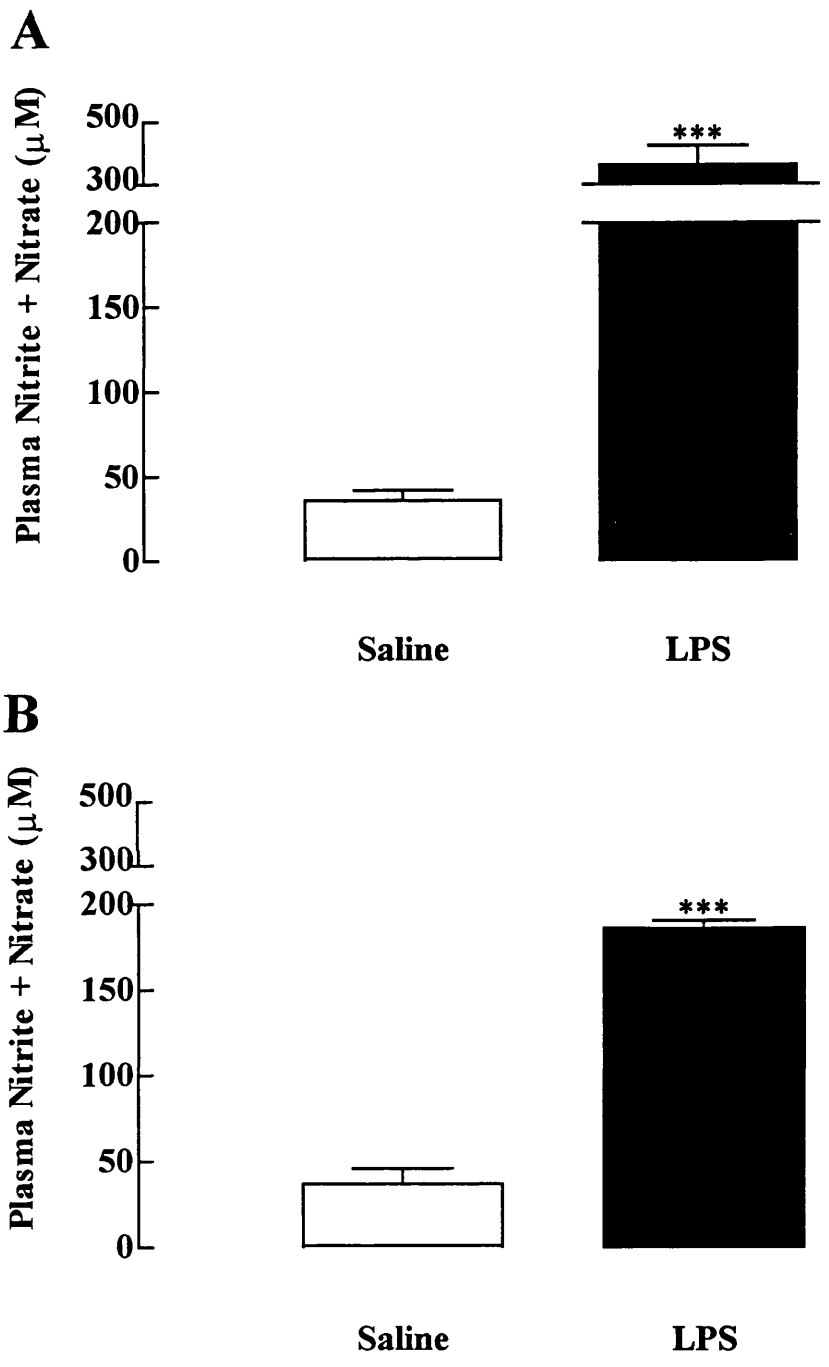


Figure 5.1. Plasma levels of nitrite/nitrate in endotoxin treated mice. Wild-type (A) or iNOS mutant mice (B; obtained from Professor Liew) were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). Twelve hours later blood samples were collected and plasma nitrite/nitrate levels were determined after reduction of plasma nitrates to nitrite upon reaction with nitrate reductase. Results are mean ± s.e.m of data obtained in 6 experiments. $^{***}P<0.0005$, saline vs LPS.

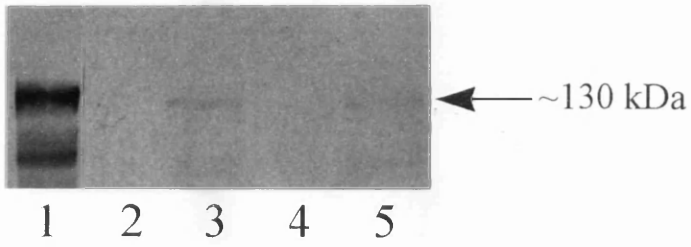


Figure 5.2. Expression of iNOS in mouse heart. Wild-type (W/T) or iNOS mutant (K/O; obtained from Professor Liew) mice were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). After 12 h tissues were harvested, homogenised and proteins separated on a 10 % denaturing acrylamide gel and probed for iNOS. 1, LPS-treated mouse macrophage lysate (positive control); 2, W/T saline, 3, W/T LPS, 4, K/O saline, 5, K/O LPS. Results are representative of those obtained in 2 separate experiments.

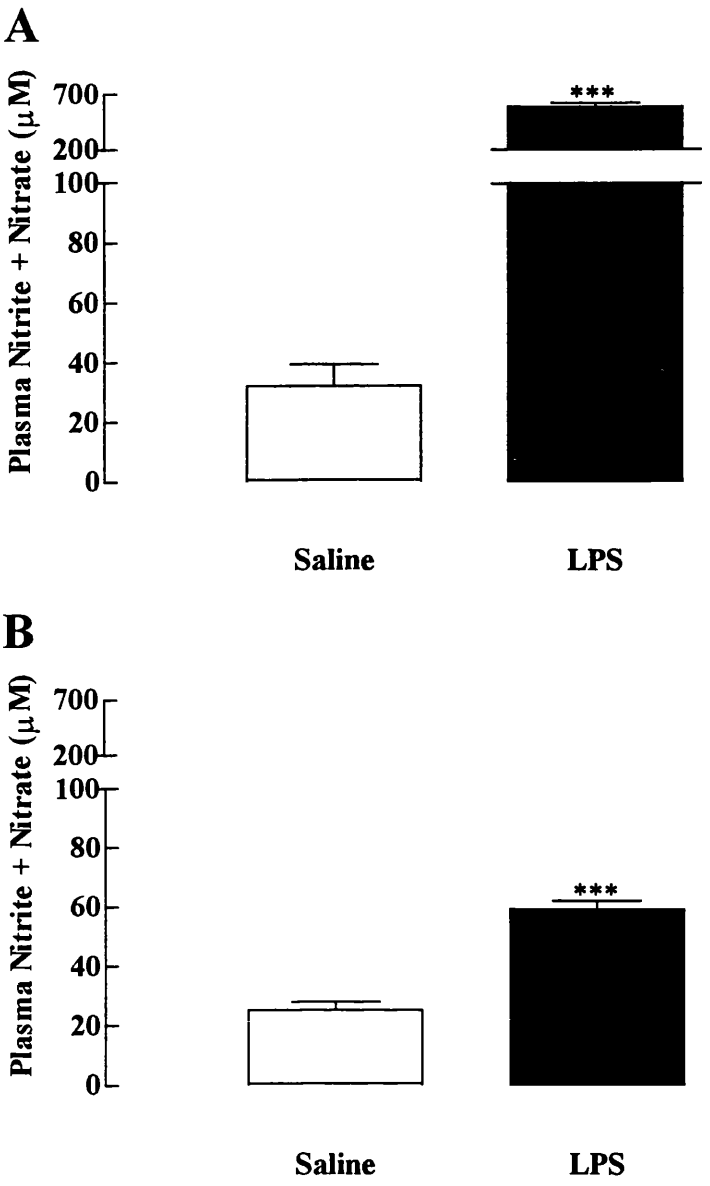


Figure 5.3. Plasma levels of nitrite/nitrate in endotoxin treated mice. Wild-type (A) or iNOS mutant mice (B; obtained from Jackson Laboratory, USA) were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). Twelve hours later blood samples were collected and plasma nitrite/nitrate levels were determined after reduction of plasma nitrates to nitrite upon reaction with nitrate reductase. Results are mean ± s.e.m of data obtained in 8 experiments. $^{***}P<0.0005$, saline vs LPS.

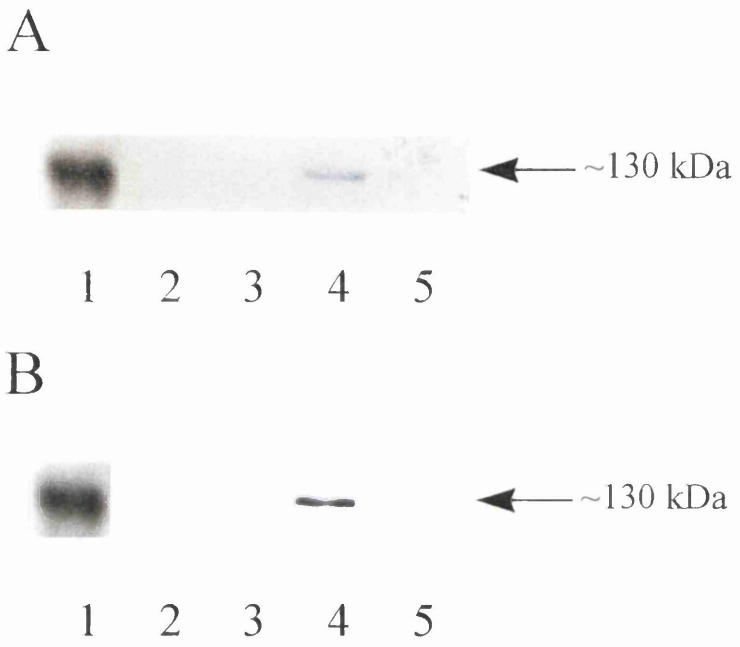


Figure 5.4. Expression of iNOS in mouse aorta (A) and heart (B). Wild-type (W/T) or iNOS mutant (K/O; obtained from Jackson Laboratory, USA) mice were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). After 12 h tissues were harvested, homogenised and proteins separated on a 10 % denaturing acrylamide gel and probed for iNOS. Panel (A); 1, LPS-treated mouse macrophage lysate (positive control); 2, W/T saline, 3, K/O saline, 4, W/T LPS, 5, K/O LPS; (B); 1, LPS-treated mouse macrophage lysate; 2, W/T saline, 3, K/O saline, 4, W/T LPS, 5, K/O LPS. Results are representative of those obtained in 3 separate experiments.

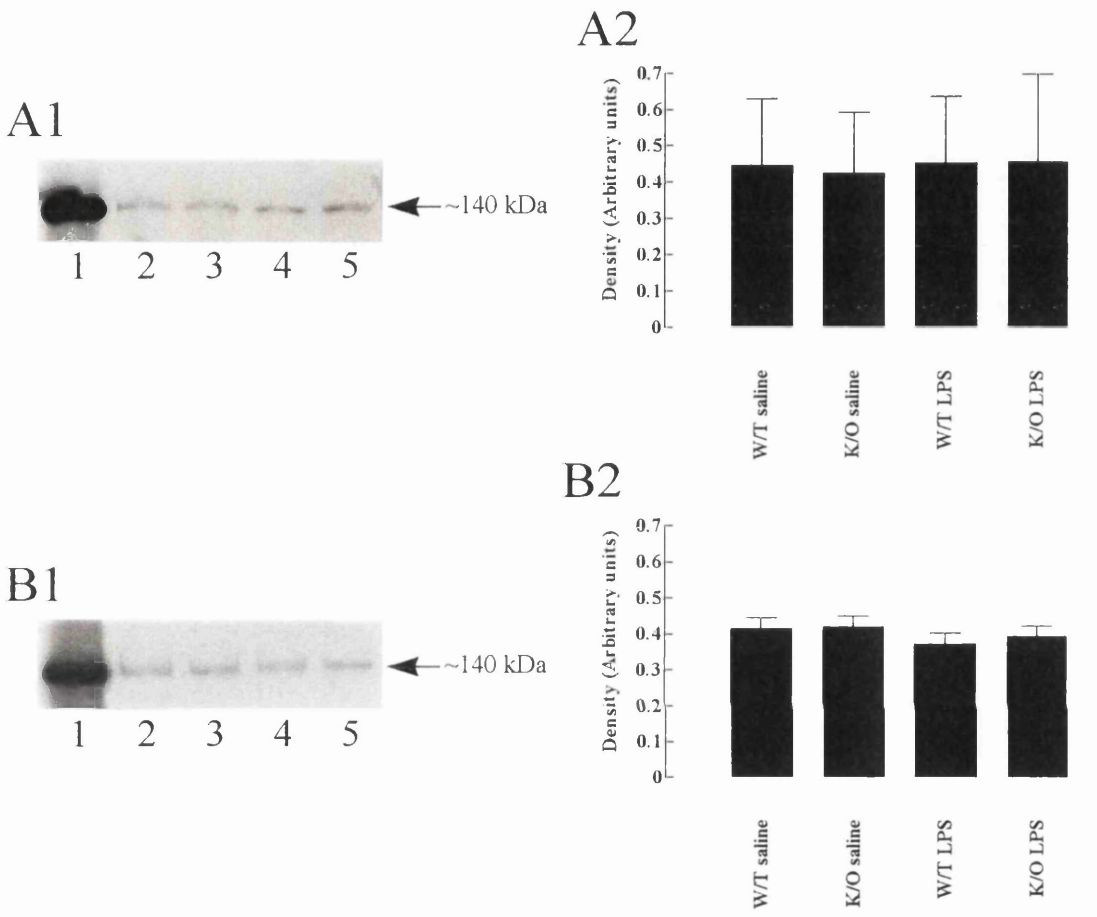


Figure 5.5. Expression of eNOS in mouse aorta (A) and heart (B). Wild-type (W/T) or iNOS mutant (K/O; obtained from Jackson Laboratory, USA) mice were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). After 12 h tissues were harvested, homogenised and proteins separated on a 10 % denaturing acrylamide gel and probed for eNOS. Panel (A1); 1, human endothelial lysate; 2, W/T saline, 3, W/T LPS, 4, K/O saline, 5, K/O LPS; (B1); 1, human endothelial lysate; 2, W/T saline, 3, K/O saline, 4, W/T LPS, 5, K/O LPS. Density of bands for aorta and heart are shown in panels A2 and B2, respectively. Results are representative of those obtained in 3 separate experiments.

