Induction of nitric oxide synthase in vascular smooth muscle

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Summary

The first aim of this study was to examine and assess the sensitivity and utility of a number of different measuring techniques for the detection of nitric oxide (NO). The systems examined did not measure NO directly. Instead, some of the systems employed measured nitrite, the major *in vitro* breakdown product of NO: these were the Griess reaction, chemiluminescence detection coupled to two reducing systems, i.e. 1,1'-dimethylferrocene/acetonitrile reflux and sodium iodide/glacial acetic acid reflux and lastly fluorometric detection. The Griess reaction was the least sensitive with a threshold detection limit of 300 pmol, but a large number of samples could be assayed each day. The 1,1'-dimethylferrocene/acetonitrile system produced chemiluminescence signals that were difficult to discern from background noise and was abandoned. The sodium iodide/glacial acetic acid reflux system did, however, produce good chemiluminescence signals, giving a threshold sensitivity of 100 pmol. This technique was slow, however, permitting only small numbers of samples to be assayed each day. The fluorometric detection system for nitrite had a similar sensitivity of around 120 pmol but as each sample was stable for around 10 min only small numbers of samples could be assayed each day. Thus, if samples contained large amounts of nitrite, the less sensitive Griess reaction (300 pmol) was by far the easiest system to use. A stronger reducing system, involving vanadium chloride/hydrochloric acid was tested which when combined with chemiluminescence could measure both of the breakdown products of NO, i.e. nitrite and nitrate. This system had a high sensitivity of around 200 pmol but in addition to being able to handle only small numbers of samples, it was much less selective since many 'false' positive signals were generated by constituents of tissue culture medium.

Having assessed the suitability of the above detection systems, the second aim of this study was to use them in conjunction with other techniques to examine the expression of iNOS in different preparations. Expression of iNOS in J774.2 murine macrophages was seen after incubation with

lipopolysaccharide (LPS; 1 - 1000 ng ml^{-1}) or interferon-γ (IFN-γ; 1 - 100 u ml^{-1}). In contrast, LPS (1 - 1000 ng ml^{-1}) failed to stimulate expression of iNOS in bovine aortic endothelial cells (BAEC) in culture.

Following an overnight incubation at 37°C in Krebs solution, phenylephrine (PE; 1 nM - 10 μM)-induced contraction of endothelium-denuded rings of rat aorta was depressed. This depression was prevented by including polymixin B (30 μg ml^{-1}) during the incubation suggesting it resulted from LPS contaminating the Krebs solution. This depression was likely to have resulted from production of NO by iNOS as it could be reversed by the addition of L-NAME (1 mM), an inhibitor of NOS, methylene blue (10 μM), an inhibitor of soluble guanylate cyclase or haemoglobin (10 μM), which binds and inactivates NO. The effects of generators of superoxide anion, which destroy NO, were also examined. The intracellular generator of superoxide anion, LY 83583 (0.1 - 1 μM), reversed the depression but the extracellular generator, hypoxanthine (100 μM)/xanthine oxidase(16 mu ml^{-1}), had no effect. These data suggest that NO is generated and acts within the same smooth muscle cells.

In contrast to rat aorta, rat aortic smooth muscle cells (RASMC) in culture did not express iNOS, as assessed by measuring nitrite accumulation and cyclic GMP content, when stimulated by LPS (1 - 100 μg ml^{-1}) alone except at exceptionally high concentrations (300 and 1000 μg ml^{-1}). Certain cytokines, i.e. IFN-γ (1 - 100 u ml^{-1}) and interleukin-1α (1 - 10 u ml^{-1}), did however, lead to expression of iNOS. The presence of serum appeared to inhibit the expression of iNOS, probably because of the presence of platelet-derived growth factor and transforming growth factor. The production of nitrite and elevated levels of cyclic GMP were blocked following incubation with inhibitors of the expression of iNOS, dexamethasone (1 μM) and cycloheximide (30 u ml^{-1}) and by the inhibitors of NOS, L-NAME (100 μM) and L-NMMA (300 μM).

Thus, it appeared that LPS alone was able to stimulate expression of iNOS in isolated rings of rat aorta but not in cultured RASMC. Whether this apparent difference resulted from differences in smooth muscle phenotype in the vessel wall and in culture or from any other differences has yet to be resolved.

Contents

List of Figures

List of Tables

Abbreviations

ACh	acetylcholine
anti-m	monoclonal antibody
ATP	adenosine triphosphate
BAEC	bovine aortic endothelial cells
cNOS	constitutive nitric oxide synthase
DAN	diaminonaphthalene
DMEM	Dulbecco's Modified Eagles Medium
DNA	deoxyribonucleic acid
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediamine tetra acetic acid
FAD	flavin adenine dinucleotide
FCS	foetal calf serum
FMN	flavin mononucleotide
GMP	guanosine monophosphate
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
Hb	haemoglobin
IFN-γ	interferon-γ
IL-1α	interleukin-1α
IL-1β	interleukin-1β
IL-2	interleukin-2
iNOS	inducible nitric oxide synthase
L-NAA	N^G-amino-L-arginine
L-NAME	N^G-nitro-L-arginine methyl ester
L-NIO	N^G-iminoethyl-L-arginine
L-NMMA	N^G-monomethyl-L-arginine
L-NOARG	N^G-nitro-L-arginine
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid

NADPH	reduced nicotinamide-adenosine dinucleotide phosphate
NANC	non-adrenergic, non cholinergic
NBS	new born calf serum
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
PE	phenylephrine
ppb	parts per billion
ppb.s	parts per billion times seconds
RASMC	rat aortic smooth muscle cells
RIA	radioimmunoassay
Scyclic GMP-TME-[^{125}I]	succinyl cyclic GMP tyrosine methyl ester-[^{125}I]
TNF-α	tumour necrosis factor-α

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Chapter 1
General Introduction

Nitric oxide (NO), a rare atmospheric gas and one of the toxic components of air pollution that contributes to acid rain and destruction of the ozone layer, is now known to be enzymatically synthesised in a number of cell types. It has been demonstrated that NO is responsible for diverse functions such as cell-cell signalling and immune-mediated cytotoxicity.

Discovery

It was first suggested in 1916 by Mitchell *et al.* that mammals produced oxides of nitrogen, when it was shown that more nitrate was excreted than was ingested. For a long time this excess was thought to be produced by intestinal micro-organisms and it was not until work in the early 1980's that Mitchell's findings were substantiated. Work studying carcinogenic actions of nitrosamines confirmed that mammals produced nitrate (NO_3^-) and this production was enhanced by infection in human subjects and after endotoxin treatment in rodents (Green *et al.*, 1981a; Green *et al.*, 1981b). Stuehr and Marletta (1985) demonstrated that murine macrophages released nitrate and this was the first evidence of the formation of nitrogen oxides from a specific cell type. Hibbs then went on to show that the nitrite and nitrate were produced from the substrate L-arginine (Hibbs *et al.*, 1987). In a separate line of work investigating the endothelium-derived relaxing factor (EDRF) described by Furchgott and Zawadzki (1980), Ignarro and Palmer independently reported this EDRF to be NO (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). Palmer's group also showed that the substrate for this NO was L-arginine (Palmer *et al.*, 1988). In yet another separate line of work examining the rat anococcygeus muscle, it was discovered that it has a motor noradrenergic and an inhibitory non-adrenergic, non-cholinergic (NANC) innervation (Gillespie, 1972). When the noradrenergic response is blocked with guanethidine, field stimulation of the preparation produces a powerful relaxation and this response can be mimicked by NO and blocked by agents which block the conversion of

L-arginine to NO (Gillespie *et al.*, 1989). Similar responses have since been established in other peripheral nerves in many systems including the gut (Bult *et al.*, 1990), respiratory tract (Tucker *et al.*, 1990), reproductive system (Liu *et al.*, 1991) and penile arteries (Liu *et al.*, 1991). NO-like activity has also been established by Garthwaite's group in the central nervous system after N-methyl-D-aspartic acid stimulation in the brain (Garthwaite *et al.*, 1988). These and other studies have demonstrated that NO plays a key role in many of the processes in the body and is produced from the amino acid L-arginine. However, from an evolutionary point of view NO is not a new biological molecule as evidence of NO and its actions have been found in the limulus crab which according to fossil records, has not evolved in 500 million years (Radomski *et al.*, 1991; McCall & Vallance, 1992; Moncada & Martin, 1993).

Controversy over identity of EDRF

There is wide debate as to whether EDRF is truly NO or an NO-releasing molecule. Reports that EDRF is NO came from Palmer's group where they used chemiluminescence in the presence of a reducing system to detect the metabolites of NO (Palmer *et al.*, 1987) and also from Kelm and Schrader (1988) who used methaemoglobin detection, where oxyhaemoglobin is oxidised to methaemoglobin in the presence of NO. However, neither of these methods measure native NO. Myers tried to measure native NO using the chemiluminescence method without reducing solutions and found the amount of NO present was insufficient to account for the activity of EDRF; it was still insufficient even when a reducing system was introduced (7 - 10 times too low) (Myers *et al.*, 1989). It was then suggested that EDRF resembled a S-nitrosothiol more closely than NO (Myers *et al.*, 1990). Other groups have since agreed with these findings; firstly, Rubanyi's group found that whilst EDRF and NO were both inactivated when passed through a haemoglobin-agarose column, only authentic NO gave a NO-haemoglobin electron

paramagnetic signal (Rubanyi *et al.*, 1991). This study suggested that S-nitrosocysteine was more likely to be EDRF as it also gave no electron paramagnetic signal (Rubanyi *et al.*, 1991). Another group (Kukreja *et al.*, 1993) in agreement with Myers found that S-nitrosocysteine produced relaxations in cerebral arteries in cats but that native NO was 10-fold less potent. In contrast, Liu found that S-nitrosothiols were unlikely to function as the nitergic neurotransmitter in bovine retractor penis muscle, since the neurotransmitter was found to be resistant to destruction by superoxide anion whereas S-nitrosoglutathione and S-nitrosocysteine were not (Liu *et al.*, 1994). Also, Feelisch found that EDRF was more likely to be NO than an S-nitrosothiol as the activities of EDRF and NO were inhibited by L-cysteine but the actions of S-nitrosothiols were not (Feelisch *et al.*, 1994). Another possibility is that NO binds to iron haem moieties forming dinitrosyl iron (Vanin, 1991). This dinitrosyl iron has been detected by electron spin resonance spectroscopy (Vanin *et al.*, 1993). It is possible that the release of these complexes accounts for the activity of EDRF (Mülsch *et al.*, 1993). Thus, there is still debate as to the true identity of EDRF.

An area of general agreement, however, is that NO derived from L-arginine is the active component of EDRF even if it is released bound to a thiol or some other molecule such as an iron haem moiety.

Inorganic chemistry of NO

NO is a radical (i.e., it has an unpaired electron) that is reactive with other substances but does not self-react, perhaps because its bond length is intermediate between double and triple bond lengths (table 1.1). NO is not as unstable as many other radicals, but has a half life of 3 - 50 s in aqueous solution at biological pH (McCall & Vallance, 1992). NO readily reacts with O_2

Molecular weight	30.006 (1 mole = 0.03006 kg)
N - O bond distance[a]	1.1508 Å
Partition coefficient[b]	≈ 20
Saturated NO solution[c]	≈ 3 mM
Oxidation products	nitrite, nitrate
Dissociation $t_{1/2}$ Hb-NO[d]	≈ 3 h
Absolute density	101.325 kPa at 25°C
Solubility in H_2O at 0, 20 and 60°C	7.34, 4.6 and 2.37 ml/100 ml
Physico-chemical properties	High lipid solubility Highly diffusible Gas or in solution at body temperature
Redox-activated forms	Nitric oxide (NO) Nitrosonium cation (NO^+) Nitroxyl anion (NO^-)
Half-life and fate	very short (3 to 50 s) Gaseous - oxidised to NO_2 and higher oxides of nitrogen Solution - oxidised to nitrite and nitrate

Table 1.1: Properties of nitric oxide (nitrogen oxide, NO).

[a] The bond distance of N - O is intermediate between that of a typical double and triple bond (1.18 and 1.06 Å, respectively).

[b] Partition coefficient = molecules per unit volume gas/molecules per unit volume solution.

[c] Saturated NO solutions contain approximately 3 nmol NO/µl when in the absence of oxygen.

[d] The dissociation of NO from haemoglobin is much slower than the dissociation of carbon monoxide (CO) from Hb ($T_{1/2}$ 35 s), accounting for the much higher affinity of Hb for NO than O_2 or CO.

(Wennmalm *et al.*, 1992; Ignarro *et al.*, 1993). In aqueous solution containing O_2, NO is oxidised mainly to NO_2^- with little or no formation of NO_3^- by the following reaction (Ignarro *et al.*, 1993):

$$4NO^{\bullet} + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+$$

NO in gaseous mixtures containing oxygen, however, forms NO_2 by the following reaction (Ignarro *et al.*, 1993):

$$NO^{\bullet} + O_2 \rightarrow OONO$$
$$OONO + NO^{\bullet} \rightarrow ONOONO \rightarrow 2NO_2 \rightarrow O_2NNO_2\ (N_2O_4)$$

The N_2O_4 formed dismutases spontaneously in water (pH 7.4) to yield both nitrite and nitrate by the following reaction (Ignarro *et al.*, 1993):

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$$

Wennmalm *et al.* (1992) found in blood with high oxygen saturation of haemoglobin that nitrate is the main product by the following reaction:

$$Hb(Fe^{2+})O_2 + NO \rightarrow Hb(Fe^{3+}) + NO_3^-$$

NO also reacts with superoxide radical to yield peroxinitrite ($OONO^-$) by the following reaction (Gryglewski *et al.*, 1986):

$$NO + O_2^- \rightarrow ONOO^-$$

Peroxynitrite ($ONOO^-$) when formed is a powerful oxidant which is freely diffusible and can cause tissue damage (Ignarro *et al.*, 1987; Freeman, 1994; Stamler *et al.*, 1992).

NO is poorly soluble in water, which is indicated by its high partition coefficient (table 1.1), and tends to exist as a gas. It has great potential as a biological messenger due to its small size which facilitates diffusion across biological membranes, thus permitting its entry into and exit from cells (Ignarro *et al.*, 1987). Once within the cell it avidly binds to transition metals such as Fe, Cu, Co and Mn (Henry *et al.*, 1991; Stamler *et al.*, 1992), which are crucial to the function of many cytochromes and oxidases.

The reactivity and properties of NO depend on its interrelated redox forms (Stamler *et al.*, 1992): neutral nitric oxide ($NO^{\bullet}$); nitrosonium cation (NO^{+}, with the removal of an electron at its outer orbit) and nitroxyl anion (NO^{-}, with the addition of an electron to its outer orbit). The reactions of $NO^{\bullet}$ have been discussed above. NO^{+} reacts with nucleophiles such as electron-rich bases, aromatic compounds and hydrogen peroxide. A variety of nitrosocompounds that form efficiently under neutral physiological conditions can be viewed as NO^{+} carriers and these include metal-nitrosyl complexes, thionitrites, nitrosamines, alkyl and aryl nitrites, and dinitrogen tri- and tetra-oxides (N_2O_3 and N_2O_4). NO^{-} reverts rapidly to N_2O through dimerization and dehydration. NO^{-} also undergoes reversible addition to low molecular weight and protein thiols leading to sulfhydryl oxidation.

The properties of NO are summarised in table 1.1.

NO biosynthesis

It is accepted that NO is derived from either of the chemically equivalent guanidino nitrogens of the amino acid L-arginine (Palmer *et al.*, 1988) following activation of the enzyme nitric oxide synthase (NOS). This reaction involves a five-electron oxidation of L-arginine to yield NO. The reaction scheme for the production of NO is not fully understood but N^{G}-hydroxyl-L-arginine appears to

be an intermediate in the reaction (Stuehr *et al.*, 1991c). A second amino acid L-citrulline is produced as a by-product (Hibbs *et al.*, 1987). It has also been shown that the oxygen in NO and the ureido oxygen in L-citrulline are derived from molecular oxygen (Leone *et al.*, 1991). The reaction mechanism is shown in fig 1.1. NADPH is required as a cosubstrate and FAD, FMN (Stuehr *et al.*, 1991a) and tetrahydrobiopterin (Mülsch & Busse, 1991; Schmidt *et al.*, 1992) are required as cofactors. An additional cofactor required is calmodulin (Förstermann *et al.*, 1991; Pollock *et al.*, 1991; Schmidt *et al.*, 1991). In the presence of elevated calcium, the calcium/calmodulin complex binds to constitutive NOS (cNOS), which exists in endothelial cells (Sessa *et al.*, 1992) and certain neurones (Bredt *et al.*, 1990). Another form of NOS is inducible NOS (iNOS). Originally it was thought that iNOS was calcium/calmodulin-independent, however, iNOS has now been found to bind calmodulin so tightly that calcium is not required for activation (Cho *et al.*, 1992).

Isotypes of NOS

NOS, thus far, is known to exist in at least three distinct isoforms representing three distinct gene products which have been isolated, purified and cloned. These three isoforms vary in subcellular location, amino acid sequence, regulation and functional roles. Two isoforms are present in the cell under normal resting conditions and are termed cNOS, whereas the third isotype is only expressed under pathological conditions following stimulation by certain cytokines, microbes or microbial products and is thus termed iNOS.

One of the two isotypes of cNOS has been localised to the endothelium and is mostly membrane bound via a myristylation site (Sessa *et al.*, 1992; Sessa, 1994). A second cNOS has been localised to the cytosol of central and peripheral neurones but is also found in extraneuronal sites such as skeletal

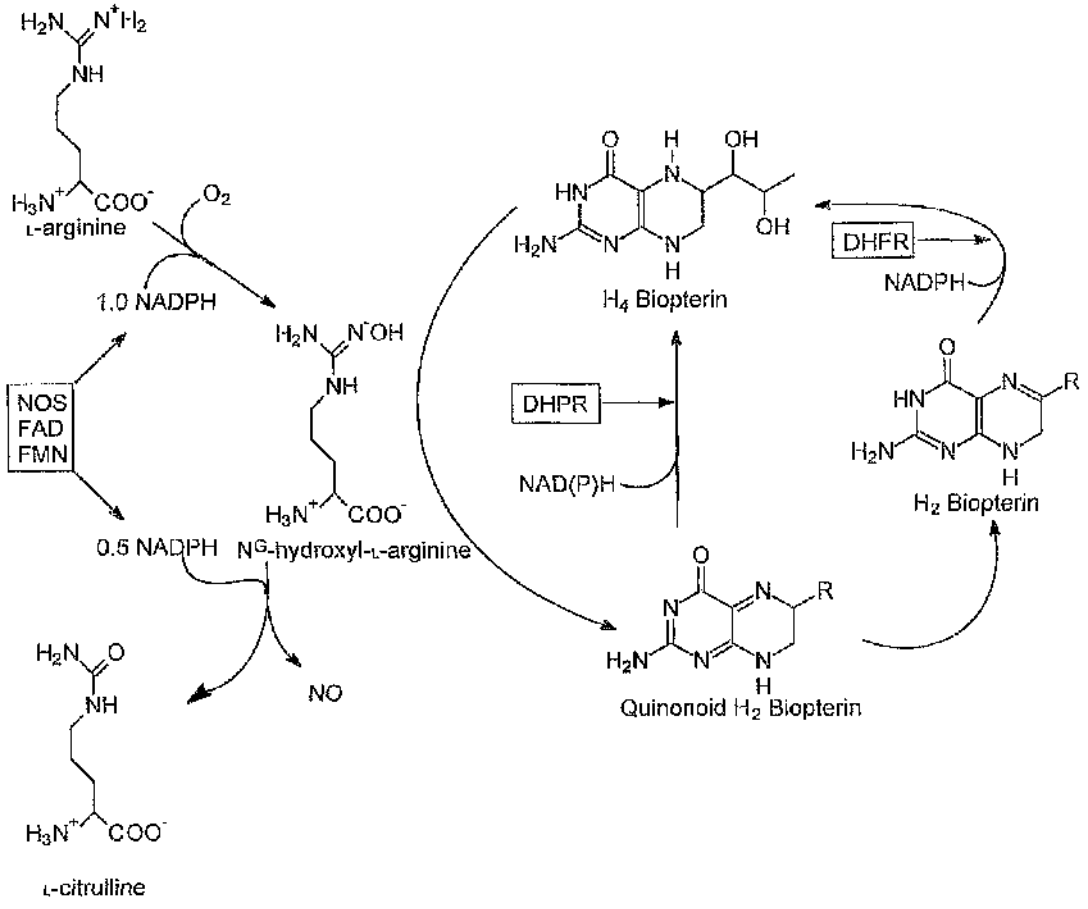


Figure 1.1: Biosynthesis of nitric oxide. A guanidino nitrogen of L-arginine undergoes a five electron oxidation to yield the gaseous radical nitric oxide (NO) via an N^G-hydroxyl-L-arginine intermediate. NADPH donates two electrons for the formation of this intermediate and one electron for its further oxidation. Both steps are catalyzed by the FAD- and FMN-containing enzyme, nitric oxide synthase (NOS). Molecular oxygen is incorporated into both the ureido group of the by-product L-citrulline and NO itself. Tetrahydrobiopterin (H_4 biopterin) is also required; the amounts needed are substoichiometric with respect to the amount of NO generated, provided that tetrahydrobiopterin can be regenerated from its oxidised form, quinonoid dihydrobiopterin (H_2 biopterin). Regeneration of tetrahydrobiopterin proceeds both through the methotrexate-sensitive enzyme dihydrofolate reductase (DHFR) and through the methotrexate-resistant enzyme, dihydropteridine reductase (DHPR). Modification of a diagram by Nathan (1992).

muscle, pancreas and kidney (Bredt *et al.*, 1990; Nathan, 1992; Nakane *et al.*,1993). This isoform may also be termed neuronal NOS (nNOS). When activation occurs calcium/calmodulin binding permits electron transfer from NADPH via flavin groups within the enzyme to a heme-containing active site (Stuehr *et al.*, 1991a; Abu-soud & Stuehr, 1993) resulting in small amounts (pmol) of NO being generated by these two types of cNOS. This activation is short lived, and the NO produced serves as a diffusible signalling molecule, mediating numerous physiological processes, including vasodilation and neurotransmission.