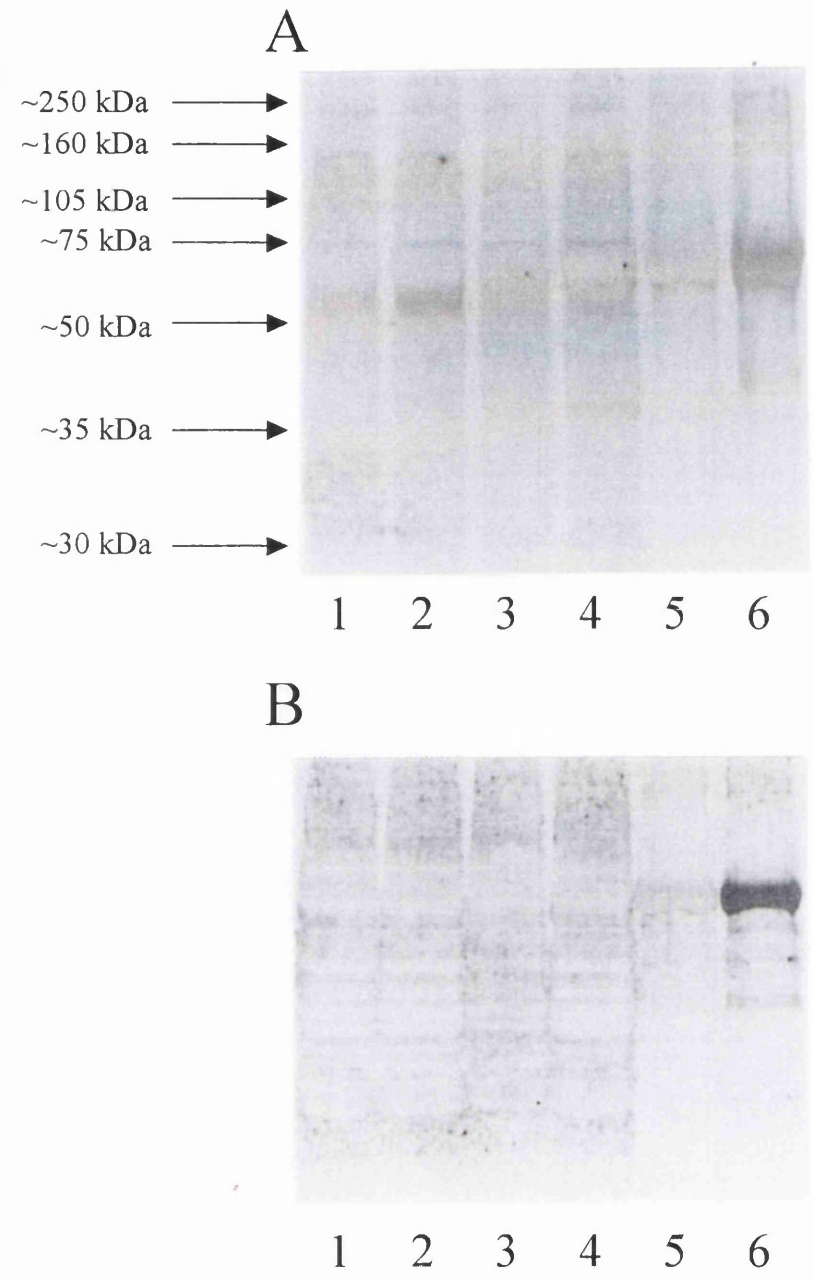


Figure 5.6. Expression of nitrotyrosine in mouse aorta (A) and heart (B). Wild-type (W/T) or iNOS mutant (K/O; obtained from Jackson Laboratory, USA) mice were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). After 12 h tissues were harvested, homogenised and proteins separated on a 10 % denaturing acrylamide gel and probed for nitrotyrosine. Panel (A); 1, W/T saline, 2, W/T LPS, 3, K/O saline, 4, K/O LPS; 5, BSA; 6, BSA + SIN-1 (10 mM) (B); 1, W/T saline, 2, K/O saline, 3, W/T LPS, 4, K/O LPS; 5, BSA; 6, BSA + SIN-1. Results are representative of those obtained in 3 separate experiments.

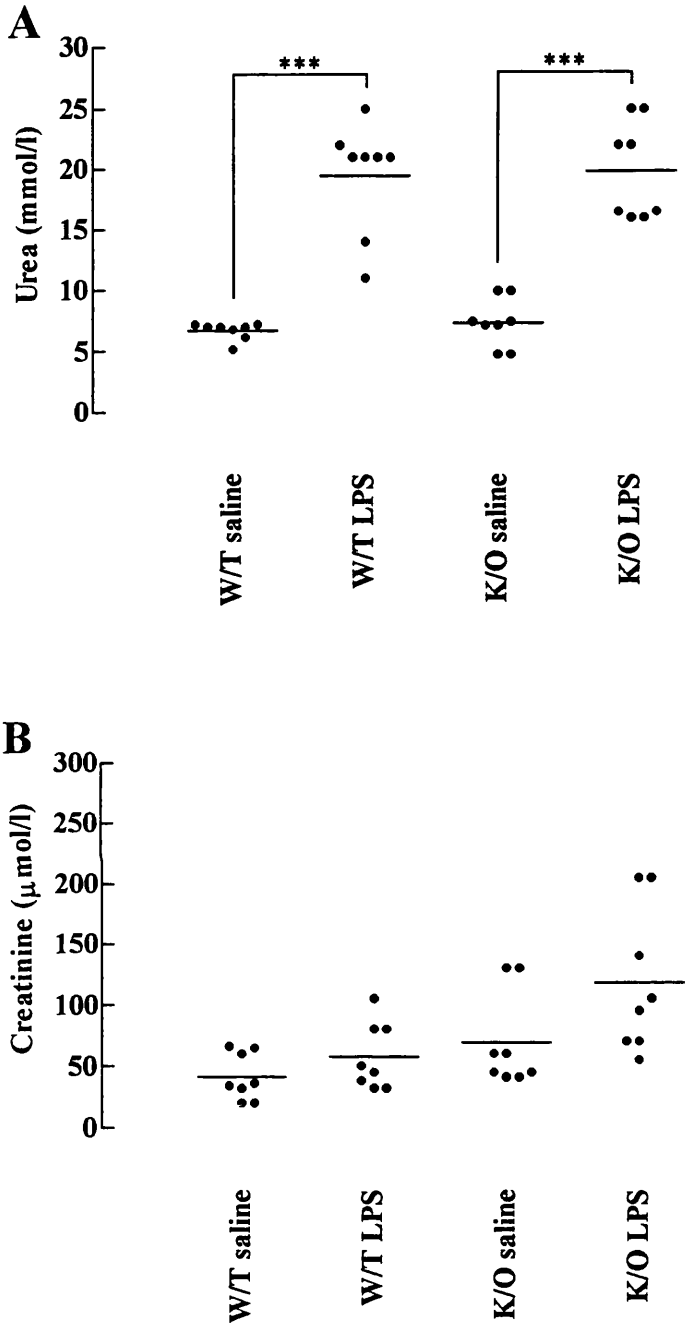


Figure 5.7. Levels of plasma urea (A) and creatinine (B) in endotoxin-treated mice. Wild-type (W/T) or iNOS mutant (K/O) mice (obtained from Jackson Laboratory, USA) were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). Twelve hours later blood was removed from mice and plasma prepared as described in chapter 2, section 2.3.2. Measurements of plasma urea and creatinine were performed commercially by Vet Diagnostics Ltd.

TABLE 5.1. LEVELS OF TISSUE AND PLASMA NITROTYROSINE IN ENDOTOXIN-TREATED MICE

	Wild-Type		iNOS mutant	
	Control (*μg/mg protein*)	*LPS* (*μg/mg protein*)	*Control* (*μg/mg protein*)	*LPS* (*μg/mg protein*)
Aorta	ND	ND	ND	ND
Heart	0.027 ± 0.009	0.036 ± 0.009^{NS}	0.031 ± 0.007	0.032 ± 0.002^{NS}
Plasma	0.016 ± 0.003	0.009 ± 0.001^{NS}	0.018 ± 0.003	0.013 ± 0.001^{NS}

Wild-type or iNOS mutant mice (obtained from Jackson Laboratory, USA) were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). Twelve hours later aorta, heart and blood were isolated from mice. Measurements of nitrotyrosine in plasma, aortic and heart homogenates were made be ELISA. Results are mean ± s.e.m of data obtained in 4 experiments. ND and NS denotes not detected (0.133 μg/ml) and not significantly different (P>0.05), saline vs LPS, respectively.

5.4. Discussion

The main observations from this study were that (i) LPS induces iNOS expression and NO production in wild-type mice and (ii) in iNOS mutant mice there was no induction of iNOS protein but levels of nitrite/nitrate were still increased, albeit to a lesser extent. Peroxynitrite can be generated through the interaction between $O_2^{\cdot-}$ and NO. The NO can result from induction of iNOS (Szabo *et al.*, 1995; Xia & Zweier, 1997) or release from an exogenous source from an NO donor (Chapter 4; Fig. 4.5). Endotoxin treatment has been used to mimic the effects of sepsis and to cause inflammation. These processes have been shown to result in induction of iNOS, increased formation of NO, $ONOO^-$, and lead to protein nitration. Therefore, measurement of tyrosine nitration has commonly been used as an index of $ONOO^-$ formation (Haddad *et al.*, 1994; Kooy *et al.*, 1995). However, it is unclear whether tyrosine nitration can occur in an iNOS independent manner. Therefore, this study investigated the importance of NO generation independent of iNOS in the formation of nitrotyrosine, in a model of endotoxin shock in the conscious mouse (Rees *et al.*, 1998).