In contrast to cNOS, iNOS is not expressed under normal conditions but as already stated requires induction by certain cytokines, microbes or microbial products (Nussler & Billiar, 1993). Furthermore, there is often strong synergy between stimulating agents. For example, murine macrophages express high levels of iNOS upon exposure to lipopolysaccharide (LPS), with strong synergy occurring with the additional presence of interferon-γ (IFN-γ) (Stuehr & Marletta, 1987a). Regulation of iNOS activity seems to occur primarily at transcriptional level but may also be affected by mRNA stability (Beasley *et al.*, 1991; Xie *et al.*, 1992). Changes in iNOS mRNA probably reflect changes in iNOS gene translation (Beasley *et al.*, 1991). After stimulation, NO synthesis is delayed for several hours by *de novo* synthesis of iNOS protein and by the production of essential cofactors (Beasley *et al.*, 1991). iNOS expression results in a sustained production of NO, which exerts both cytostatic/cytotoxic and antimicrobial activity towards certain pathogens (Nussler & Billiar, 1993). Unlike the cNOS isoform, iNOS remains activated and continuously produces NO for the life of the active enzyme and, as already stated, it is not regulated by calcium (Cho *et al.*, 1992). The capacity to express iNOS exists in nearly every tissue in the body from immune cells such as macrophages (Stuehr & Marletta, 1985; Stuehr & Marletta, 1987a) to cells such as hepatocytes (Geller *et al.*, 1993b), vascular smooth muscle cells (Busse & Mülsch, 1990; Fleming

et al., 1991a), endothelial cells (Radomski *et al.*, 1990b; Gross *et al.*, 1991), chondrocytes (Palmer *et al.*, 1993), cardiac myocytes (Schulz *et al.*, 1992) and neurones (Minc-Golomb *et al.*, 1994), when appropriately stimulated.

The complementary DNA for the three isoforms of NOS has been cloned and a comparison of these is made in table 1.2. These isotypes appear to come from distinct genes, however there is a high degree of homology among them and also with cytochrome P450 reductase (Bredt *et al.*, 1991). For each isoform (cNOS or iNOS) there is a high conservation of amino acid identity between species (50 - 94%). Figure 1.2 shows a schematic alignment of the amino acids for the 3 major forms of NOS and their similarity to cytochrome P450 reductase.

Actions of NO

NO, once produced, is involved in many processes in the body from intercellular signalling to cytotoxic actions on invading organisms.

NO in the cardiovascular system

NO is responsible for active vasodilation in the vasculature. Endothelial cells continually release small amounts of NO exerting a tonic basal vasodilation tone. Furthermore, the release of NO can be increased by shear stress exerted by the flow of blood (Rubanyi *et al.*, 1986; Rees *et al.*, 1989) or by agonists such as acetylcholine (ACh) and bradykinin (Furchgott & Zawadzki, 1980; Palmer *et al.*, 1988). A list of agonists is given in table 1.3. These agonists act by binding to their receptors on the cell membrane of endothelial cells, causing a transient increase in intracellular calcium. Calcium binds to calmodulin and this complex activates cNOS increasing the production of NO.

NOS isoform	species	cell type[a]	size of transcript, kb[b]	Protein Mr (deduced)	Ca^{2+} dependence of NOS activity
Neuronal (cNOS)	Human[1]	Cerebellum	10	161037	+++
	Rat[2]	Cerebellum	10.5	160458	+++
Cytokine inducible (iNOS)	Human[3]	Hepatocyte	4.5	131000	?[c]
	Murine[4]	Macrophage	4.5	130556	-
	Rat[5]	Hepatocyte	4.5	130653	-
	Rat[6]	Vascular smooth muscle	4	131000	-
Endothelial (cNOS)	Human[7]	Aortic EC	4.4-4.8	133000	+++
	Bovine[8]	Aortic EC	4.4-4.8	133286	+++

Table 1.2: Comparison of cloned NOS isoforms. Modification of a table by Sessa (1994).

[a] Source of cDNA library

[b] Approximate size of mRNA under high stringency hybridisation and wash conditions

[c] Partial calcium-dependence of expressed cDNA based on the ability of EDTA to inhibit NOS activity

[1] (Nakane *et al.*, 1993)

[2] (Bredt *et al.*, 1991)

[3] (Geller *et al.*, 1993a)

[4] (Lyons *et al.*, 1992; Marczin *et al.*, 1992)

[5] (Wood *et al.*, 1993)

[6] (Nunokawa *et al.*, 1993)

[7] (Janssens *et al.*, 1992)

[8] (Sessa *et al.*, 1992; Lamas *et al.*, 1992; Nishida *et al.*, 1992)

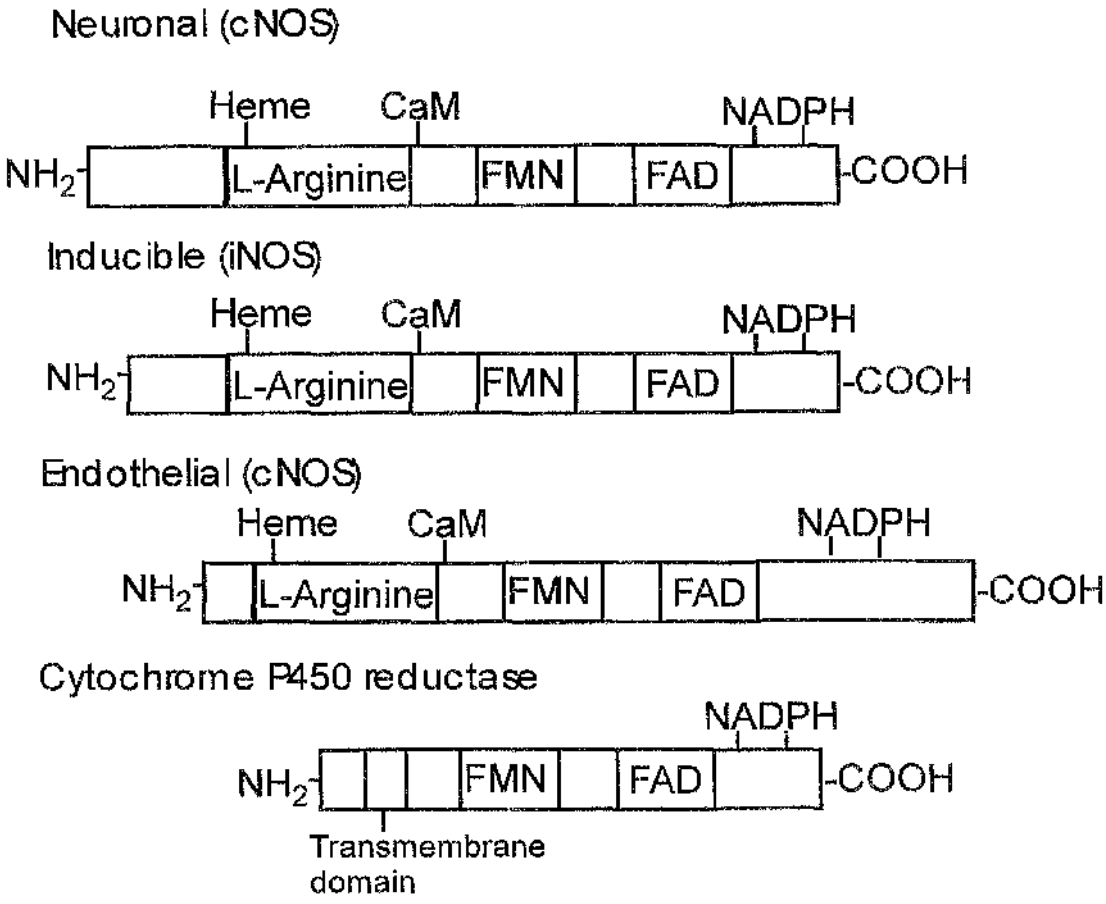


Figure 1.2: Schematic alignment of the deduced amino acid sequences of neuronal, inducible and endothelial nitric oxide synthase (NOS) with cytochrome P450 reductase. Consensus sequence sites include binding sites for heme, L-arginine, calmodulin (CaM), FMN, FAD and reduced FAD phosphate (NADPH). Endothelial NOS is the only isoform that contains a unique N-terminal myristylation site permitting adherence to cell membrane. Modification of a figure by Sessa (1994).

Activators of cNOS in vascular endothelium	Acetylcholine ATP Bradykinin Calcium inophores Electrical field stimulation Excitatory amino acids Formylated peptides Histamine Leukotrienes Noradrenaline Phorbol myristate acetate Platelet activating factor Thrombin 5HT

Table 1.3: Some activators of endothelial constitutive nitric oxide synthase (cNOS). It should be noted that receptors do exist for all agents in every endothelial cell type.

This NO then diffuses out of the endothelium and into adjacent vascular smooth muscle cells where it binds to the haem moiety of soluble guanylate cyclase. This causes the activation of soluble guanylate cyclase resulting in an increased production of cyclic guanosine monophosphate (cyclic GMP) (Ignarro *et al.*, 1987). This in turn, decreases the intracellular calcium concentration in the vascular smooth muscle cell, mediated by the activity of cyclic GMP-dependent protein kinases (Warner *et al.*, 1994). This activity of cyclic GMP-dependent kinases results in the blockade of intracellular calcium release and calcium entry together with stimulation of calcium extrusion via calcium pumps, thus leading to relaxation of the smooth muscle (Collins *et al.*, 1986; Rapoport, 1986; Warner *et al.*, 1994). NO also activates the sodium-potassium pump, which leads to transient hyperpolarisation of the vascular smooth muscle cell membrane (Archer *et al.*, 1994). This latter mechanism is thought to mediate shear stress induced, or flow-dependent, vasodilation. Fig 1.3 shows the production of NO by cNOS in the endothelium and the mechanism of vasodilation of the smooth muscle. It also shows the potential for production of NO by vascular smooth muscle cells following expression of iNOS.

NO automatically regulates blood flow in response to local changes in some regions of the vasculature. The actions of NO in ischemia followed by reperfusion are complex. In rat brain, for example, occlusion of the middle cerebral artery increased cerebral NO levels, but levels subsequently declined and increased again upon reoxygenation as measured using a NO-sensitive microelectrode (Malinski *et al.*, 1993). NO appears to function in a protective role to reduce infarct size and preserve cerebral blood flow (Morikawa *et al.*, 1992). In contrast, NO has also been implicated in the myocardial injury associated with ischemia/reperfusion. Specifically, in anaesthetised pigs, NO production was decreased by hypoxia and increased upon reoxygenation

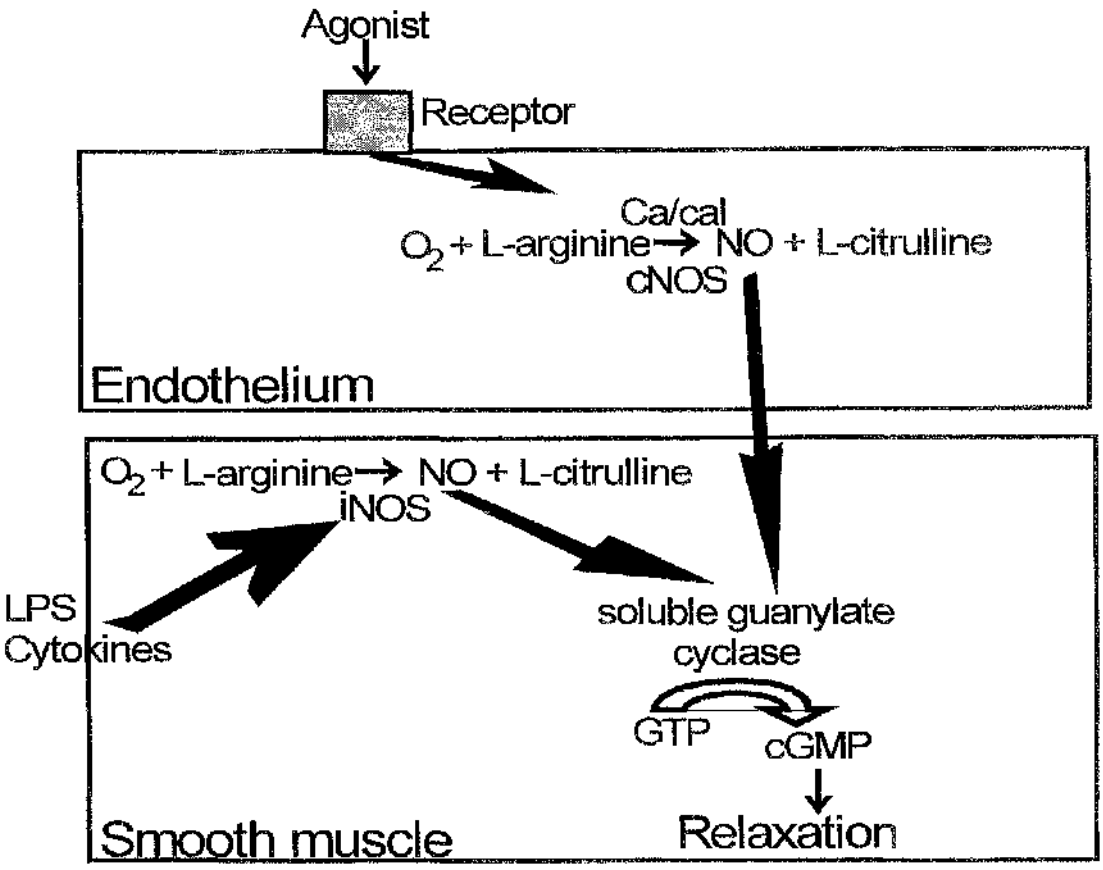


Figure 1.3: Schematic diagram illustrating the relaxation of vascular smooth muscle via the generation of nitric oxide (NO) by inducible and constitutive nitric oxide synthase (i and cNOS, respectively). cNOS: agonists bind to their membrane bound receptors on the endothelium causing a transient increase in intracellular calcium. Calcium binds to calmodulin and this complex (Ca/cal) activates cNOS increasing the production of NO. This NO then diffuses out of the endothelium and into adjacent smooth muscle cells where it binds to soluble guanylate cyclase. This causes activation of the soluble guanylate cyclase, thus increasing cyclic GMP (cGMP) levels leading to relaxation. iNOS: lipopolysaccharide (LPS) and cytokines can stimulate the expression of iNOS in smooth muscle. Expression of iNOS leads to the production of NO which activates soluble guanylate cyclase and in turn leads to relaxation.

(Matheis *et al.,* 1992). Furthermore, inhibitors of NOS protected against reoxygenation-elicited injury, measured as a decrease in contractility and an increase in lipid peroxidation (Matheis *et al.,* 1992). In addition, inhibition of NOS also appears to limit infarct size in the rabbit heart (Patel *et al.,* 1993).

The release of NO into the vasculature is also controlled by the autonomic nervous system. Parasympathetic nerves containing NOS terminate in the adventitia of certain large vessels, such as the cerebral and retinal arteries (Nozaki *et al.,* 1993). NO is released from the nerves and diffuses into the muscular media from the outside of the vessel, causing vasorelaxation.

Dysfunction of the L-arginine/NO pathway is believed to contribute to many vascular pathologies. For example, impaired NO-related, endothelium-dependent vasorelaxation has been observed in essential hypertension (Panza *et al.,* 1990). Clearly the endothelium is abnormal in patients with hypertension because ACh causes less vasodilation in hypertensive than in healthy patients (Panza *et al.,* 1990). In contrast, the vascular smooth muscle is normal in patients with hypertension because intravenous nitroprusside, which releases NO, causes comparable vasodilation in both normal and hypertensive patients (Panza *et al.,* 1990). Also, the blood pressure of healthy patients increases when NOS inhibitors are infused, but that of patients with hypertension increases to a much less degree (Panza *et al.,* 1993).

Other diseases where endothelial dysfunction is characteristic are atherosclerosis/hypercholesterolaemia (Drexler *et al.,* 1991) and the pulmonary arterial hypertension of chronic obstructive lung disease (Dinh-Xuan *et al.,* 1991). Scavenging of NO by advanced glycosylation end products (Bucala *et al.,* 1991) and oxidised lipoproteins (Chin *et al.,* 1992) may contribute to defective vasoregulation in diabetes and atherosclerosis.

As already mentioned, NO can also be produced within the smooth muscle cells themselves following expression of iNOS (Busse & Mülsch, 1990) by the administration of LPS (Kilbourn *et al.*, 1990b; Thiemermann & Vane, 1990) or cytokines (Kilbourn *et al.*, 1990a). Following expression of iNOS large quantities of NO are produced for prolonged periods and this may be responsible for the hypotension seen in septic shock (Kosaka *et al.*, 1992). The effects of septic shock on the cardiovascular system are discussed in more depth in the next chapter.

NO can also act on other areas of the cardiovascular system including the heart. Here NO exerts negative chronotropic and negative inotropic effects on cardiac muscle cells (Brady *et al.*, 1993) by an action similar to its relaxation of smooth muscle cells, i.e. activation of cGMP-dependent protein kinase (Méry *et al.*, 1991). In addition to production of NO in coronary endothelium, NO has been shown to be synthesised by calcium-dependent enzymes in the endocardial endothelial cell (Schulz *et al.*, 1990) as well as in the cardiomyocyte itself (Schulz *et al.*, 1992).

The NO produced by endothelial cells can diffuse not only towards the vessel wall but also into the lumen and thus enter blood platelets. NO acts as an endogenous antithrombic via a direct action on blood platelets. This occurs through inhibition of platelet aggregation (Radomski *et al.*, 1990a) and adhesion (Radomski *et al.*, 1987b). The molecular basis of this action is not yet fully established, but possible mechanisms include activation of platelet guanylate cyclase (Radomski *et al.*, 1987b), inhibition of platelet phospholipase C (Durante *et al.*, 1992), or ADP-ribosylation of platelet glyceraldehyde 3-phosphate dehydrogenase (Brüne *et al.*, 1990). The anti-aggregating actions of NO act in synergy to those of prostacyclin (Radomski *et al.*, 1987a).

NO also inhibits leukocyte adhesion (Kubes *et al.,* 1991) to the vascular endothelium, endothelin production by endothelial cells (Boulanger & Lüscher, 1990) and smooth muscle proliferation (Nakaki *et al.,* 1990).

Immune effects of NO

NO plays an important role in the host defence mechanism. The large amounts of NO produced in the immune system arise from the expression of iNOS in macrophages and other cell types. The NO thus produced can kill or inhibit the growth of many pathogens, including bacteria (Adams *et al.,* 1991), fungi (Alspaugh & Granger, 1991) and parasites (Green *et al.,* 1990; Liew *et al.,* 1990). Recent papers suggest NO may also block viral replication (Croen, 1993; Karupiah *et al.,* 1993) and kill tumour cells (Hibbs *et al.,* 1988). NO also regulates lymphocyte function and may have a role in inhibiting certain subsets of T helper cells (Liew *et al.,* 1991). Lymphocytes and neutrophils also synthesize and release NO although its role in the normal function of these cells is unknown. NO may, in addition, play a role in auto-immune diseases in which NOS maybe inappropriately induced (Weinberg *et al.,* 1994). These cytotoxic and cytostatic actions of NO are mediated by a variety of mechanisms but one of the main mechanisms may be via the ability of NO to react with the iron containing enzymes, thus leading to inhibition of their activity (Lancaster & Hibbs, 1990). NO is known to inhibit adenosine triphosphate production at three separate stages, i.e. by blocking glycolysis through transferring ADP-ribose to glyceraldehyde-3-phosphate dehydrogenase, disrupting the Krebs cycle by binding to the heme group of cis-aconitase, and inhibiting oxidative phosphorylation by binding to the heme group of ubiquinone reductase (Lepoivre *et al.,* 1991; Kwon *et al.,* 1991). Furthermore, NO inhibits DNA synthesis by inactivating ribonucleotide reductase and can damage DNA directly by deamination (Wink *et al.,* 1991).

The mechanisms by which macrophages avoid these toxic effects of NO are unknown.

NO also contributes to the inflammatory response (Nussler & Billiar, 1993). The associated vasodilatation may be mediated by inflammatory mediators stimulating endothelial NOS or by induction of the inducible isoform in endothelium, smooth muscle, and inflammatory cells in the vessel wall. The NO produced is thought to contribute to local vascular leakage, oedema, (Hughes *et al.,* 1990) and cell damage (Palmer *et al.,* 1992).

NO as a neurotransmitter

NO is involved in neurotransmission both peripherally and centrally.

In the peripheral autonomic nervous system NO mediates neurotransmission by certain NANC nerves. Early evidence of the involvement of NO came from experiments on the rat and mouse anococcygeus and bovine retractor penis muscles in which haemoglobin, methylene blue and free radical generators, which inhibit the actions of NO (Furchgott & Zawadzki, 1980; Martin *et al.,* 1985; Moncada *et al.,* 1986) were shown to inhibit neurogenic relaxation (Bowman *et al.,* 1982; Bowman *et al.,* 1986; Gillespie & Sheng, 1990). The actions of NO in these tissues, like the actions on vascular smooth muscle (Rapoport & Murad, 1983) have been shown to proceed through elevation of cyclic GMP (Bowman & Drummond, 1984). The most compelling evidence that NO acts as the neurotransmitter is the finding that inhibitors of NOS produce inhibition of neurogenic relaxation in the anococcygeus of the mouse and rat (Gillespie *et al.,* 1989; Liu *et al.,* 1991; Martin *et al.,* 1993).

NOS has also been found in the myenteric plexus throughout the gastrointestinal tract (Young *et al.,* 1992). Activation of these NOS-containing nerves causes gastric relaxation in response to a food bolus as well as

relaxation of the ileocolonic junction and anal sphincter (Bult *et al.*, 1990; Desai *et al.*, 1991). Pelvic plexus neurones also contain NOS and NO is the mediator of penile erection (Burnett *et al.*, 1992).

NOS is also found in the central nervous system. In the brain, immunoactive NOS has been found in discrete populations in the striatum, hypothalamus, posterior pituitary, midbrain, basal forebrain and cerebellum of the rat (Bredt *et al.*, 1990). NO is produced in response to stimulation of excitatory amino acid receptors and also by action potentials (Garthwaite *et al.*, 1988). Evidence has been presented suggesting that it may be a mediator of long-term synaptic depression and long-term potentiation, and thus be responsible for learning and memory (O'Dell *et al.*, 1991). NOS-containing neurones also innervate the adventitia of cerebral blood vessels (Bredt *et al.*, 1990).

Inhibition of NOS and the actions of NO

The L-arginine/NO system can be influenced at a number of key stages by drugs. For example, many compounds are available which can inhibit the expression of iNOS, the activity of both cNOS and iNOS or the actions of NO. Table 1.4 provides a summary of these agents. The next few sections will discuss some of these in more detail.