The induction of endotoxin shock by a sub-lethal dose of bacterial LPS, resulted in a significant increase in plasma nitrite/nitrate levels in wild-type mice. This result is consistent with the idea that induction of iNOS occurs throughout the animal. Following LPS treatment, iNOS expression has been demonstrated in murine and human macrophages (Bogle *et al.*, 1994; Plum *et al.*, 1999) and elevated plasma nitrite/nitrate concentrations occur in sepsis (Doughty *et al.*, 1998; Rees *et al.*, 1998; Stoclet *et al.*, 1999). To investigate whether iNOS induction is essential for these responses, iNOS mutant mice from two independent sources were treated with endotoxin. After LPS treatment, an increase in plasma nitrite/nitrate was observed in both endotoxin-treated mice, although the increase in plasma nitrite/nitrate in mice obtained from Professor Liew was unexpectedly high. This was accompanied by iNOS protein expression, as shown by western blotting. These results could be explained if during the mouse breeding program, the homozygous allele had been lost due to mating of breeding pairs which included a heterozygous mouse expressing the iNOS gene, thus generating a colony of mice which would be iNOS (+/-) deficient. The absence of iNOS protein accompanied by an increase in plasma nitrite/nitrate in iNOS mutant mice obtained from

Jackson Laboratory could be accounted for either by i) an increase in nNOS and/or eNOS expression, ii) an increase in eNOS activity possibly due to upregulation of cofactor(s) availability or (iii) changes in nitrite/nitrate excretion. Experiments to determine whether eNOS enzyme activity was altered were not performed since isolated enzyme assays require the addition of excess amounts of co-factors such as BH_4 thus making interpretation difficult. Functional studies using isolated blood vessels from LPS-treated mice might enable eNOS activity to be determined. Endothelial NOS protein levels in tissue homogenates from saline or endotoxin-treated wild-type or iNOS mutant mice were unchanged, suggesting that an increase in eNOS expression did not account for the increased nitrite/nitrate levels in these experiments. These observations are consistent with previous findings of eNOS expression and increased NO-mediated dilatation, in the absence of iNOS in human hand veins treated with cytokines (Bhagat *et al.*, 1999). In these studies it was suggested that cytokines could induce upregulation of cofactor production such as BH_4 which increases eNOS activity and results in increased NO production. Interestingly, the increase in nitrite/nitrate levels measured following LPS treatment of iNOS mutant mice were similar to those reported in human sepsis (3-fold elevation in serum levels of nitrite and nitrate in septic patients compared to control; Evans *et al.*, 1993). Thus, it appears that LPS induces NO generation independent of iNOS induction.

The possibility that changes in the excretion of nitrite/nitrate during sepsis could account for increased plasma levels of nitrite/nitrate was addressed by measuring plasma levels of urea and creatinine, as markers of renal function. LPS treatment resulted in a consistent rise in plasma urea in wild-type and iNOS mutant mice. In contrast, creatinine levels were not significantly altered by LPS treatment. These results suggest that LPS induces renal dysfunction in wild-type and iNOS mutant mice, at least through measurement of urea. Changes in plasma urea are likely to result from a period of renal hypotension associated with the systemic hypotension elicited by LPS. This might result in reduced clearance of nitrite/nitrate and recent evidence suggests that serum nitrite/nitrate levels correlate with creatinine clearance in intensive therapy unit patients (MacKenzie *et al.*, 1996). Thus a component of the increased nitrite/nitrate levels detected after LPS treatment may be due to reduced clearance.

Immunoblotting was used in order to assess the levels of nitrotyrosine in tissue proteins as a potential biomarker for peroxynitrite. Nitrotyrosine staining was present in aorta and heart from endotoxin-treated wild-type mice. These findings are consistent with previous observations of increased iNOS expression and formation of nitrotyrosine in rat aorta (Szabo *et al.*, 1995). LPS treatment to wild-type mice or iNOS mutant mice resulted in an apparent increase in nitrotyrosine staining. A further attempt to quantify nitration in endotoxin-treated animals was made by use of an alternative detection method. A competitive enzyme-linked immunosorbent assay (ELISA) which has been used previously to detect nitrotyrosine in human plasma (Khan *et al.*, 1998) was chosen as the ELISA for nitrated proteins is a sensitive semi-quantitative method for determination of relative amounts of nitrated proteins. Consistent with results from immunoblotting, levels of nitrotyrosine were detected at very low levels, at or below the limit of detection for the ELISA method, resulting in difficulty of interpretation of results, although this assay has been reported to be a sensitive method for the detection of nitrotyrosine (Khan *et al.*, 1998). Although competitive ELISA has been previously used for the determination of relative amounts of nitrated plasma proteins from human patients, the unsuccessful detection of significant levels of nitrotyrosine by this method could be accounted for by the relatively small amounts present in murine tissue or the poor specificity of the antibody used. Alternatively, the inability to detect significant levels of nitrotyrosine in tissues or plasma could be accounted for if a nitration of tyrosine residues of proteins were a reversible process, as suggested by a recent study (Kamisaki *et al.*, 1998). The use of alternative methods for the detection of nitrotyrosine, such as gas chromatography mass spectroscopy (GC/MS; Moore, 1999; personal communication), high pressure liquid chromatography (HPLC; Kaur & Halliwell, 1994) or immunohistochemistry (Szabo *et al.*, 1995) may have enabled the quantification or visualisation of modified proteins. However, analytical techniques such as GC/MS and HPLC have the disadvantages that the samples have to undergo extensive and lengthy processing which can give rise to artefacts. For example, in the presence of contaminating nitrite, acid hydrolysis to release free nitrotyrosine or even mild acid treatment, may result in artifactual nitration and the overestimation of the nitrotyrosine content (Shigenaga *et al.*, 1997). In summary, the results of the studies in this chapter

show that serum nitrite + nitrate levels increase following endotoxin treatment of iNOS mutant mice. Although reduced excretion might contribute to some of the change, it is unlikely to account for it in total. Furthermore, the finding that nitrotyrosine, as assessed by western blotting, appears to increase following LPS treatment is consistent with increased NO generation. Due to the possibility of incomplete deletion of the iNOS gene in the mice obtained from Professor Liew, all subsequent studies were conducted using mice from The Jackson Laboratory, USA.

CHAPTER 6

REGULATION OF NITRIC OXIDE SYNTHASE BY TETRAHYDROBIOPTERIN

6.1. Introduction

In man, infection with gram negative organisms may lead to septic shock, a syndrome which is characterised by progressive hypotension, hyporeactivity to vasopressor agents and multiple organ failure. This syndrome is associated with an increase in NO generation, which may account for the hypotension and impaired vascular reactivity. In rodents administration of a single dose of LPS (a component of the gram negative bacterial cell wall) produces a shock-like state. Nitrite and nitrate in plasma is derived, in part, from the oxidation of NO and increases in the level of plasma nitrite/nitrate have been used as an index for the overproduction of NO associated with shock. Animal studies suggest that the increased generation of NO following LPS and/or cytokine treatment results from the *de novo* expression of an inducible, Ca^{2+}-independent NO synthase (iNOS). Conventionally, this isoform is considered to generate large quantities of NO over long periods in contrast to eNOS which generates NO as a continuous background that can be increased on demand. The mechanism(s) involved in the generation of NO in human sepsis is less clear. Induction of iNOS can be demonstrated in human cells (Ogura and Esumi, 1996) and tissues (Lafond-Walker *et al.*, 1997) and plasma nitrite/nitrate is elevated in septic patients (Evans *et al.*, 1993) and those with other inflammatory/infectious conditions (tuberculosis). However, sepsis in humans results in more modest rises in nitrite/nitrate compared to that seen in rodents and in many human cell types induction of iNOS has not been achieved even with multiple cytokines.

Recent evidence suggests that increased NO generation by human blood vessels or cells following exposure to cytokines may occur in the absence of iNOS expression (Bhagat *et al.*, 1999; Rosenkranz-Weiss *et al.*, 1994). Several additional studies have now also demonstrated an increase in NO production following inflammation in the absence of iNOS expression (Bhagat *et al.*, 1999; Cepinskas *et al.*, 1999; Weist *et al.*, 1999). An alternative route to increases in NO could involve activation of eNOS, by greater provision of the essential NOS cofactor, BH_4, levels of which are governed by the activity of the enzyme GTPCH-1 (rate-limiting enzyme responsible for the biosynthesis of BH_4) and whose expression is increased in inflammation. The expression of GTPCH-I mRNA is upregulated by LPS and certain inflammatory

cytokines (Hattori *et al.*, 1997b; Hussain *et al.*, 1997; Katusic *et al.*, 1998). Co-induction of GTPCH-I and NO synthesis has also been demonstrated in vascular cells and tissues (Hattori *et al.*, 1997a; Togari *et al.*, 1998). Evidence also suggests that NO generation from eNOS increases in response to increased supply of BH_4 (Kukor & Toth, 1994). Moreover, there is accumulating evidence that BH_4 acts as a molecular switch between $O_2^{\cdot-}$ and NO production by NOS, is involved in demerization/stabilization and may play a role in catalysis (Reif *et al.*, 1999; Riethmuller *et al.*, 1999; Vasquez-Vivar *et al.*, 1999). Several studies have provided evidence for a regulatory function of BH_4 in NO production in various tissues. (Hattori *et al.*, 1997a; Cepinskas *et al.*, 1999; Weist *et al.*, 1999). In chapter 5 it was demonstrated that increased NO production to LPS occurred in the absence of iNOS.

The aim of our study was to confirm whether inflammmatory stimuli could increase NO production in the absence of iNOS and test whether GTPCH-I is upregulated. To achieve these aims, experiments were conducted in mice in which the gene for iNOS had been deleted by gene targeting.

6.2. Methods

6.2.1. Induction of endotoxin shock in mice

Experiments were conducted in male C57BL/6 wild-type (+/+) and homozygous (-/-) iNOS-deficient mice, purchased from The Jackson Laboratory, USA (from a different batch to those used in chapter 5). Mice were injected with saline (3 ml/kg) or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.) as described previously in section 2.2.2. Twelve hours later blood samples were collected and plasma prepared as described in chapter 2, section 2.2.2. In some experiments, DAHP (a selective GTPCH-I inhibitor) was administered orally as described in chapter 2, section 2.2.3.

6.2.2. Measurement of plasma nitrite/nitrate

Blood samples obtained by cardiac puncture were assayed for plasma nitrite/nitrate as described in chapter 2, section 2.3.2.

6.2.3. iNOS, eNOS & GTPCH-I detection by Western blotting

Collected heart and liver tissues from treated wild-type and iNOS mutant mice were homogenised as described in chapter 2, section 2.3.11. Homogenates from tissue samples were assayed for protein content using a Coomassie blue protein reagent as described in chapter 2, section 2.3.8). Equivalent amounts of each sample (25 μg protein) were loaded onto a SDS-PAGE gel, and probed for iNOS, eNOS or GTPCH-I using a polyclonal rabbit anti-iNOS, eNOS or GTPCH-I antibody, respectively, as described in chapter 2, section 2.3.11.

6.2.4. Production of cDNA probe for GTPCH-I

The cDNA probe for GTPCH-I was prepared according to the methodology in chapter 2, section 2.4.2.

6.2.5. Expression of mRNA for GTPCH-I, β-actin and HPRT by Northern blotting

Poly(A)$^+$ mRNA was isolated from mouse liver and heart using a Micro FastTrack mRNA isolation kit according to the manufacturer's instructions, as described in chapter 2, section 2.4.1. The mRNA samples (3 μg quantified by u.v.

spectrophotometry) were separated by electophoresis on a 1 % agarose/formaldehyde gel and probed for GTPCH-I, β-actin or HPRT by northern blotting, as described in chapter 2, section 2.4.3.

6.2.6. GTPCH-I activity assay

Enzymatic activity in hearts isolated from treated wild-type and iNOS mutant mice were determined according to the methods of Ziegler (1985), as described in chapter 2, section 2.3.13.

6.2.7. HPLC determination of tissue biopterin

Total tissue biopterin levels in hearts isolated from treated wild-type and iNOS mutant mice were assessed according to the methods of Ziegler (1985), as described in chapter 2, section 2.3.14.

6.2.8. HPLC determination of plasma biopterin

Plasma total biopterins from control and LPS-treated wild-type and iNOS mutant mice were determined by HPLC according to the methods of Fukushima & Nixon (1980), as described in chapter 2, section 2.3.15 (Fig. 6.7).

6.3. Results

6.3.1. Effects of LPS on plasma nitrite/nitrate

Basal levels of nitrite/nitrate in the plasma of wild-type and iNOS mutant mice were 74 ± 17 and 24 ± 3 μM ($P<0.01$; n=8), respectively. Administration of LPS (12.5 mg/kg; i.v; bolus dose) significantly ($P<0.0001$) increased plasma nitrite/nitrate by 14-fold in the wild-type and 2-fold in the iNOS mutant mice at 12 h (Fig. 6.1).

6.3.2. Effects of LPS on iNOS, eNOS & GTPCH-I protein expression

Wild-type mice were treated with LPS (12.5 mg/kg) and 12 h later hearts removed and protein extracted as described in methods section 2.3.11. LPS treatment resulted in the appearance of a 130 kDa immunoreactive band in the heart tissue which was not present in control animals (n=3). This corresponded to the molecular weight of iNOS (Fig. 6.2) which was detected with a specific iNOS antibody. In iNOS mutant mice, 130 kDa protein immunoreactivity was not observed in control or LPS treated mouse hearts.

In other experiments, samples isolated from mouse heart were blotted against a specific eNOS antibody. This resulted in the appearance of a 140 kDa band which corresponds to the molecular weight of eNOS. Expression of the protein was unaltered between wild-type and iNOS mutant control or LPS treated mice (Fig. 6.3; n=3).

Using a selective antibody for GTPCH-I experiments were performed to determine the expression of this protein. No specific immunoreactivity was detected in liver and heart (Fig. 6.4).

6.3.3. Detection of GTPCH-I mRNA expression

Since the antibody for GTPCH-I did not detect specific GTPCH-I protein, further experiments were conducted to examine the presence of GTPCH-I mRNA in liver and heart tissues isolated from wild-type and iNOS mutant mice treated with saline or LPS. As shown in figures 6.5 and 6.7, two transcripts of 1.4 and 3.6 kb specific to GTPCH-I mRNA were detected in liver and heart, respectively. Following LPS treatment the level of the 1.4 kb fragment was constant, however there was a reduction

in the expression of 3.6 kb fragment in liver. In contrast, in the heart the level of expression of the two transcripts remained constant following LPS treatment in both wild-type and iNOS mutant mice.

6.3.4. Detection of β-actin and HPRT mRNA expression

As shown in figure 6.6, a 2 kb band corresponding to the molecular weight of β-actin mRNA was detected in liver tissue isolated from wild-type and iNOS mutant mice. Following LPS treatment, there was a significant ($P<0.0005$) 3.7 and 3.1-fold increase in the level of expression of this gene in wild-type and iNOS mutant mice treated with LPS, respectively. As β-actin could not be used as an internal control to account for mRNA lane loading, due to its regulation by endotoxin, an attempt was made to use an alternative gene. A 1.2 kb band corresponding to the molecular weight of HPRT was detected in liver and heart tissue isolated from wild-type, which was not altered by LPS. Therefore, HPRT was used as control for mRNA lane loading from blots probed for GTPCH-I.

6.3.5. Measurement of tissue GTPCH-I activity and biopterin levels

As shown in Table 6.2, GTPCH-I activity was detected in heart from wild-type and iNOS mutant mice. There was a significant ($P<0.0003$; n=3) 2 and 3-fold increase in GTPCH-I activity following LPS treatment in wild-type and iNOS mutant mice, respectively. Basal levels of total biopterins in hearts from control wild-type and iNOS mutant mice were 4.3 ± 0.2 and 4.0 ± 0.4 pmol/mg protein ($P>0.05$; n=3). After endotoxaemia, there was a significant ($P<0.0003$) 4 and 3-fold increase of total biopterin levels in wild-type and iNOS mutant mice, respectively. Basal levels of total biopterins in plasma from control wild-type and iNOS mutant mice were 120 ± 8 and 106 ± 7 pmol/mg protein. After endotoxaemia, there was a significant ($P<0.0001$) 3-fold increase of total biopterin levels, in both wild-type and iNOS mutant mice, respectively.

6.3.6. Measurement of plasma biopterin levels

Figure 6.8 shows a representative HPLC chromatogram of plasma from a saline-treated wild-type mouse. A well-defined peak at the retention time of biopterin can be seen in a standard aqueous mixture of neopterin and biopterin.

6.3.7. Effect of DAHP on plasma nitrite/nitrate levels

As shown in Table 6.1, pre-treatment of wild-type or iNOS mutant mice with DAHP (1 g/kg/day; p.o.) for 2 days prior to LPS administration did not affect the rise in plasma nitrite/nitrate levels. Mice treated with DAHP alone showed no significant (P>0.05) change in nitrite/nitrate levels.

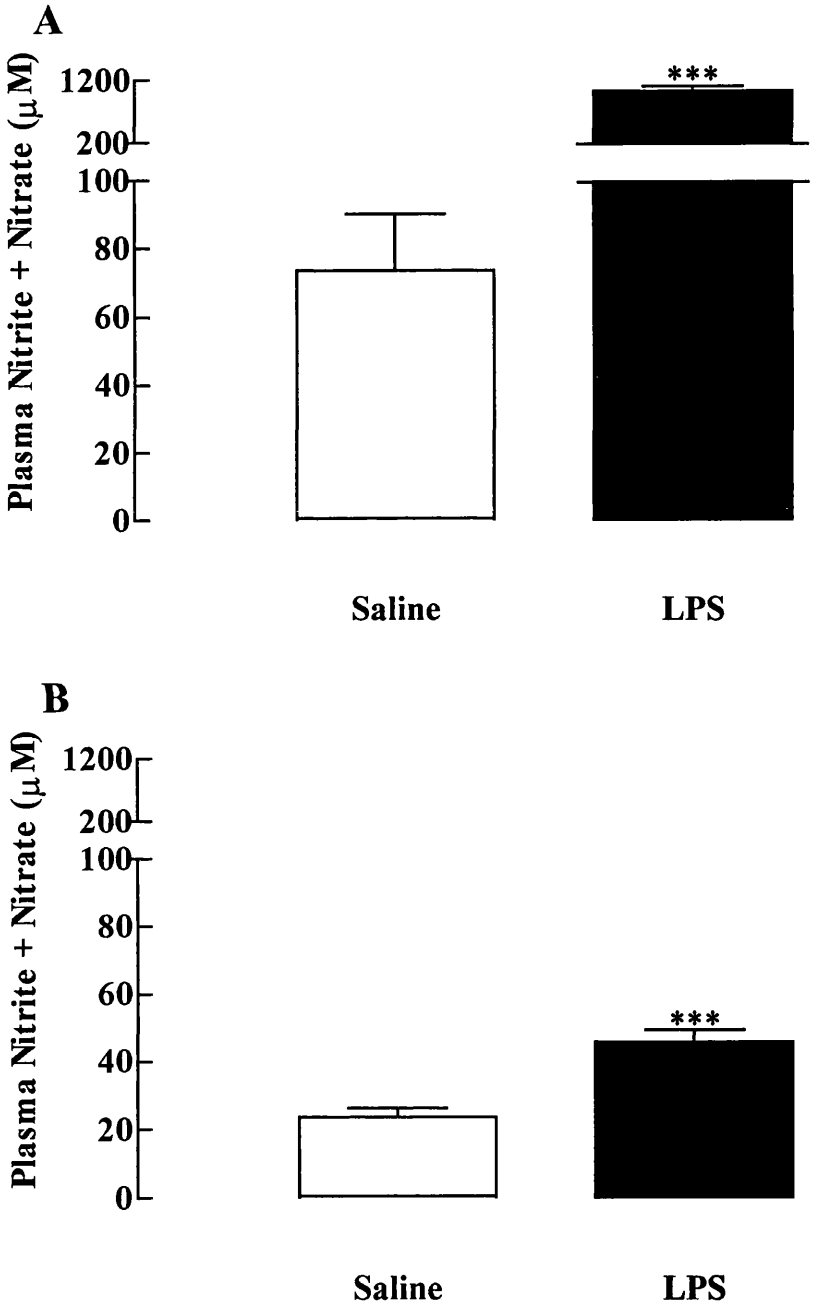


Figure 6.1. The effects of LPS on plasma nitrite + nitrate. Effect of endotoxin (*E. coli*, 026:B6, 12.5 mg kg^{-1}, i.v.) on concentrations of plasma nitrite/nitrate in wild-type mice (Panel A) and mice lacking the inducible NO synthase (Panel B). Results are mean ± s.e.m of data obtained in 8 experiments. $^{***}P<0.0001$, saline vs LPS.

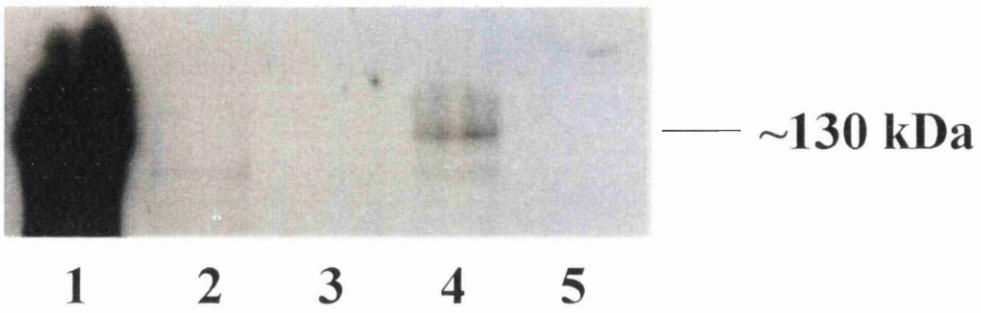


Figure 6.2. Expression of iNOS in mouse heart. Wild-type (W/T) or iNOS mutant (K/O) mice were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). After 12 h tissues were harvested, homogenised and proteins separated on a 10 % denaturing acrylamide gel and probed for iNOS. 1, LPS-treated mouse macrophage lysate (positive control); 2, W/T saline, 3, K/O saline, 4, W/T LPS, 5, K/O LPS. Results are representative of those obtained in 3 separate experiments.

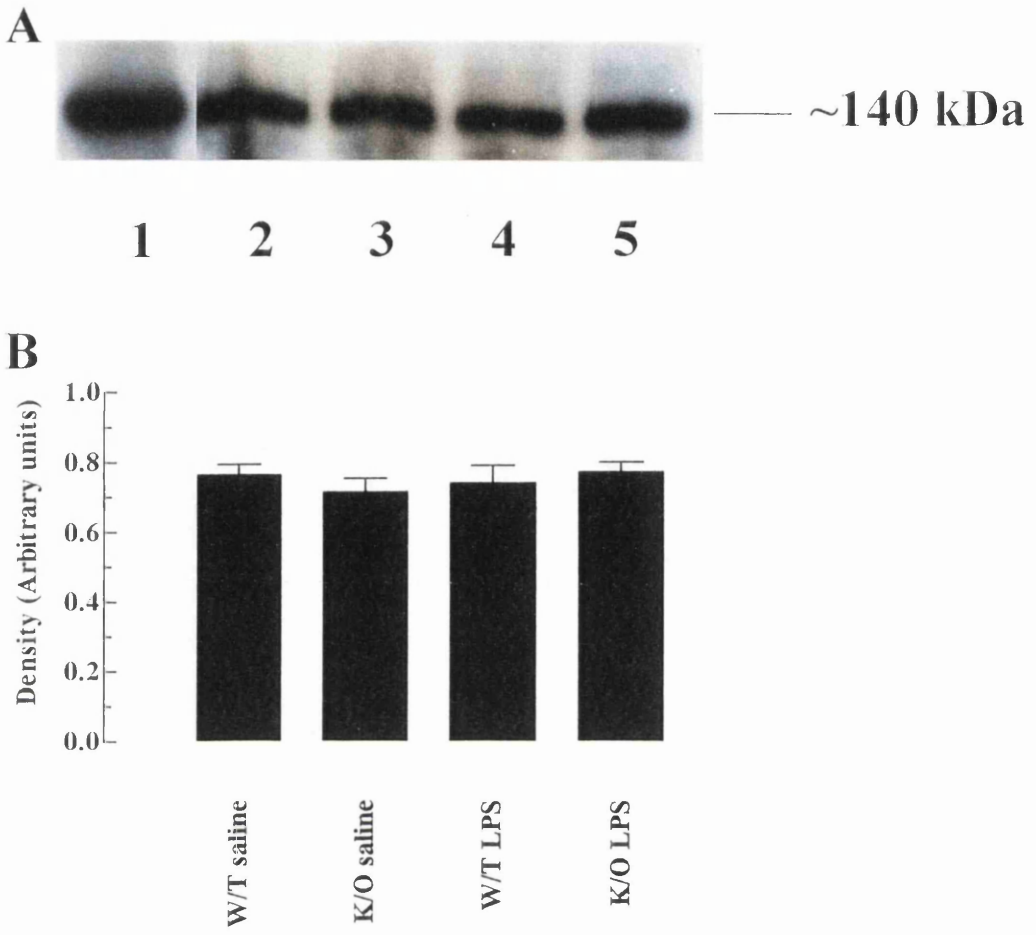


Figure 6.3. Expression of eNOS in mouse heart. Wild-type (W/T) or iNOS mutant (K/O) mice were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). After 12 h tissues were harvested, homogenised and proteins separated on a 10 % denaturing acrylamide gel and probed for eNOS. Panel (A); 1, human endothelial lysate (positive control); 2, W/T saline, 3, K/O saline, 4, W/T LPS, 5, K/O LPS. Panel B: densitometric analysis of n=3 experiments. This gel is representative of those obtained in 3 separate experiments.