Inhibition of NOS

The activity of NOS is inhibited by analogues of L-arginine including N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NOARG), N^G-nitro-L-arginine methyl ester (L-NAME), N-iminoethyl-L-ornithine (L-NIO) and N^G-amino-L-arginine (L-NAA). The structure of these analogues is shown in fig 1.4. These enantimer-specific analogues competitively inhibit all types of

class	cNOS	iNOS
Inhibitors of action		
Substrate analogues of L-arginine	N^G-nitro-L-arginine N^G-iminoethyl-L-ornithine N^G-amino-L-arginine N^G-monomethyl-L-arginine N^G-nitro-L-arginine methyl ester	N^G-nitro-L-arginine N^G-iminoethyl-L-ornithine N^G-amino-L-arginine N^G-monomethyl-L-arginine N^G-nitro-L-arginine methyl ester Aminoguanidine L-Canavanine
Flavoprotein binders	Diphenylene iodonium Iodonium diphenyl Di-2-thienyl iodonium	Diphenylene iodonium Iodonium diphenyl Di-2-thienyl iodonium
Calmodulin binders	Calcineurin Trifluoroperazine N-(4-aminobutyl)-5-chloro-2-napthalenesulfonamide	None described
Inhibitor of guanylate cyclase	Methylene blue	Methylene blue
Destroys NO	Superoxide anion	Superoxide anion
Bind NO	Haemoglobin Carboxy PTIO Hydroxocobalamin	Haemoglobin
Heme binder	Carbon monoxide L-Thiocitrulline	Carbon monoxide L-Thiocitrulline
Depleter of tetrahydrobiopterin		2,4-Diamino-6-hydroxypyrimidine
Inhibitors of induction	None described	Dexamethasone Cycloheximide Hydrocortisone Transforming growth factor-β-1,-2,3 Platelet derived growth factor $_{AB}$ and $_{BB}$ Interleukin-4 Interleukin-10 Macrophage deactivating factor Thaliporphine

Table 1.4: Inhibitors of the actions of nitric oxide, of nitric oxide synthase or of the expression of inducible nitric oxide synthase.

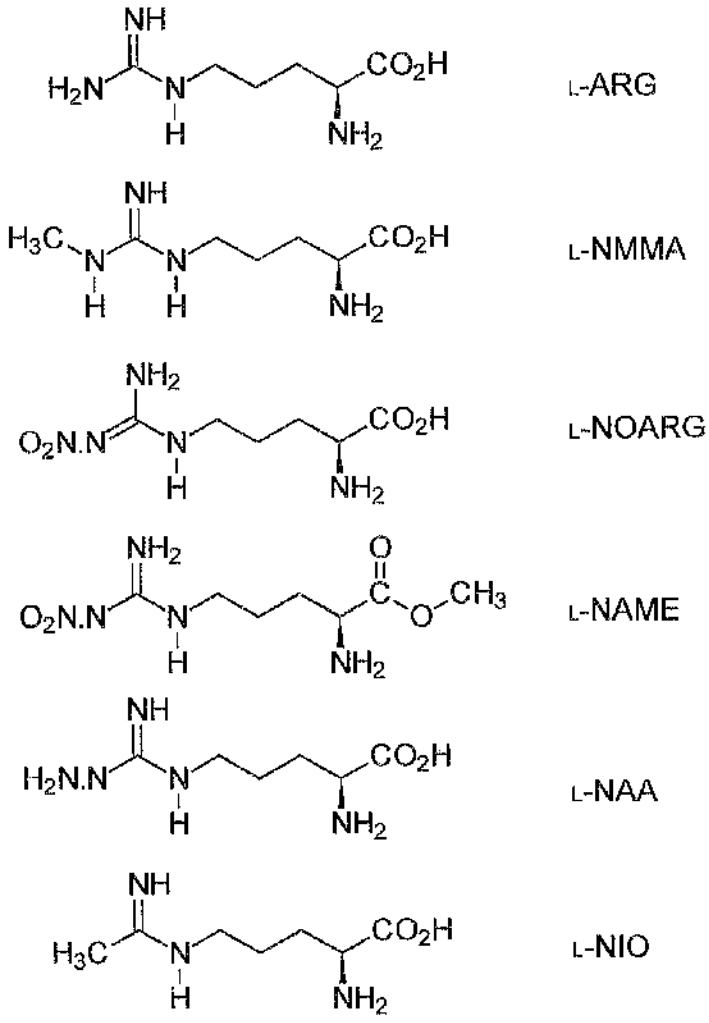


Figure 1.4: Structure of L-arginine (L-ARG) and N^G-substituted analogues of L-arginine, N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NOARG), N^G-nitro-L-arginine methyl ester (L-NAME), N^G-amino-L-arginine methyl ester (L-NAA), and N^G-iminoethyl-L-ornithine (L-NIO).

NOS. The inhibition can be reversed by L-arginine but the potency of these inhibitors differs between the isotypes. Against endothelial cell cNOS, L-NAME and L-NOARG are more potent than L-NIO and L-NMMA (Gross *et al.*, 1990; Moore *et al.*, 1990; Rees *et al.*, 1990b), whereas against macrophage iNOS, L-NMMA and L-NAA are more potent than L-NOARG, with L-NAME having little effect (Gross *et al.*, 1990; McCall *et al.*, 1991).

There is also a difference in the ability of L-NMMA to block the basal and agonist-induced production of NO in rat aortic rings (Frew *et al.*, 1993a; Frew *et al.*, 1993b). Specifically, L-NMMA can augment phenylephrine induced tone in a similar manner to other NOS inhibitors, i.e. L-NOARG, but it does not inhibit ACh- or ATP-induced relaxations. In fact, it inhibits the ability of L-NOARG to block these relaxations to ACh (Frew *et al.*, 1993a; Frew *et al.*, 1993b). Similarly in the bovine retractor penis muscle, L-NMMA protects nitrergic relaxation against the inhibitory actions of L-NOARG (Martin *et al.*, 1993). These actions of L-NMMA may be explained by the finding that L-NMMA can act as an alternative substrate for NOS, thus leading to enhanced production of NO (Olken & Marletta, 1993). This proposal is supported by recent experiments involving chemiluminescence detection showing that L-NMMA, but not L-NOARG, enhances NO production by rat aorta and pulmonary artery (Archer & Hampl, 1992).

Some inhibitors appear to show some degree of selectivity for iNOS, including aminoguanidine, guanidines and L-canavanine (Stuehr *et al.*, 1991b; Hasan *et al.*, 1993; Misko *et al.*, 1993). Aminoguanidine is equipotent to L-NMMA as an inhibitor of iNOS but is 10 to 100-fold less potent as an inhibitor of cNOS (Hasan *et al.*, 1993; Misko *et al.*, 1993). The amount of aminoguanidine required to increase blood pressure is 10 - 40 times greater than that of L-NMMA (Hasan *et al.*, 1993). L-canavanine also inhibits iNOS more

selectively than cNOS, being 30% less effective than L-NMMA on cNOS (Misko *et al.*, 1993).

Binding of NO

The actions of NO itself can be blocked by the use of haemoglobin which binds NO with high affinity and thus inactivates it (Gibson & Roughton, 1957). Haemoglobin is effective at inhibiting NO produced by cNOS, i.e. inhibits ACh induced relaxation in rabbit aortic rings (Martin *et al.*, 1985). Haemoglobin also inhibits glyceryl trinitrate, which induces relaxation by releasing NO (Harrison & Bates, 1993), suggesting that the inhibition occurs once NO has been released (Martin *et al.*, 1985). The actions of NO produced by iNOS are also inhibited, i.e. haemoglobin inhibits interleukin-1 (IL-1) induced increases in cyclic GMP in rat vascular smooth muscle cells (Beasley *et al.*, 1991).

Other compounds which bind NO include carboxy PTIO and hydroxocobalamin. Carboxy-PTIO has been shown to inhibit exogenous NO- and agonist-induced (ACh and ATP) relaxation of rat aortic rings (Rand & Li, 1995). Similarly, hydroxocobalamin also inhibits exogenous NO- and agonist-induced (ACh) relaxation of rat aortic rings (Rajanayagam *et al.*, 1993).

Inhibition of guanylate cyclase

Inhibition of the effects of NO can be produced by inhibiting soluble guanylate cyclase, the second messenger system which leads to relaxation of smooth muscle cells (Rapoport & Murad, 1983). Methylene blue has been reported to inhibit guanylate cyclase by oxidising the haem moiety of soluble guanylate cyclase (Martin *et al.*, 1985). There is, however, controversy as to the precise mechanism of action of methylene blue. Some recent studies now suggest that it may act additionally to inhibit NOS by oxidation of the haem moiety on

the enzyme (Marczin *et al.*, 1992), or via an interaction with molecular oxygen to generate superoxide anion (McCord & Fridovich, 1970; Keaney *et al.*, 1994), which then inactivates NO (Gryglewski *et al.*, 1986).

Interactions between NO and superoxide anion

The interactions between NO and superoxide anion represent an important mechanism of inactivation of NO (Gryglewski *et al.*, 1986). Superoxide anion reacts with NO to form peroxinitrite, a powerful cytotoxic oxidant by the following reaction:

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

Peroxynitrite itself is unstable and spontaneously rearranges to form nitrate:

$$ONOO^{-} \rightarrow NO_3^{-}$$

Destruction of NO leads to the loss of vasodilation as shown by cascade bioassay (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986; Moncada *et al.*, 1986). The ability of superoxide anion to inhibit basal and agonist-induced activity of NO, however, differs in rat aortic rings. Basal activity of NO is more sensitive to destruction by superoxide anion than agonist-induced activity (Mian & Martin, 1995). Superoxide anion can be formed spontaneously by tissues and may play a role in the vascular damage associated with ischaemia-reperfusion and other pathologies (Downey, 1990).

The destruction of NO by superoxide anion can be examined by adding a number of different compounds which are capable of generating the free radical. These systems include pyrogallol (Moncada *et al.*, 1986), which autoxidises leading to formation of the radical (Marklund & Marklund, 1974).

Other quinone compounds such as LY 83583 (Mülsch *et al.,* 1988; Mülsch *et al.,* 1989) also generate superoxide anion but only in the presence of strong reducing enzymes (Mülsch *et al.,* 1988; Mülsch *et al.,* 1989). Superoxide anion is probably generated from the reaction of a 1 electron reduced form of LY 83583, probably the semiquinone radical, with molecular oxygen (Mülsch *et al.,* 1988; Mülsch *et al.,* 1989), as described for other quinone compounds (Hassan & Fridovich, 1979). LY 83583 can produce superoxide both intracellularly and extracellularly.

The enzyme system hypoxanthine/xanthine oxidase is also commonly used to generate superoxide anion (McCord & Fridovich, 1968). The production of superoxide anion by this enzyme system generates the radical only extracellularly. This reaction involves xanthine oxidase acting on hypoxanthine to produce xanthine and superoxide anion. The product, xanthine, is itself metabolised generating further superoxide anion.

All of these superoxide anion generating systems have been shown to destroy EDRF activity in cascade bioassay systems (Gryglewski *et al.,* 1986; Downey, 1990) and in isolated vascular preparations (Cosentino *et al.,* 1994; Mülsch *et al.,* 1988; Katusic & Vanhoutte, 1989; Ohlstein & Nichols, 1989; Mian & Martin, 1995). In this study we will examine two superoxide generating systems, LY 83583 and hypoxanthine/xanthine oxidase.

Superoxide anion itself is destroyed by superoxide dismutase (SOD) to form hydrogen peroxide by the following reaction:

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

Hydrogen peroxide itself is highly toxic to cells and results in impairment of EDRF-dependent relaxation of rabbit aortic rings (Dowell *et al.*, 1993). Hydrogen peroxide is destroyed by catalase by the following reaction:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

As already stated, superoxide anion is itself destroyed by superoxide dismutase. The effects of destroying superoxide anion can be studied by adding exogenous superoxide dismutase but this is a large enzyme which will only be effective extracellularly. The role of endogenous superoxide dismutase can be examined by using inhibitors of this enzyme which include diethyldithiocarbamate (Heikkila *et al.*, 1976; Mian & Martin, 1995). Diethyldithiocarbamate is a powerful copper chelator which irreversibly binds and inactivates the copper/zinc-containing but not the manganese-containing isoforms of superoxide dismutase (Heikkila *et al.*, 1976).

Inhibitors of expression of iNOS

The expression of iNOS stimulated by LPS in macrophages can be inhibited by steroids such as dexamethasone and hydrocortisone but not progesterone (Di Rosa *et al.*, 1990). Similarly, expression of iNOS by LPS in the lung, liver and aorta of rats was inhibited by dexamethasone and hydrocortisone (Knowles *et al.*, 1990b; Rees *et al.*, 1990a). This inhibition prevents the *de novo* synthesis of proteins probably at the level of transcription of mRNA (Baydoun *et al.*, 1993). Cycloheximide also inhibits the expression of iNOS by LPS in macrophages (Rees *et al.*, 1990a; Baydoun *et al.*, 1993) by its ability to inhibit protein synthesis. These inhibitors have no effect on cNOS (Knowles *et al.*, 1990a) and do not affect the activity of iNOS once expressed (Di Rosa *et al.*, 1990).

The expression of iNOS in rat smooth muscle cells can also be inhibited by certain growth factors such as transforming growth factor-β1 and platelet derived growth factor$_{AB}$ and $_{BB}$ (Junquero *et al.*, 1992; Schini *et al.*, 1992; Scott-Burden *et al.*, 1993). Transforming growth factor-β1 has also been shown to inhibit expression of iNOS in murine macrophages (Chesrown *et al.*, 1994). Furthermore, inhibition of the expression of iNOS by retinoids (all-*trans*-retinoic acid and its active analogues) may be mediated by induction of transforming growth factor-β1 (Hirokawa *et al.*, 1994b). These agents act by preventing the induction of iNOS mRNA (Perrella *et al.*, 1994). Transforming growth factor-β1 and platelet derived growth factor$_{AB}$ and $_{BB}$ are present in serum, and thus the presence of serum in the tissue culture medium may inhibit the expression of iNOS in smooth muscle cells grown in culture.

Certain cytokines can also inhibit the expression of iNOS in murine macrophages and these include interleukin-4 and interleukin-10 (Oswald *et al.*, 1992; Liew *et al.*, 1991; Chesrown *et al.*, 1994). However, there is now controversy over the actions of interleukin-10, as recent findings indicate that in combination with IFN-γ it produces a dramatic augmentation in mRNA levels for iNOS (Chesrown *et al.*, 1994).

Thaliporphine, an aporphine derivative, also inhibits the expression of iNOS stimulated by LPS in macrophages (Yu, 1994), but has no effect on cNOS (Yu, 1994).

None of the above inhibitors directly block the activity of iNOS or cNOS.

Inhibition of tetrahydrobiopterin

As already stated, tetrahydrobiopterin is a cofactor in the production of NO from L-arginine (Mülsch & Busse, 1991; Schmidt *et al.*, 1992). Consequently,

inhibiting the synthesis of tetrahydrobiopterin would be expected to inhibit the synthesis of NO. Furthermore, enhanced *de novo* synthesis of tetrahydrobiopterin has been reported to occur concomitantly with expression of iNOS in smooth muscle (Hattori & Gross, 1993). The rate-limiting enzyme in the synthesis of tetrahydrobiopterin, GTP-cyclohydrolase I, can be inhibited by 2,5-diamino-6-hydroxypyrimidine (Schmidt *et al.*, 1992; Sakai *et al.*, 1992; Schoedon *et al.*, 1993), leading to reduced production of NO by iNOS in murine macrophages (Bogdan *et al.*, 1995).

Haem binders

L-Thiocitrulline binds to the haem of NOS and thus prevents L-arginine binding and the production of NO (Frey *et al.*, 1994). Carbon monoxide also binds to haem and can prevent L-arginine binding. However, carbon monoxide can also bind to the haem of soluble guanylate cyclase, leading to activation of the enzyme, and smooth muscle relaxation which is NO-independent (Furchgott & Jothianandan, 1991).

Flavoprotein binders

As already stated, FMN and FAD are essential cofactors for NOS (Stuehr *et al.*, 1991a). Inhibitors of flavoproteins would thus be expected to block the actions of NOS. Diphenyleneiodonium, di-2-thienyl iodonium and iodonium diphenyl have been shown to inhibit the actions of cNOS in endothelial cells and iNOS in macrophages (Stuehr *et al.*, 1991b).

Calmodulin inhibitors

Calmodulin is required as an additional cofactor for cNOS (Förstermann *et al.*, 1991; Pollock *et al.*, 1991; Schmidt *et al.*, 1991). This accounts for the ability of

the calmodulin antagonists, calmidazolium and trifluoperazine, and the calmodulin binding protein calcineurin to inhibit NO formation in bovine aortic endothelial cells (Förstermann *et al.*, 1991). Since in iNOS calmodulin is tightly bound, these inhibitors have no effect (Cho *et al.*, 1992).

Chapter 2
Endotoxic Shock

Sepsis and its associated syndromes (table 2.1) represent a potentially devastating systemic inflammatory response estimated to occur in 1% of hospitalised patients, 10 - 20% of whom die. Mortality approaches 60 - 90% in those who develop septic shock, a figure which has changed little since intensive care was first developed (Hess *et al.*, 1981). Septic shock has traditionally been recognised as a consequence of Gram-negative bacteraemia, but it may also be caused by Gram-positive organisms, fungi and possibly viruses and parasites (Bone *et al.*, 1987; Ispahani *et al.*, 1987; Calandra *et al.*, 1988; Glauser *et al.*, 1991). The mortality of septic shock does not depend on the type of organism responsible (Bone *et al.*, 1987; Ispahani *et al.*, 1987; Calandra *et al.*, 1988 & Glauser *et al.*, 1991).

Lipopolysaccharide (LPS)

The prime initiator of Gram-negative bacterial septic shock is endotoxin, a lipopolysaccharide (LPS) component of the bacterial outer membrane. The structure of LPS is shown in fig 2.1. The outermost part of the endotoxin molecule consists of a series of oligosaccharides that are structurally and antigenically diverse. The internal part of the endotoxin molecule is the core oligosaccharides, and these are structurally rather similar in common Gram-negative bacteria. To the core oligosaccharide is bound a lipid component, lipid A. The structure of lipid A is highly conserved, and it is lipid A that is responsible for most of the toxicity of endotoxin (Glauser *et al.*, 1991; Rietschel *et al.*, 1993).

Disease state

Septic shock is predominantly responsible for circulatory failure which is characterised by severe hypotension, hyporeactivity to endogenous and

1 Sepsis
'The systemic response to infection' Includes two or more of the following Temperature > 38°C or < 36°C Heart Rate > 90 beats/min Respiratory rate > 20 breaths/min or $PaCO_2$ < 4.3 kPa Leucocyte count > 12000/mm^3 < 4000/mm^3, or > 10% band (immature) forms
2 Sepsis syndrome
'Sepsis with evidence of altered organ perfusion' Altered organ perfusion includes one or more of the following PaO_2/FiO_2≤280 (without other cardiopulmonary disease) Elevated lactate level (> upper limit of normal for the laboratory) Oliguria < 0.5 ml/kg body weight
3 Systemic inflammatory response syndrome
'The response to a variety of severe clinical insults (not necessarily infection), which is indistinguishable from sepsis'
4 Septic shock
'Sepsis with hypotension (sustained decrease in systolic blood pressure < 90 mmHg, or drop > 40 mmHg, for at least 1 h) despite adequate fluid resuscitation, in the presence of perfusion abnormalities that may include, but not be limited to lactic acidosis, oliguria or an acute alteration in mental status.' Patients who are on inotropic or vasopressor agents may not be hypotensive at the time perfusion abnormalities are measured.

Table 2.1: Definitive descriptions of sepsis and septic shock. Descriptions are from Bone *et al.* (1992) except that for sepsis syndrome, which is adapted from Bone (1991). Table is adapted from Curzen *et al.* (1994). Abbreviations: PaO_2, Arterial partial pressure of oxygen; $PaCO_2$, Arterial partial pressure of carbon dioxide; FiO_2, fraction of inspired oxygen.

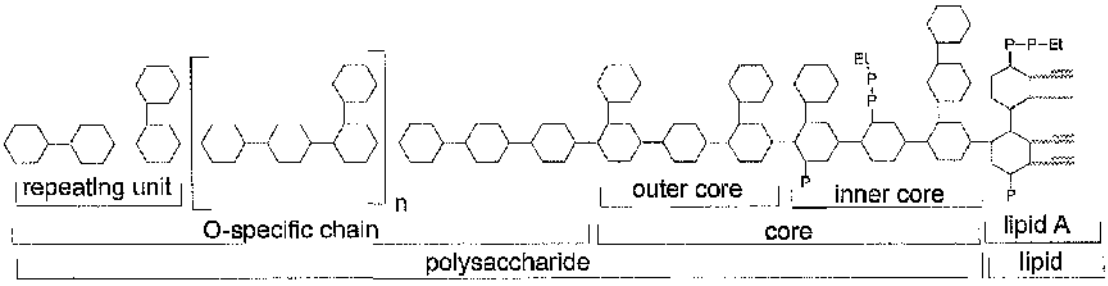


Figure 2.1: Diagramatic representation of the structure of endotoxin (lipopolysaccharide). ⬡ represents monosaccharide, P represents phosphate, Et represents ethanolamine and 〰 represents long chain (hydroxyl) fatty acid. Modification of a diagram by Glauser *et al.* (1991).

exogenous vasoconstrictor agents, myocardial dysfunction, maldistribution of organ blood flow, and reduced tissue oxygen extraction, which ultimately lead to multiple organ failure and death (Thiemermann, 1994). Septic shock affects other systems in addition to the circulation, and these are listed in table 2.2.

It has been suggested that the circulatory failure may occur due to the hyperproduction of the potent endogenous vasodilator nitric oxide (NO). LPS and cytokines, as already stated, can induce the expression of inducible nitric oxide synthase (iNOS) in a variety of cell types and thus increase the production of NO. The role of NO in circulatory failure is discussed in the next few sections.

Hypotension

Septic shock, induced by LPS in mice and rats, is associated with an increased production of NO assessed as an increase in nitrosylated haemoglobin measured by electron paramagnetic resonance (EPR; Kosaka *et al.*, 1992). This increased production of NO contributes to the hypotension caused by endotoxin in the anaesthetised rat as it is reversed by intravenous infusion of the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) (Thiemermann & Vane, 1990). Similarly, in the anaesthetised dog infusion of LPS causes a decrease in peripheral vascular resistance and a fall in mean arterial blood pressure, i.e. hypotension, which is reversed by L-NMMA (Kilbourn *et al.*, 1990b). The finding that the increase in blood pressure caused by L-NMMA in dogs with the early stages of endotoxic shock is significantly greater than that elicited in control animals (Kilbourn *et al.*, 1990b; Klabunde & Ritger, 1991) supports the view that even brief periods of endotoxemia are associated with an enhanced formation of NO.

Central nervous system	Encephalopathy Agitation
Gastrointestinal	Cholestatic jaundice Gastric erosions in 100% (gross bleeding rare)
Blood	Early neutropenia, then neutrophilia disseminated intravascular coagulation, especially with Gram-negative bacteremia Thrombocytopenia in 50%
Renal	Proteinuria Focal proliferative glomerulonephritis Acute tubular necrosis
Metabolic	Hyper-, hypoglycemia Skeletal muscle proteolysis Hypertriglyceridemia Lactic acidosis
Pulmonary	Respiratory alkalosis Respiratory muscle fatigue/failure (terminal)

Table 2.2: Non-cardiovascular effects of septic shock. Table adapted from Dal Nogane (1991).