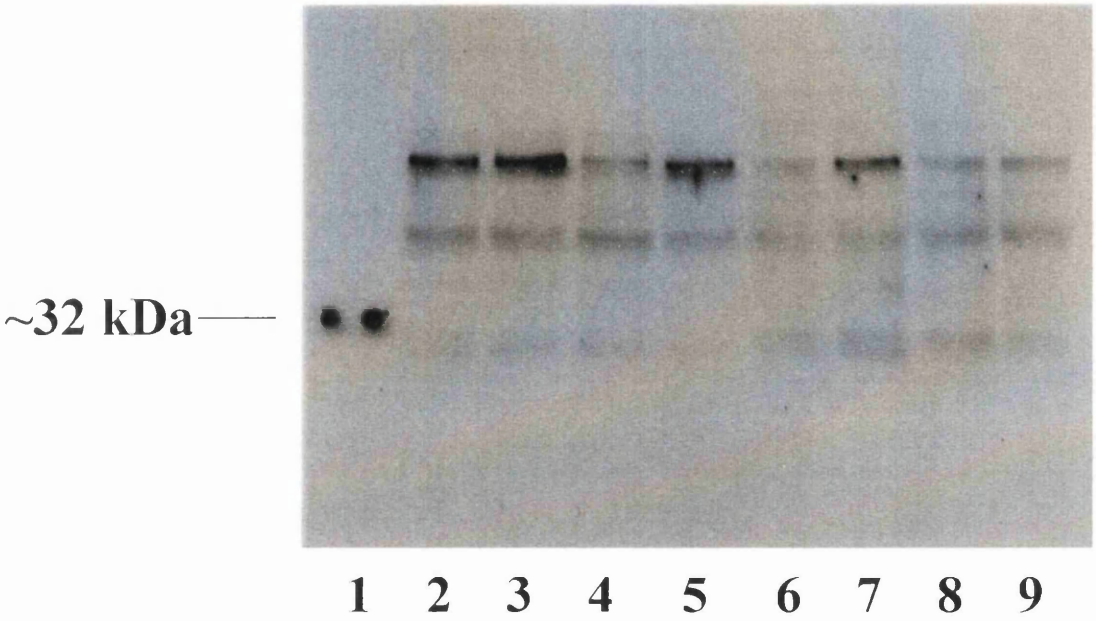


Figure 6.4. Expression of GTPCH-I protein in mouse heart and liver. Wild-type (W/T) or iNOS mutant (K/O) mice were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). After 12 h tissues were harvested, homogenised and proteins (3 μg) separated on a 10 % denaturing acrylamide gel and probed for GTPCH-I. 2-5 heart and 6-9 liver; 1, recombinant murine GTPCH-I (positive control); 2,6 W/T saline, 3,7 W/T LPS, 4,8 K/O saline, 5,9 K/O LPS. Results are representative of those obtained in 3 separate experiments.

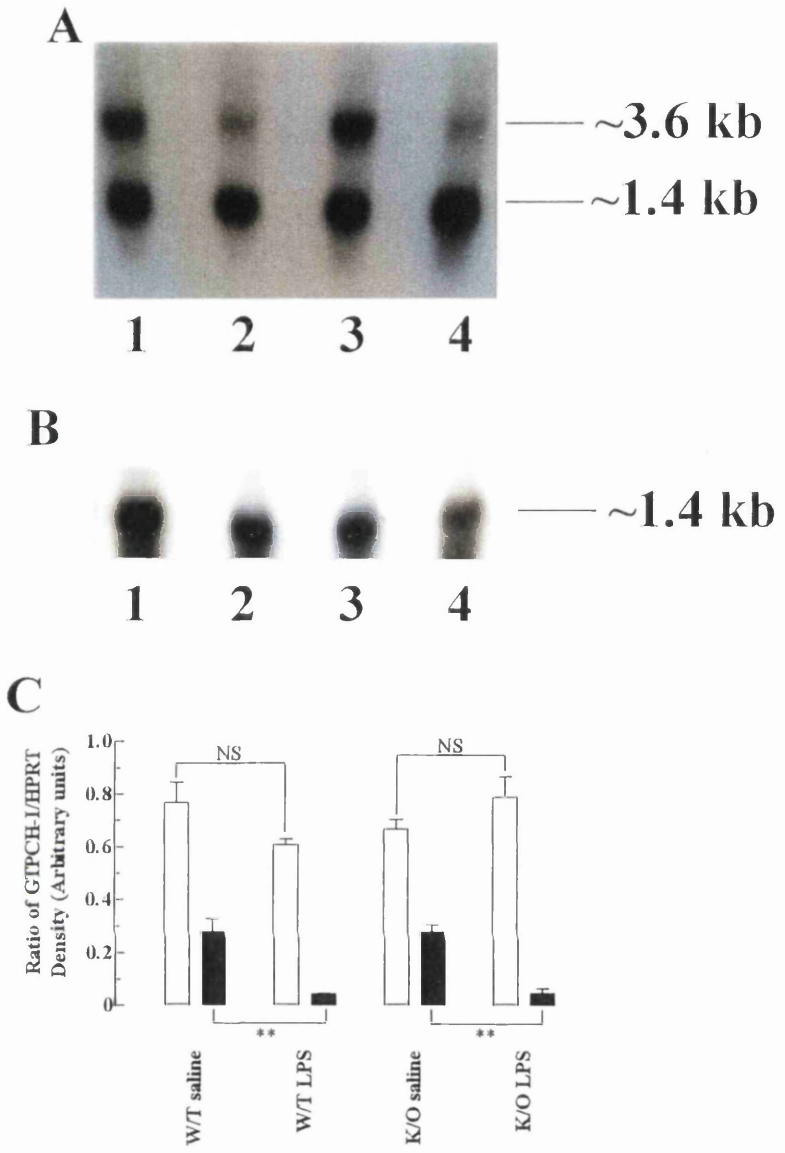


Figure 6.5. Expression of GTPCH-I and HPRT mRNA in mouse liver. Wild-type (W/T) or iNOS mutant (K/O) mice were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). After 12 h tissues were harvested, and poly(A)$^+$-selected mRNA was isolated from mouse liver and electophoresed on a 1 % agarose/formaldehyde gel and probed for GTPCH-I. Panel (A); 1, W/T saline, 2, W/T LPS, 3, K/O saline, 4, K/O LPS. Panel (B), expression of HPRT mRNA. Panel (C): densitometric analysis of n=3 experiments expressed as the ratio of the density of GTPCH-I/HPRT bands for liver; 1.4 kb (open bars) and 3.6 kb (closed bars) GTPCH-I mRNA. This gel is representative of those obtained in 3 separate experiments. $^{**}P<0.005$, NS denotes no significance.

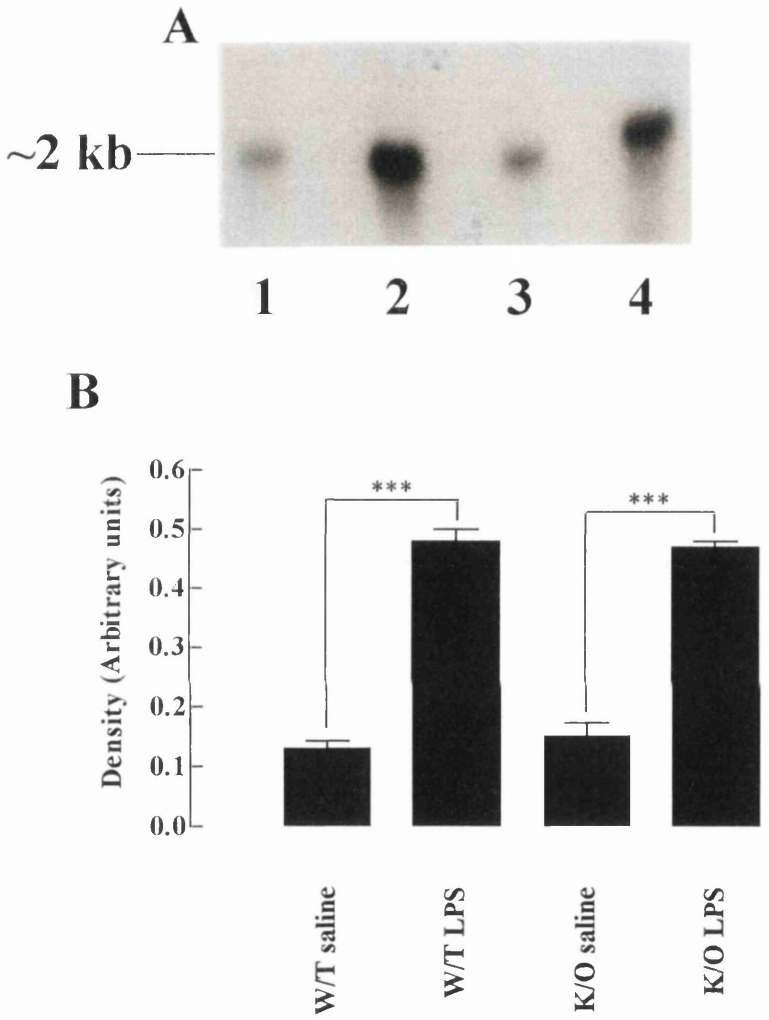


Figure 6.6. Expression of β-actin mRNA in mouse liver. Wild-type (W/T) or iNOS mutant (K/O) mice were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). After 12 h tissues were harvested, and poly(A)$^+$-selected mRNA was isolated from mouse liver and electophoresed on a 1 % agarose/formaldehyde gel and probed for β-actin. Panel (A); 1, W/T saline, 2, W/T LPS, 3, K/O saline, 4, K/O LPS. Panel B: densitometric analysis of n=3 experiments. This gel is representative of those obtained in 3 separate experiments. $^{***}P<0.0005$.

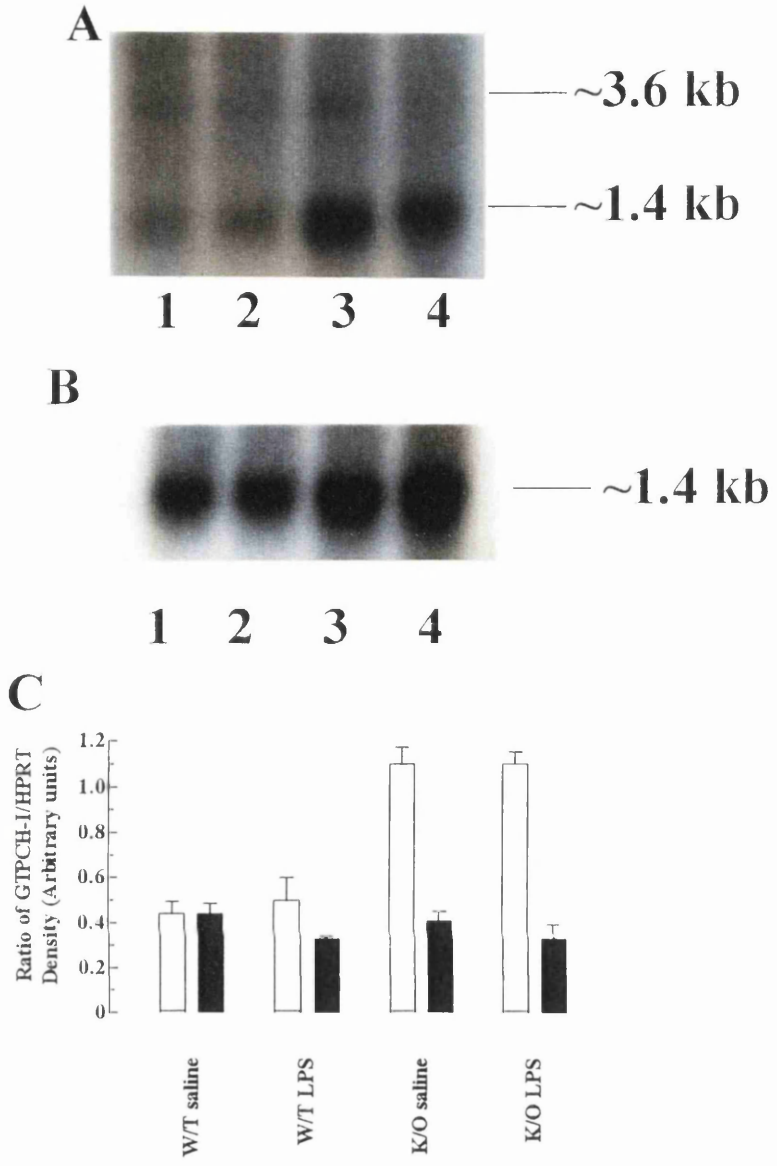


Figure 6.7. Expression of GTPCH-I and HPRT mRNA in mouse heart. Wild-type (W/T) or iNOS mutant (K/O) mice were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). After 12 h tissues were harvested, and poly(A)$^+$-selected mRNA was isolated from mouse liver and electophoresed on a 1 % agarose/formaldehyde gel and probed for GTPCH-I. Panel (A); 1, W/T saline, 2, W/T LPS, 3, K/O saline, 4, K/O LPS. Density of 1.4 kb (open bars) and 3.6 kb (closed bars) bands for heart. Panel (B), expression of HPRT mRNA. Panel (C): densitometric analysis of n=3 experiments expressed as the ratio of the density of GTPCH-I/HPRT bands for liver; 1.4 kb (open bars) and 3.6 kb (closed bars) GTPCH-I mRNA. This gel is representative of those obtained in 3 separate experiments.

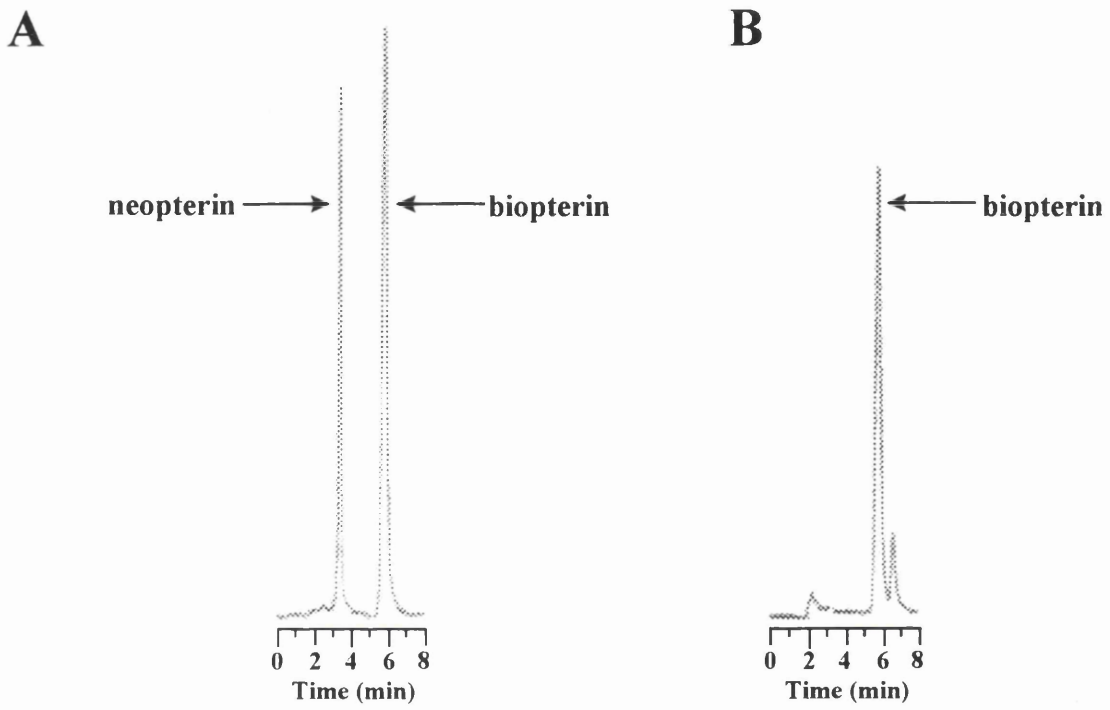


Figure 6.8. Representative HPLC chromatogram of biopterin. After deproteination of plasma from saline-treated wild-type mouse with 60 % perchloric acid and oxidised with 3 % iodine, 6 % potassium iodide solution for 1 h in the dark at room temperature. Oxidation was terminated by addition of excess ascorbate, and total biopterins were measured. HPLC separation of (A) standard aqueous solution of neopterin and biopterin (retention times of 3.36 and 5.82 min, respectively) and (B) plasma sample from saline-treated wild-type mouse. Spike on trace B represents biopterin running at a retention time identical to that seen in the standard solution.

TABLE 6.1. EFFECTS OF DAHP ON PLASMA NITRITE + NITRATE LEVELS

Treatment	Nitrite + Nitrate (μM)	
	Wild-Type	**iNOS mutant**
Control	35 ± 11	22 ± 3
LPS	$976 \pm 58^{***}$	$46 \pm 3^{**}$
LPS + DAHP	934 ± 59^{NS}	40 ± 3^{NS}
LPS + CMC	986 ± 59^{NS}	40 ± 4^{NS}
DAHP	$34 \pm 5^{NS*}$	$19 \pm 2^{NS*}$

Wild-type or iNOS mutant mice were treated with DAHP 1 g/kg for 48 h with doses administered 2 times per day. At 48 h saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.) was administered. Twelve hours later blood was collected from mice and plasma nitrite/nitrate levels were determined as described in chapter 2, section 2.3.2. CMC (carboxymethylcellulose) is the vehicle control for DAHP. Results are mean ± s.e.m of data obtained in 4 experiment. $^{**}P<0.0001$ and $^{***}P<0.005$, control vs LPS; NS denotes no significant difference between treatment group vs LPS alone; NS* denotes no significance difference between treatment group vs control.

TABLE 6.2. LEVELS OF GTPCH-I ACTIVITY AND TOTAL BIOPTERIN

	Wild-Type		iNOS mutant	
	Control	***LPS***	***Control***	***LPS***
GTPCH-I activity (nmol/g/min)	13 ± 1	28 ± 1***	12 ± 2	29 ± 1**
Total tissue biopterin (pmol/mg)	4 ± 0.2	15 ± 2**	4 ± 0.4	11 ± 0.4***
Total plasma biopterin (nmol/L)	120 ± 8	419 ± 48**	106 ± 7	320 ± 19***

Wild-type or iNOS mutant mice were treated with saline or LPS (*E. coli*, 026:B6, 12.5 mg/kg, bolus dose; i.v.). 12 h later heart and blood were isolated from mice. Measurements of GTPCH-I activity in hearts and total biopterin in heart and plasma were performed, as described in chapter 2, sections 2.3.13-2.3.15. Results are mean ± s.e.m of data obtained in 3 experiments. **$P<0.005$, ***$P<0.0005$ control vs LPS.

6.4. Discussion

Evidence over recent years has demonstrated that both naturally-occurring and experimental inflammatory stimuli, e.g. LPS exposure, lead to an overproduction of NO commonly associated with septic shock (Vallance *et al.*, 1989; Evans *et al.*, 1993; Buttery *et al.*, 1994). The classical paradigm for these observations is that the marked increase in NO production is the result of an elevation of the *de novo* expression of iNOS. Recent reports have challenged the assertion that increases in the production of NO as a result of inflammatory stimuli, are always due to an induction of iNOS (Bhagat *et al.*, 1999), suggesting that the induction of GTPCH-I may in part be responsible for the increased production of NO mediated by elevated BH_4 availability to eNOS resulting in greater enzymatic activity.

To test directly whether an increase in NO production can be achieved in the absence of iNOS, experiments were performed in mice in which the gene for iNOS had been deleted. Initial experiments demonstrated a significant increase in the production of NO following exposure of wild-type and iNOS mutant mice to LPS, consistent with previous observations from chapter 5. This finding was accompanied by observations of the absence of iNOS protein and an insignificant change in the level of eNOS protein expression in LPS-treated, confirmed by western blotting, suggesting that the marked increase in the production of NO in LPS-treated iNOS mutant mice could in-part be accounted for by an increased activity of eNOS.

To further confirm the presence of a functional pterin pathway in LPS-treated wild-type and iNOS mutant mice, the activity of GTPCH-I was assessed. In agreement with plasma nitrite/nitrate measurements, a significant 2-fold increase in GTPCH-I activity was evident. This finding also mirrored an increase in tissue and plasma biopterin (product of GTPCH-I activity) levels, consistent with the idea that BH_4 might stimulate eNOS. Whilst activity of GTPCH-I rose, the functional significance of this is less clear. The K_m of eNOS for BH_4 is 100 nM (Knowles & Moncada, 1994; Pollock *et al.*, 1991) which is above the plasma concentration of approximately 7 nM (Leeming *et al.*, 1976). Following LPS exposure, BH_4 levels increase and this might be expected to result in greater enzymatic activity. Measurements of end-product (nitrite + nitrate) suggests that this occurs *in vivo*. Furthermore, evidence suggests that under conditions when BH_4

concentrations are limiting, the K_m of NOS for arginine increases (Brand *et al.*, 1995). The mechanism for this effect is not known, but BH_4 is reported to be an allosteric modifier of phenylalanine hydroxylase (Doskeland *et al.*, 1987) and has been reported to increase the affinity of purified nNOS for the inhibitor, *N*-nitroarginine (Klatt *et al.*, 1994a).

To test the hypothesis that induction of GTPCH-I might contribute to the LPS-induced NO generation, western blotting of GTPCH-I was undertaken. Immunoreactivity to GTPCH-I was not evident in liver or heart tissue, suggesting that either GTPCH-I was not expressed in the tissue samples or at least expressed at levels below the limit of detection for immunoblotting. However, GTPCH-I protein must have been present, as activity and end-product of this enzyme was present. Recent evidence demonstrating the expression of GTPCH-I mRNA in murine liver (Gütlich *et al.*, 1994), rat aortic smooth muscle cells (Hattori & Gross, 1993) and protein in murine liver (Cha *et al.*, 1991) suggests that a likely explanation for the failure to detect this protein in liver and heart could be due to the level of expression of GTPCH-I protein, although low levels of GTPCH-I in heart and liver, have been previously detected by western blotting (Milstein *et al.*, 1996) with a different antibody.

Further experiments were conducted to examine whether mRNA for GTPCH-I could be detected. Northern blotting showed the presence of two transcripts corresponding to 1.4 and 3.6 kb, consistent with previous reports in murine liver (Gütlich *et al.*, 1994) and rat liver, kidney, bone marrow, spleen and brain (Gütlich *et al.*, 1992). Both transcripts were detected in mouse liver and heart, although higher levels were detected in the liver compared with heart. The functional importance of the two mRNA transcripts are not clear. There was a significant decrease in the expression of a 3.6 kb GTPCH-I mRNA in liver tissue isolated from wild-type and iNOS mutant mice treated with LPS. The significance of this is not clear. This effect may represent a dominant negative effect of the protein encoded that might inhibit GTPCH-I activity, but further studies would be required to test this directly. However, molecular studies were not consistent with transcriptional up-regulation of GTPCH-I expression, suggesting that alternative mechanisms such as post-translational modification of the enzyme may contribute to the increased activity seen following LPS treatment.

An attempt to standardise mRNA loading by use of a murine β-actin probe proved was unsuccessful, as it appears that the expression of this gene is upregulated by endotoxin. Moreover, the use of an alternative gene such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was not possible, as evidence suggests that this gene is also regulated by NO (Nomura *et al.*, 1996; Galli *et al.*, 1998). However, use of murine hypoxanthine phosphoribosyl-transferase (HPRT) as a control for mRNA loading proved successful as this gene appeared not to be regulated by endotoxin.

To test the functional significance of the increased GTPCH-I activity, experiments were designed to inhibit the induction of GTPCH-I by use of DAHP, an inhibitor of this enzyme (Kobayashi *et al.*, 1991; Bune *et al.*, 1996). Contrary to recent findings demonstrating inhibition of LPS-induced increased tissue BH_4 levels and NO synthesis in response to DAHP administration *in vivo* (Bune *et al.*, 1996), results of studies from this chapter demonstrate no significant change in the levels of plasma nitrite/nitrate compared to mice treated with LPS alone. One possibility for these observations are that DAHP does not cause substantial inhibition of GTPCH-I *in vivo*. However, there were problems encountered with the use of DAHP. This inhibitor was insoluble and was given to animals as a slurry in cellulose. The levels of GTPCH-I activity in tissue were not directly measured from DAHP-treated animals to determine whether the levels of the inhibitor were sub-therapeutic. Interestingly, recent evidence demonstrating inhibition of GTPCH-I via GTPCH-I feedback regulatory protein (GFRP) in tissues expressing high levels of GFRP (Xie *et al.*, 1998) may in part account for the failure to inhibit GTPCH-I, probably due to a low level of expression of GFRP in the tissues analysed. In addition the selectivity of DAHP as a GTPCH-I inhibitor has recently been questioned. It has been demonstrated in interferon-γ-activated murine peritoneal macrophages, that DAHP suppresses the expression of iNOS mRNA and protein in a BH_4-independent manner and, thus, acts by a novel mechanism (Bogdan *et al.*, 1995).

In summary, results of this chapter demonstrate an elevation in NO production and tetrahydrobiopterin in after LPS-treatment. Further studies highlighting the GTPCH-I dependent mechanism responsible for the elevation in NO production in the absence of iNOS could involve the use of (i) a selective inhibitor for the enzyme; (ii) detection of the protein by immunoblotting using a specific primary antibody; (iii) use of

mice deficient in the gene for and iNOS and GTPCH-I, and (iv) the development and use of a GTPCH-I anti-sense.