Continuous infusion of endotoxin for 24 h in anaesthetised Marino ewes resulted in a hyperdynamic model of circulatory shock characterised by reduced peripheral resistance, increased cardiac output, and increased pulmonary vascular resistance (Meyer *et al.*, 1992). Intravenous administration of the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), 24 h after commencing the endotoxin infusion, completely restored these alterations in peripheral resistance, cardiac output and blood-flow distribution in pulmonary vasculature (Meyer *et al.*, 1992). In rats, L-NMMA inhibited the increase in plasma levels of both nitrite and nitrate, the stable products of NO (Wennmalm *et al.*, 1992; Ignarro *et al.*, 1993), prevented the fall in blood pressure and improved the survival rate associated with LPS administration (Nava *et al.*, 1992). Furthermore, in a murine model of advanced sepsis caused by intraperitoneal application of live *Escherichia coli* bacteria, L-NMMA dose-dependently improved the survival rate when co-administered with a conventional antibiotic treatment, imipenem-cilastatin (Teale & Atkinson, 1992). Imipenem is a beta-lactam antibiotic which can be metabolised and partially inactivated in the kidney and is therefore administered in combination with cilastatin, a specific enzyme inhibitor, which blocks its renal metabolism. Thus, taken together, these studies indicate a crucial role for overproduction of NO in septic shock.

Although these findings support the view that inhibition of NOS with L-NMMA exerts beneficial hemodynamic effects and improves survival in endotoxic shock in various species, there is also convincing evidence that at higher doses NOS inhibitors exacerbate the circulatory failure caused by endotoxin. For instance, in anaesthetised rabbits subjected to endotoxemia for 3 h, intravenous bolus injection of a high dose of L-NMMA resulted in severe hypotension, intense vasoconstriction, and increased mortality (Wright *et al.*, 1992). Similarly, in a canine model of endotoxic shock, L-NMMA increased systemic vascular resistance, but significantly decreased cardiac output

(Klabunde & Ritger, 1991). The detrimental hemodynamic effects of high-dose L-NMMA in rabbits (Wright *et al.*, 1992) and rats (Nava *et al.*, 1992) with endotoxic shock are prevented by co-administration of the NO donor S-nitroso-N-acetylpenicillamine suggesting that the formation of NO by the endothelium is important for the maintenance of organ blood flow and ultimately tissue viability. Thus, although inhibition of NO production from iNOS is vital to prevent the hypotension of septic shock, inhibition of endothelial cNOS must not be allowed to occur.

Selective inhibitors of iNOS, having little or no effect on cNOS, would be advantageous in treating septic shock. Such inhibitors include aminoguanidine and L-canavanine (Stuehr *et al.*, 1991b; Hasan *et al.*, 1993; Misko *et al.*, 1993) but these have still to be evaluated in animal models of septic shock.

Administration of dexamethasone, an inhibitor of the expression of iNOS, prior to LPS, prevents the expected decrease in blood pressure, again suggesting the hypotension is a result of increased production of NO (Nava *et al.*, 1992; Hom *et al.*, 1995).

There is increasing evidence that septic shock in man is associated with enhanced formation of NO. The relationship between plasma levels of nitrate, the major metabolite of NO (Wennmalm *et al.*, 1992; Ignarro *et al.*, 1993), and the observed hemodynamic alterations were recently investigated in 39 critically ill trauma and septic patients (Ochoa *et al.*, 1992). These findings indicated a good correlation between the levels of nitrate and the severity of symptoms. Two case studies have now examined the beneficial effects of inhibition of NOS in patients with septic shock. In the first study, two patients with septic shock received L-NMMA or L-NAME (Petros *et al.*, 1991). Both patients had severe hypotension, which was resistant to conventional therapy. Injection of L-NMMA caused a dose-dependent increase in blood pressure and

peripheral vascular resistance. In the second study, a randomised double blind placebo-controlled investigation of 12 patients with septic shock, L-NMMA caused an increase in vascular tone and increased blood pressure but it also caused a fall in cardiac output (Petros *et al.,* 1994). Thus, NO appears to be an important mediator in septic shock, but more studies are required to examine the effects of NOS inhibitors on cardiac output, morbidity and mortality.

It is not only LPS that can be used experimentally to mimic clinical septic shock. Agents known to stimulate the expression of iNOS, including cytokines such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and interleukins-1 and -2 (IL-1, IL-2), have been shown to induce shock-like symptoms in experimental animals (Kilbourn *et al.,* 1990a; Gray *et al.,* 1991; Hibbs *et al.,* 1992; Ochoa *et al.,* 1992; Szabó *et al.,* 1993c). Similarly, these septic shock-like symptoms can be reversed by treatment with NOS inhibitors, L-NMMA or L-NAME (Kilbourn *et al.,* 1990a; Gray *et al.,* 1991).

Vascular hyporeactivity to vasoconstrictor agents

It is well documented that the peripheral vascular failure associated with septic shock in animals (Parratt, 1973) and man (Teale & Atkinson, 1992) is also associated with a reduction of the pressor response caused by vasoconstrictor agents, ultimately resulting in therapy-resistant hypotension and multiple organ failure. Similarly, isolated vascular rings either treated with LPS or obtained from animals subjected to prolonged periods of endotoxemia show a reduced contractile response to noradrenaline (Parratt, 1973), vasopressin (Schaller *et al.,* 1985), angiotensin II (Schaller *et al.,* 1985), serotonin (Wakabayashi *et al.,* 1987), histamine and potassium chloride (Wakabayashi *et al.,* 1987). The finding that inhibition of NOS with L-NMMA reverses the depression of the pressor effects of noradrenaline observed in rats with endotoxic shock

suggests that an enhanced formation of NO accounts for this vascular hyporeactivity (Gray *et al.*, 1991; Julou-Schaeffer *et al.*, 1990). There is now increasing evidence that excessive formation of NO by vascular smooth muscle cells exposed to LPS for several hours (3 - 24) accounts for the vascular hyporeactivity to vasoconstrictor agents (Fleming *et al.*, 1990; Rees *et al.*, 1990a).

In addition to conductance and resistance vessels, endotoxin also induces NOS in various venous vessels *in vitro* (Parratt, 1973). This NOS induction contributes to the hyporeactivity to vasoconstrictor agents demonstrated in the jugular vein from rabbits treated with endotoxin (Vallance *et al.*, 1992). Thus, it is conceivable that enhanced formation of NO in the venous circulation contributes to the development of venous pooling and reduced venous return associated with endotoxemia. Although the pressor responses elicited by systemic application of various vasoconstrictor agents are reduced in animal models of septic shock, induction of NOS does not necessarily result in vascular hyporeactivity in all vascular beds. For instance, NOS induction in the heart is associated with a NO-mediated vascular hyporeactivity to the thromboxane analogue U46619 (Smith *et al.*, 1991). In contrast, the vasoconstrictor effects caused by U46619 in the mesenteric artery and vein are not diminished despite induction of NOS and this may be due to an increased sensitivity of the receptors (Mitchell *et al.*, 1993).

Endotoxemia for 3 - 24 h in anaesthetised rats resulted in the induction of a calcium-independent NOS isoform in various organs including lung, spleen, mesentery, liver, kidney, heart and aorta (Knowles *et al.*, 1990b; Salter *et al.*, 1991). The increase in a calcium-independent NOS activity after a single injection of endotoxin is maximal within approximately 6 h and returns to baseline within 24 h (Knowles *et al.*, 1990b; Salter *et al.*, 1991). Moreover, endotoxin and certain cytokines induce the *de novo* biosynthesis of a calcium-

independent NOS in endothelium-denuded segments of rabbit (Busse & Mülsch, 1990) and rat aorta (Rees *et al.,* 1990a), and this effect is prevented by dexamethasone (Rees *et al.,* 1990a) and by inhibition of protein biosynthesis with cycloheximide (Busse & Mülsch, 1990; Rees *et al.,* 1990a). These findings suggest that enhanced formation of NO by iNOS accounts for both the hypotension and vascular hyporeactivity to vasoconstrictor agents elicited by endotoxin. Indeed, pre-treatment of rabbits (Wright *et al.,* 1992) and rats (Szabó *et al.,* 1993b) with dexamethasone prevents the hypotension (rabbit and rat) and attenuation of the pressor response to noradrenaline (rat) caused by prolonged periods of endotoxemia. However, enhanced formation of NO also accounts for the hypotension and vascular hyporeactivity observed after brief periods of endotoxemia (60 min) in anaesthetised rats, where expression of iNOS had not yet occurred (Julou-Schaeffer *et al.,* 1990). Thus, both activation of endothelial cNOS (early phase) and expression of iNOS (delayed phase) contribute to enhanced formation of NO in endotoxemia. The mechanism by which endotoxin activates the cNOS is not well characterised. It is possible that endotoxin may stimulate the release of autacoids, including bradykinin and platelet-activating factor, which in turn may activate cNOS in the endothelium (Fleming *et al.,* 1992).

Agents known to stimulate expression of iNOS including cytokines such as IFN-γ, TNF-α, IL-1β and IL-2 also cause depression of the contractile effects of noradrenaline and phenylephrine (Busse & Mülsch, 1990; Alonso *et al.,* 1993). The NOS inhibitors L-NAME and L-NMMA reverse this depression (Busse & Mülsch, 1990; Alonso *et al.,* 1993), confirming that it results from enhanced formation of NO.

The smooth muscle layer has been reported to be the major site at which iNOS is expressed in the vascular wall (Knowles *et al.,* 1990a), and indeed, expression of iNOS can be stimulated in vascular smooth muscle cells in

culture following treatment with IL-1β, TNF-α, IFN-γ or a combination of IFN-γ and LPS (Busse & Mülsch, 1990; Beasley *et al.*, 1991; Kanno *et al.*, 1993; Koide *et al.*, 1994). Although LPS alone has been reported to stimulate the expression of iNOS in rat aortic smooth muscle cells in culture (Marczin *et al.*, 1993; Szabó *et al.*, 1993a), this is disputed by Sirsjö *et al.* (1994), who suggested that the additional presence of a cytokine was required.

Myocardial dysfunction

Prolonged periods of circulatory shock are associated with a reduction in cardiac contractility and, hence, cardiac output. In rats, prolonged periods of endotoxemia are associated with time-dependent induction (minimum: 6h) of calcium-independent NOS activity in cytosolic preparations of the myocardium (Schulz *et al.*, 1992). NOS induction also occurs in rat cardiac myocytes treated with TNF-α or IL-1β (Schulz *et al.*, 1992). The negative inotropic effect caused by TNF-α, IL-1β or IL-6 in isolated papillary muscle of the hamster is reversed by L-NMMA, suggesting that the reduction in myocardial contractility caused by these cytokines is mediated by NO (Finkel *et al.*, 1992). Moreover, porcine ventricular endocardial cells express a calcium-independent NOS activity when treated with endotoxin, TNF-α or IL-1 (Smith *et al.*, 1993). Isolated cardiac myocytes obtained from endotoxin-treated guinea pigs exhibit a significant reduction in the contractile response elicited by electrical stimulation. This depressed cardiac contractility is due to enhanced formation of NO by iNOS for it is prevented by pre-treatment of the animals with dexamethasone or reversed by L-NMMA or L-NAME *in vitro* (Brady *et al.*, 1992). Expression of iNOS in ventricular myocytes also attenuates the positive inotropic effects elicited by isoprenaline (Balligand *et al.*, 1993). Thus, enhanced formation of NO by iNOS may contribute to the myocardial dysfunction associated with endotoxemia. However, an improvement of

cardiac performance by NOS inhibitors in animal models of endotoxemia has yet to be documented.