CHAPTER 7

GENERAL DISCUSSION

7.1. Introduction

During episodes of sepsis and acute inflammation, profound physiological changes are observed, including systemic vasodilation and activation of the immune system. These changes are brought about by many intra and intercellular mediators, including endotoxin, various cytokines, adhesion molecules and eicosanoids. Since the first reports of NO as a product of mammalian cells, it has been shown that NO plays an important role as one of these mediators of sepsis. In view of the importance of the role of NO in the pathogenesis of inflammatory conditions, I have attempted to further explore the understanding of the regulation of NOS and determine the contribution of this free radical in the inflammatory response.

7.2. Role of calmodulin in the regulation of inducible nitric oxide synthase

Expression of iNOS is likely to be of importance in the generation of NO that occurs during inflammation. Calmodulin binding to iNOS is an essential step leading to the expression of an active enzyme and hence NO production and agents that interfere with this process might lead to the expression of a functionally inactive iNOS which could then be activated by calcium/calmodulin in a manner similar to eNOS and nNOS. Previous work has demonstrated that anti-fungal imidazoles inhibit the induction, but not the activity of iNOS in the J774 murine macrophage cell line (Bogle *et al.*, 1994) and that anti-fungal imidazoles inhibit nNOS activity by reducing the responsiveness of the enzyme to calmodulin (Wolff *et al.*, 1993), suggesting that calmodulin antagonists might be important in modifying the iNOS response. To develop this idea, focusing in particular on the regulation of NOS induction in a model of iNOS expression in J774 macrophages. Experiments were designed to examine the effects of calmodulin antagonists on the induction of iNOS. A range of structurally unrelated compounds were used in an established model of iNOS induction, namely LPS-stimulated J774 cells.

The important findings from these studies were that calmodulin antagonists inhibited NO production in J774 cells, with a rank order of potency that correlated closely with reported inhibitory potency of these agents on isolated calcium/calmodulin kinase II. Of all the antagonists, ophiobolin A was the most potent inhibitor of iNOS induction, consistent with previous findings. To dissect out whether calmodulin

antagonists inhibited induction or activity, experiments were performed with calmodulin antagonists added to cells pre-stimulated with LPS that already had iNOS induced. Once the iNOS was expressed, the calmodulin antagonists were less effective although the difference were quite small for most compounds. The direct effect of the antagonists on iNOS examined by measuring iNOS activity *in vitro*, showed that none of the agents inhibited the activity of the enzyme. The inhibition of NO production by the calmodulin antagonists was found to correlate closely with the effects of the compounds on cell viability suggesting that some of the iNOS induction observed was due to cell death. In summary the data suggested that the selected antagonists appear to inhibit the induction of NOS. With the exception of ophiobolin A, inhibitory effects do not appear to be significantly selective or non-toxic, making calmodulin antagonists difficult tools for the study of regulation of NOS induction *in vitro*. One useful compound however was ophiobolin A, which might be used as a tool for future studies on the regulation of NOS induction. It is unclear whether the effects of the antagonists on cell viability were due to calmodulin binding or other non-specific actions, as this calcium binding protein is a component of a number of calcium-dependent enzymes and may mediate the effects of calcium on a wide variety of cellular functions such as microtubule assembly (Marcum *et al.*, 1978). Moreover, it has been demonstrated that trifluoperazine inhibits growth of J774 cells in culture (Horwitz *et al.*, 1981).

7.3. Interactions between superoxide anion and nitric oxide

It is believed that within the blood vessel wall $O_2^{\cdot-}$ is generated in amounts similar to that of NO. In contrast to the well characterised biology of NO in vessels, the biosynthetic pathway and interaction of $O_2^{\cdot-}$ with NO remains unclear. Blood vessels generate $O_2^{\cdot-}$ which under certain conditions can react with NO to form $ONOO^-$ which decomposes to form a nitrating species. Moreover, the formation of $ONOO^-$ has been proposed in a number of pathophysiological conditions that are associated with overproduction of NO and/or $O_2^{\cdot-}$. Based on this hypothesis, the aims of these experiments were firstly, to measure $O_2^{\cdot-}$ production from blood vessels *in vitro* and to identify the possible enzymatic source of this radical species and secondly, to test whether an increase in NO generation from an NO donor would be sufficient to

combine with endogenous $O_2^{\cdot-}$ to form $ONOO^-$. Using an isolated preparation of rabbit aortic rings these studies showed that endogenous generation of $O_2^{\cdot-}$ was present upon inhibition of intrinsic SOD activity with DDC. No $O_2^{\cdot-}$ was detected in the absence of DDC, suggesting that metabolism of $O_2^{\cdot-}$ by SOD is an important regulator of basal $O_2^{\cdot-}$ levels. The production of this free radical was further increased in the presence of NADH or NADPH, potential substrates for a putative $O_2^{\cdot-}$ generating system commonly termed as the NAD(P)H oxidase. When added to this system, the NO donors GSNO and SNP quenched $O_2^{\cdot-}$ chemiluminescence from aortic rings. These findings were confirmed in an artificial cell-free generating system (xanthine/xanthine oxidase). Determination of the relationship between NO release and quenching of $O_2^{\cdot-}$ found that the quantity of NO released from GSNO in this acellular system was proportional to the quenching of $O_2^{\cdot-}$ chemiluminescence. To assess $ONOO^-$ formation, evidence of protein tyrosine nitration was explored using a specific antibody against nitrotyrosine. Basally and in the presence of NADH, a single band was detected. Incubation of aortic rings with either GSNO alone or GSNO with NADH resulted in the appearance of additional nitrotyrosine bands. To try and understand the delicate balance between NO and $O_2^{\cdot-}$ an *in vitro* system was used, where bovine serum albumin was nitrated in an cell-free preparation. Incubation of serum albumin with GSNO alone did not cause nitrotyrosine formation, confirming that the presence of NO alone is not sufficient to cause nitrotyrosine formation. In contrast, incubation with SIN-1 (a co-generator of NO and $O_2^{\cdot-}$ and therefore $ONOO^-$) resulted in marked nitration of albumin. This was reduced in the presence of oxyhaemoglobin or SOD. Incubation of albumin with GSNO and pyrogallol, also resulted in protein nitration. Addition or exogenous NO results in nitrotyrosine formation in rabbit aortic rings. Nitrotyrosine formation is likely to result from the reaction of exogenous NO and basal endogenous $O_2^{\cdot-}$ resulting in the formation of $ONOO^-$. It is important to realise that the reactions discussed above may be different in an *in vivo* setting, e.g. in a complex milieu of a cell, the reaction of SOD with $ONOO^-$ may become important because the SOD combines rapidly with $ONOO^-$ and directs a more selective nitration of tyrosines on certain proteins (Beckman and Koppenol, 1996). Formation of $ONOO^-$ and nitration of tyrosine residues potentially could lead to vascular damage and might represent unexpected adverse effects of long-term nitrate therapy (Mihm *et al.*, 1999).

Moreover, evidence of nitrotyrosine formation in neuronal tissue from patients with multiple sclerosis, suggests that $ONOO^-$ production is a major consequence of NO generation and implicates a role for this powerful oxidant in the pathogenesis of multiple sclerosis (Cross *et al.*, 1998).

7.4. Regulation of endothelial nitric oxide synthase in septic shock

The production of $ONOO^-$ has been demonstrated in many inflammatory conditions including, atherosclerosis (Beckman *et al.*, 1994), septic shock (Szabo *et al.*, 1995) or adult respiratory distress syndrome (Haddad *et al.*, 1994). However, the role of constitutive NOS in the generation of $ONOO^-$ remains unclear. So far the paradigm for septic shock is that endotoxin leads to the expression of iNOS, and production of NO and $ONOO^-$, evidenced by increased immunoreactivity to nitrotyrosine. However, in the absence of iNOS, the constitutive eNOS might be a system that can be upregulated to produce NO and lead to the chemical interaction between $O_2^{\cdot-}$ and NO leading to $ONOO^-$ formation. Mice in which the gene encoding iNOS had been deleted were used to explore the potential contribution of eNOS.

Induction of endotoxic shock by LPS resulted in increases in plasma nitrite/nitrate levels in wild-type mice. To investigate whether eNOS was involved in the nitrite/nitrate responses associated with endotoxic shock, iNOS mutant mice were treated with endotoxin, resulting in a reproducible increase in plasma nitrite/nitrate. Western blotting showed the presence or absence of the protein in wild-type and iNOS mutant mice, respectively, demonstrating the induction of iNOS in the wild-type animals. Constitutive eNOS protein levels were unchanged in tissue homogenates from LPS-treated iNOS mutant mice. However, in iNOS mutant mice nitrite/nitrate levels were increased, suggesting that this response might have been a result of eNOS activation through induction of co-factors such as GTPCH-I. Alternatively, clearance of nitrite/nitrate might have been modified by sepsis. Experiments were undertaken to examine this by measurement of plasma levels of urea and creatinine. It was found that endotoxin shock led to increased urea and creatinine, indicating renal failure in these animals. This may be an important mechanism for increases in nitrite/nitrate. An attempt to explore the importance of GTPCH-I activity in the regulation of NO production was

made by pharmacological inhibition of the enzyme by use of DAHP. Contrary to findings of inhibition by DAHP of LPS-induced increased tissue BH_4 levels and NO production (Bune *et al.*, 1996), there appeared to be no significant change in the levels of plasma nitrite/nitrate in animals treated with the inhibitor to those administered with LPS, suggesting that GTPCH-I may not have been responsible for the LPS-induced changes in plasma nitrite/nitrate levels or that DAHP was a poor selective inhibitor of the enzyme, in agreement with previous reports (Bogdan *et al.*, 1995; Xie *et al.*, 1998). Evidence for the activation of the pterin pathway was further explored by attempting to detect expression of the gene encoding for GTPCH-I. In agreement with previous reports (Gütlich *et al.*, 1992; Gütlich *et al.*, 1994), two mRNA transcripts for GTPCH-I were detected, confirming the presence of GTPCH-I. However, it appeared that LPS treatment resulted in a decrease in the level of expression of the 3.6 kb transcript, suggesting a dominant negative effect of the protein product of this gene, at least in liver tissue.

In agreement with the findings of expression of GTPCH-I mRNA, a significant increase in GTPCH-I activity was evident in mice treated with LPS, mirrored by an increase in tissue and plasma biopterin levels, further confirming the activation of the pterin pathway, suggesting a role for GTPCH-I in the upregulation of NO production through increased BH_4.

7.5. Overall Conclusion

This thesis explores the regulation of NO production and its possible consequences. The major findings are: (i) iNOS expression can be inhibited by calmodulin antagonists and thus blocks NO generation. Although many of the calmodulin antagonists exhibited cytotoxicity, ophiobolin A was identified as an antagonist with minimal cytotoxicity that potentially inhibited iNOS induction, making it a useful drug for future experimental studies, (ii) increased NO generation in response to LPS can also arise in the absence of iNOS, most likely mediated through eNOS or nNOS activity, (iii) increased BH_4 generation may account for the increased NO production. However, due to the lack of specific inhibitors to GTPCH-I, it was not possible to test this directly. The increase in GTPCH-I activity appears to result from

post-translational modification rather than transcriptional regulation, and (iv) an increase in NO production from any source may cause vascular damage through interaction with $O_2^{\cdot -}$ to form $ONOO^-$. In summary, increased NO leading to $ONOO^-$ formation may arise from NO donors or possibly from eNOS. An understanding of alternative routes of NO generation (other than iNOS) may be important for understanding pathology.

Further research is required to enhance the present understanding of the regulation of NO production *in vivo*, and the interaction between NO and $O_2^{\cdot -}$ and the biological significance of this. An advancement in our knowledge of the regulation of NOS and the pathology associated with an overproduction of NO, may prove extremely important therapeutically, since the L-arginine:NO pathway has, to date, been implicated in the control of many physiological functions, and selective antagonists of this and other interacting systems may prove efficacious medicines, particularly if the differences in the isoforms of NOS can be exploited.

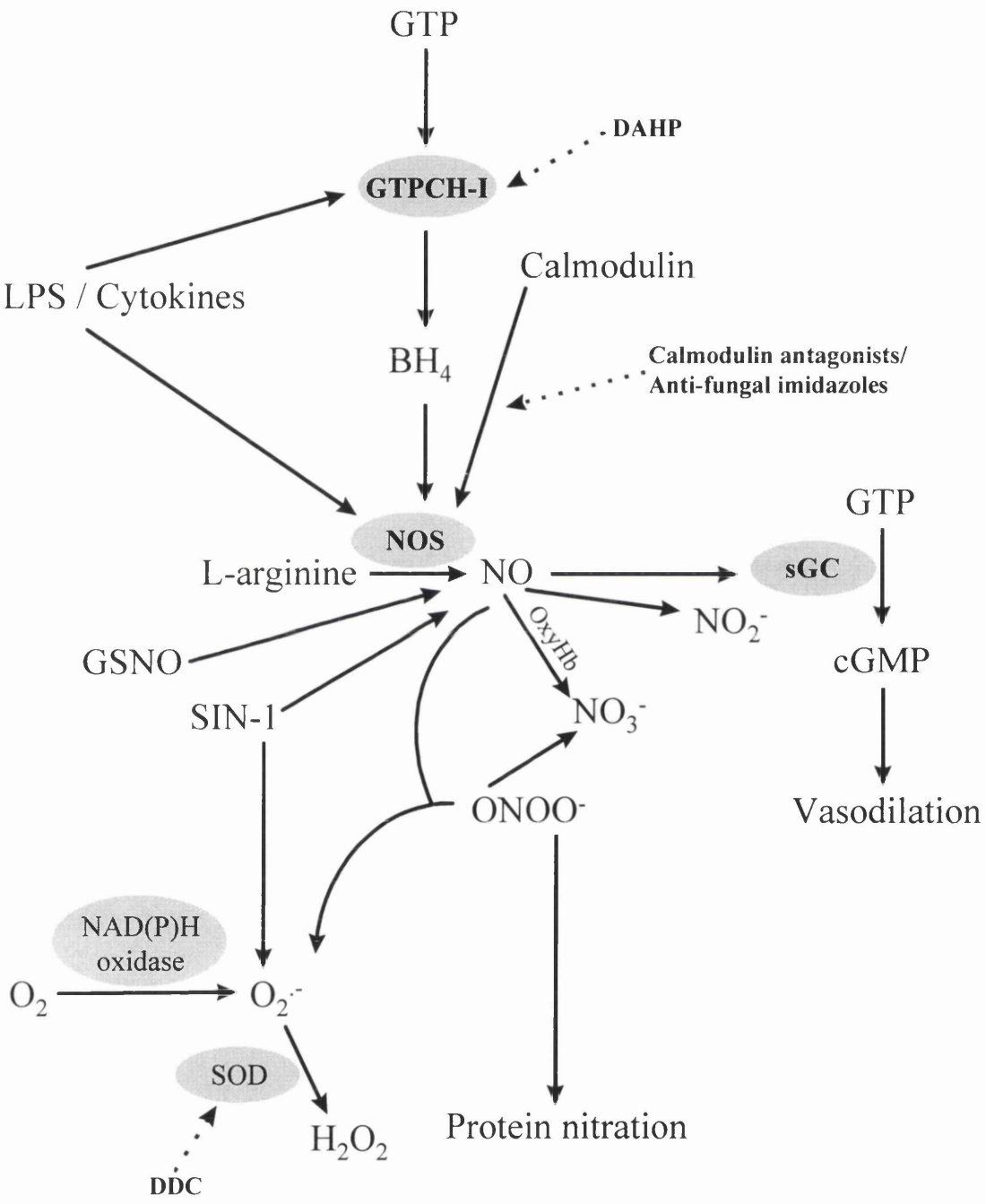


Figure 7.1. Synthesis, interactions and biological consequences of NO.

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APPENDIX

Materials

Agents	*Source*
Acrylamide/bis-acrylammide	Sigma-Aldrich Chem. Co., Dorset, U.K.
Agarose	Sigma-Aldrich Chem. Co., Dorset, U.K.
Aminoguanidine	Sigma-Aldrich Chem. Co., Dorset, U.K.
Ammonium persulphate	Sigma-Aldrich Chem. Co., Dorset, U.K.
Anti-eNOS IgG (rabbit polyclonal)	Transduction Laboratories, KY, U.S.A.
Anti-iNOS IgG (rabbit polyclonal)	Transduction Laboratories, KY, U.S.A.
Antimycin A	Sigma-Aldrich Chem. Co., Dorset, U.K.
Anti-nitrotyrosine IgG (rabbit polyclonal)	Upstate Biotechnology Inc., NY, U.S.A.
Anti-rabbit IgG-HRP	Santa Cruz Biotechnology Inc., CA, U.S.A.
L-Arginine	Bio-Rad Ltd, Hertfordshire, U.K.
Bovine serum albumin	Sigma-Aldrich Chem. Co., Dorset, U.K.
Bromophenol blue	Sigma-Aldrich Chem. Co., Dorset, U.K.
Calcium chloride	Merck Ltd, Leicestershire, U.K.
Carboxymethylcellulose	Sigma-Aldrich Chem. Co., Dorset, U.K.
Clotrimazole	Sigma-Aldrich Chem. Co., Dorset, U.K.
Cytochrome *c* (from horse heart)	Sigma-Aldrich Chem. Co., Dorset, U.K.
3,3'-Diaminobenzidine	Sigma-Aldrich Chem. Co., Dorset, U.K.
2,4-Diamino-6-hydroxypyrimidine	Sigma-Aldrich Chem. Co., Dorset, U.K.
Diethyldithiocarbamate	Sigma-Aldrich Chem. Co., Dorset, U.K.
Diethyl pyrocarbonate	Sigma-Aldrich Chem. Co., Dorset, U.K.
Disodium hydrogen orthophosphate	Merck Ltd, Leicestershire, U.K.
Dithiothreitol	Sigma-Aldrich Chem. Co., Dorset, U.K.
DMEM	Life Technologies Ltd, Renfrewshire, U.K.
DNA (From Salmon Testes)	Sigma-Aldrich Chem. Co., Dorset, U.K.
Dowex-50	Bio-Rad Ltd, Hertfordshire, U.K.
dRhodamine terminator cycle sequencing kit	Applied Biosystems, Warrington, U.K.
ECL western blotting detection reagents	Amersham Ltd, Buckinghamshire, U.K.
Econazole	Sigma-Aldrich Chem. Co., Dorset, U.K.
Ethylenediaminetetraacetic acid	Sigma-Aldrich Chem. Co., Dorset, U.K.

ExpressHyb hybridization solution	Clontech Labs Ltd, Hampshire, U.K.
Foetal calf serum	Harland Sera-Lab Ltd, Leicestershire, U.K.
Formaldehyde	Sigma-Aldrich Chem. Co., Dorset, U.K.
Formamide	Sigma-Aldrich Chem. Co., Dorset, U.K.
Gel loading solution	Sigma-Aldrich Chem. Co., Dorset, U.K.
D-Glucose	Sigma-Aldrich Chem. Co., Dorset, U.K.
Glucose-6-phosphate	Sigma-Aldrich Chem. Co., Dorset, U.K.
Glucose-6-phosphate dehydrogenase	Sigma-Aldrich Chem. Co., Dorset, U.K.
L-Glutamine	Life Technologies Ltd, Renfrewshire, U.K.
Glutathione	Sigma-Aldrich Chem. Co., Dorset, U.K.
Glycerol	Sigma-Aldrich Chem. Co., Dorset, U.K.
Glycine	Sigma-Aldrich Chem. Co., Dorset, U.K.
GSNO	Dr. Madge, The Cruciform Project, U.K.
Haemoglobin (human, double-crystallised)	Sigma-Aldrich Chem. Co., Dorset, U.K.
HEPES	Sigma-Aldrich Chem. Co., Dorset, U.K.
Hybond-N+ nylon membrane	Amersham Ltd, Buckinghamshire, U.K.
Hydrogen peroxide	Sigma-Aldrich Chem. Co., Dorset, U.K.
Hyperfilm MP autoradiography film	Amersham Ltd, Buckinghamshire, U.K.
Interferon-γ (recombinant murine)	Insight Biotechnology, Middlesex, U.K.
Isoflurane	Abbott Labs Ltd, Kent, U.K.
Leupeptin	Sigma-Aldrich Chem. Co., Dorset, U.K.
Lipopolysaccharide (serotype 055:B5)	Sigma-Aldrich Chem. Co., Dorset, U.K.
Lipopolysaccharide (serotype 026:B6)	Difco Labs Ltd, Oxfordshire, U.K.
Lucigenin	Sigma-Aldrich Chem. Co., Dorset, U.K.
Magnesium sulphate	Sigma-Aldrich Chem. Co., Dorset, U.K.
Methanol	Merck Ltd, Leicestershire, U.K.
2-Mercaptoethanol	Sigma-Aldrich Chem. Co., Dorset, U.K.
Miconazole	Sigma-Aldrich Chem. Co., Dorset, U.K.
Micro-FastTrack mRNA isolation system	Invitrogen Corp., CA, U.S.A.
Molecular weight filter (10,000 kDa)	Millipore Ltd, Herts, U.K.
MOPS	Sigma-Aldrich Chem. Co., Dorset, U.K.

MTT formazan	Sigma-Aldrich Chem. Co., Dorset, U.K.
β-NADH	Roche Diagnostics Ltd, East Sussex, U.K.
β-NADPH	Sigma-Aldrich Chem. Co., Dorset, U.K.
Napthylethylenediamine dihydrochloride	Sigma-Aldrich Chem. Co., Dorset, U.K.
Nick spin columns	Amersham Ltd, Buckinghamshire, U.K.
Nitrate reductase	Roche Diagnostics Ltd, East Sussex, U.K.
Nitrocellulose membrane	Millipore Ltd, Hertfordshire, U.K.
3-nitro-L-tyrosine	Sigma-Aldrich Chem. Co., Dorset, U.K.
[α-^{32}P] dCTP	Amersham Ltd, Buckinghamshire, U.K.
Ophiobolin A	Sigma-Aldrich Chem. Co., Dorset, U.K.
Penicillin	Life Technologies Ltd, Renfrewshire, U.K.
Pepstatin A	Sigma-Aldrich Chem. Co., Dorset, U.K.
Phenylmethylsulphonyl fluoride	Sigma-Aldrich Chem. Co., Dorset, U.K.
Phosphate buffered saline	Life Technologies Ltd, Renfrewshire, U.K.
Phosphoric acid	Sigma-Aldrich Chem. Co., Dorset, U.K.
Potassium chloride	Sigma-Aldrich Chem. Co., Dorset, U.K.
Potassium dihydrogen orthophosphate	Merck Ltd, Leicestershire, U.K.
Protein assay reagents	Bio-Rad Ltd, Hertfordshire, U.K.
Pyrogallol	Sigma-Aldrich Chem. Co., Dorset, U.K.
Random Primed cDNA labeling kit	Roche Diagnostics Ltd, East Sussex, U.K.
RNA molecular size standards	Life Technologies Ltd, Renfrewshire, U.K.
Sephadex G-25	Sigma-Aldrich Chem. Co., Dorset, U.K.
SIN-1	Alexis Corp. Ltd, Nottinghamshire, U.K.
SNP	Sigma-Aldrich Chem. Co., Dorset, U.K.
Sodium acetate	Sigma-Aldrich Chem. Co., Dorset, U.K.
Sodium chloride	Sigma-Aldrich Chem. Co., Dorset, U.K.
Sodium dihydrogen orthophosphate	Merck Ltd, Leicestershire, U.K.
Sodium dithionite	Merck Ltd, Leicestershire, U.K.
Sodium dodecyl sulphate	Sigma-Aldrich Chem. Co., Dorset, U.K.
Sodium hydrogen carbonate	Merck Ltd, Leicestershire, U.K.
Sodium nitrate	Sigma-Aldrich Chem. Co., Dorset, U.K.

Sodium nitrite	Sigma-Aldrich Chem. Co., Dorset, U.K.
Sodium pentobarbitone	Rhône Mérieux Ltd, Essex, U.K.
SSC (20X) solution	Life Technologies Ltd, Renfrewshire, U.K.
Streptomycin	Life Technologies Ltd, Renfrewshire, U.K.
Succinate	Sigma-Aldrich Chem. Co., Dorset, U.K.
Sulphanilamide	Sigma-Aldrich Chem. Co., Dorset, U.K.
Superoxide dismutase (from bovine brain)	Sigma-Aldrich Chem. Co., Dorset, U.K.
TEMED	Sigma-Aldrich Chem. Co., Dorset, U.K.
Tetrahydrobiopterin	Dr. B. Schircks Labs., Jona, Switzerland
Tiron	Sigma-Aldrich Chem. Co., Dorset, U.K.
Tris-Base	Sigma-Aldrich Chem. Co., Dorset, U.K.
Tris-HCl	Sigma-Aldrich Chem. Co., Dorset, U.K.
Triton X-100	Sigma-Aldrich Chem. Co., Dorset, U.K.
Trypan blue	Life Technologies Ltd, Renfrewshire, U.K.
Tween 20	Sigma-Aldrich Chem. Co., Dorset, U.K.
Wide range colour molecular markers	Amersham Ltd, Buckinghamshire, U.K.
Xanthine	Sigma-Aldrich Chem. Co., Dorset, U.K.
Xanthine oxidase (from buttermilk)	Sigma-Aldrich Chem. Co., Dorset, U.K.