Dysfunction of the endothelium

Although enhanced formation of NO by either cNOS (early phase of endotoxemia) or iNOS (after several h of endotoxemia) is well documented, it should be noted that prolonged periods of endotoxemia also result in an inhibition of the NO-mediated vasodilator effects elicited by endothelium-dependent vasodilators. Isolated vessels, including mesenteric, femoral and renal artery as well as the thoracic aorta obtained from animals subjected to prolonged periods of endotoxemia, exhibit reduced responses to endothelium-dependent vasodilators *ex vivo* (Julou-Schaeffer *et al.,* 1990; Parker & Adams, 1993). Interestingly, the degree of inhibition of this receptor-mediated release of NO from the endothelium is largely dependent on the vascular preparation (Li *et al.,* 1992). Specifically, the relaxation produced by acetylcholine (ACh) is unaltered in the ear artery but attenuated in the perfused kidney (Li *et al.,* 1992). Similarly, inhibition of the responses to endothelium-dependent vasodilators occurs in various vessels (middle cerebral artery, renal artery, thoracic aorta) of animals with haemorrhagic shock (Wang *et al.,* 1993; Wang *et al.,* 1994). This inhibition is already significant in the early phases of haemorrhagic shock, persists despite resuscitation with fluids (Wang *et al.,* 1993), and is prevented with heparin (Wang *et al.,* 1994).

The mechanism of this inhibition of NO release from the endothelium of animals with either endotoxic or haemorrhagic shock is not fully characterised, but may involve: (i) inactivation of NO by oxygen-derived free radicals produced, for example by activated neutrophils (Csaki *et al.,* 1991); (ii) down-regulation of cNOS in the endothelium by TNF-α or other cytokines released by endotoxin (Yoshizumi *et al.,* 1993); or (iii) endothelial cell damage due to

the cytotoxic effects of NO itself. Indeed, activation of endothelial cells with cytokines or endotoxin results in endothelial cell death, which can be prevented by L-NMMA or dexamethasone (Estrada *et al.*, 1992; Palmer *et al.*, 1992). These findings suggest that the formation of large quantities of NO following iNOS expression in endothelial cells exerts a cytotoxic effect on the endothelium. Moreover, NO irreversibly inhibits the activity of a number of enzymes, including cNOS (Mitchell *et al.*, 1991) and iNOS (Assruey *et al.*, 1993). However, the relative importance of this 'autoinhibition' of cNOS or iNOS in endotoxemia is not clear.

Our study

In this study we wished to examine the factors controlling expression of iNOS in the vascular smooth muscle cells of rat aorta. We used a similar technique to Rees *et al.* (1990a) where endothelium-denuded rings of rat aorta were incubated overnight (18 h) at 37°C in Krebs solution with and without the addition of endotoxin. The expression of iNOS was assessed indirectly by examining the depression of phenylephrine (PE)-induced contractions. To examine whether depression of contraction resulted from production of NO by iNOS we examined the effects of the inhibitor of NOS, L-NAME (Rees *et al.*, 1990b), and the inhibitor of the expression of iNOS, dexamethasone (Rees *et al.*, 1990a). We also examined the effects of the scavenger of LPS, polymixin B, to determine if the depression of PE-induced contraction obtained in the absence of added endotoxin resulted from contamination of the Krebs by endotoxin. The effects of drugs that interfere with NO activity were also examined to determine if the depression of contraction was indeed due to this mediator; these included haemoglobin which binds and inactivates NO (Gibson & Roughton, 1957; Martin *et al.*, 1985) and the superoxide anion generators, LY 83583 (Mülsch *et al.*, 1988; Mülsch *et al.*, 1989) and the xanthine/xanthine oxidase system (McCord & Fridovich, 1968).

The induction process was also examined by using antibodies to certain cytokines in order to establish if LPS had a direct effect on the vascular smooth muscle or whether it acted on other cell types in the vessel wall, such as macrophages, releasing cytokines which then stimulated expression of iNOS in the vascular smooth muscle cells. The actions of pentoxifylline which inhibits transcription of mRNA for TNF-α (Strieter *et al.*, 1988; Han *et al.*, 1990; Doherty *et al.*, 1991) were also examined.

A second part of the study was an examination of the processes involved in the expression of iNOS in rat cultured aortic vascular smooth muscle cells (RASMC). Here, iNOS expression was measured as a function of the accumulation of nitrite, the major *in vitro* breakdown product of NO (Wennmalm *et al.*, 1992; Ignarro *et al.*, 1993). The ability of LPS and cytokines (IFN-γ and IL-1α) to stimulate expression of iNOS were examined and compared with their activities on aortic rings.

Some experiments were also conducted on bovine aortic endothelial cells (BAEC) and on J774.2 murine macrophages in culture to examine the processes regulating expression of iNOS in these cell types.

Chapter 3
Nitric Oxide Detection Systems

Measurement of nitric oxide (NO) in biological samples is difficult both because of the small amounts present and its short half-life of 3 - 50 s (McCall & Valliance, 1992). The presence of NO in biological systems is usually inferred on the basis of one of its physiological effects, such as the relaxation of blood vessels, activation of guanylate cyclase activity, increased cyclic GMP concentration, production of L-citrulline or inhibition of platelet aggregation. Also, inhibitors of NO such as analogues of L-arginine or molecules which bind and inactivate NO such as haemoglobin, have been used to estimate NO production. Only the cyclic GMP or L-citrulline concentration can be used to obtain quantitative information relating to the amount of NO produced, but even these results may be misleading. The currently used instrumental techniques for NO detection are the chemiluminescence reaction with acid reflux (Palmer *et al.,* 1987; Myers *et al.,* 1990) or without acid reflux (Myers *et al.,* 1989), spectrophotometric assay involving the diazotization of sulfanilic acid (Green *et al.,* 1982; Ignarro *et al.,* 1987; Schmidt *et al.,* 1988; Tracey *et al.,* 1990), electron paramagnetic resonance (EPR) with nitroso or haemoglobin traps (Arroyo & Kohno, 1991), and spectrophotometric measurement of the conversion of oxyhaemoglobin to methaemoglobin (Ignarro *et al.,* 1987; Kelm *et al.,* 1988; Kelm & Schrader, 1988). Other detection systems include mass spectrometry (Gustafsson *et al.,* 1991), gas chromatography (Pai *et al.,* 1987), NO-sensitive electrodes (Shibuki, 1990; Malinski & Taha, 1992) and a fluorometric assay (Damiani & Burini, 1986; Misko *et al.,* 1993).

Relaxation of tissues

Relaxation of blood vessels occurs in the presence of NO as described earlier and in fig. 1.3. If a blood vessel is contracted, e.g. by phenylephrine (PE) or noradrenaline, and an agent then causes a relaxation this may be due to release of endothelial NO. To test this inhibitors of nitric oxide synthase (NOS),

such as L-NAME, L-NMMA and L-NOARG, can be used to see if they reverse the relaxation. Alternatively, compounds which bind and inactivate NO, such as haemoglobin, could be used. Attempts could be made to estimate the amount of NO produced by comparing the magnitude of vasodilation obtained with that produced by known concentrations of NO. Alternatively, a cascade bioassay system could be used, e.g. using endothelium-containing vessels as a donor and endothelium-denuded vessel downstream as a detector. Again, amounts of NO released could be assessed by comparing the degree of relaxation of the detector produced with that obtained to known standards of NO.

Another way of assessing, at least indirectly, the activity of NO is to examine depression in the contraction produced by a vasoconstrictor such as PE. This technique has been used to assess basal NO tone in endothelium-containing blood vessels (Martin *et al.*, 1986) and demonstrate the presence of iNOS in endothelium-denuded vascular rings (Rees *et al.*, 1990a; this study).

L-Citrulline assay

This assay measures the amount of L-citrulline generated by NOS. L-Citrulline is the byproduct of the production of NO as indicated by the following reaction (Hibbs *et al.*, 1987):

$$\text{L-arginine} + O_2 \xrightarrow{\text{NOS}} \text{NO} + \text{L-citrulline}$$

The L-citrulline assay has been widely used to examine NOS activity (Rees *et al.*, 1990b; Pollock *et al.*, 1991; Bush *et al.*, 1992; Yu, 1994). Typically, cell homogenates are incubated in the presence of [^{3}H] L-arginine at 37°C for a set period of time. The activity of NOS is then stopped by the addition of perchloric acid. The reaction mixture is then passed through a dowex AG 50 W-X8 Na^+

column. This column binds L-arginine but allows L-citrulline to pass through. The column is then washed and the effluent is counted by liquid scintillation for the presence of [^{3}H] L-citrulline.

Cyclic GMP assay

NO activates soluble guanylate cyclase, increasing the production of cyclic GMP. The amount of cyclic GMP formed can be measured using a radioimmunoassay and used as an index of NO production (Rapoport & Murad, 1983). This assay relies on the competition between radioactive and non-radioactive antigen for a fixed number of binding sites. As the amount of antigen, cyclic GMP, increases it displaces the radioactive antigen from the binding sites and thus reduces the amount of radioactive antigen-antibody complex. A standard curve of cyclic GMP binding can be used to determine the amount of cyclic GMP in the samples. This is a sensitive assay but unfortunately it is not entirely specific for the increase in cyclic GMP stimulated by NO, as other agents can also increase the production of cyclic GMP without involving NO. For example, agonists such as atrial natriuretic factor (Rapoport, 1986) and arachidonic acid (Ignarro & Wood, 1987) may stimulate the particulate or the soluble form of guanylate cyclase increasing cyclic GMP thereby causing vasodilation by an NO-independent mechanism.

Chemiluminescence assay

This assay was originally developed to measure NO as an atmospheric pollutant but is now used to measure NO in biological systems (Palmer *et al.*, 1987; Cox, 1980; Braman & Hendrix, 1989). Detection of NO is based on the observation that NO spontaneously reacts with ozone to emit light by the following reactions:

$NO^{\bullet} + O_3 \rightarrow NO_2^{\bullet} + O_2$

$NO_2^{\bullet} \rightarrow NO_2 + h\nu$

Chemiluminescence, measured by a sensitive photomultiplier tube, is proportional to the NO levels present. The assay takes advantage of the low solubility of NO in aqueous solutions by measuring NO in the gas phase, whether the original sample was a gas or a liquid. In the reaction chamber, ozone, generated internally by electrical discharge, is mixed with NO in front of a cooled, red-sensitive photomultiplier tube.

Nitrite and nitrate, the major breakdown products of NO (Wennmalm *et al.*, 1992; Ignarro *et al.*, 1993), can also be measured using the chemiluminescence assay as these can be reduced to NO in the presence of strong reducing agents. Nitrite can be reduced to NO in the presence of agents such as sodium iodide in glacial acetic acid (Cox, 1980) or alternatively by 1'1'-dimethylferrocene in acetonitrile (Termin *et al.*, 1992). Nitrate can be reduced to NO in the presence of stronger reducing agents such as titanium trichloride or ammonium molydbate in sulphuric acid (Cox, 1980), or alternatively vanadium (III) chloride in hydrochloric acid (Braman & Hendrix, 1989). This is a quantitative and highly-sensitive technique, with a threshold detection level of 100 pmol (Braman & Hendrix, 1989; Cox, 1980).

Griess reaction

This two step assay is based on the observation that the adduct of nitroxides and sulfanilic acid reacts with N-(1-naphthyl) ethylenediamine, generating a product that is readily monitored by spectrophotometry at an absorbance of 548 nm (Green *et al.*, 1982; Tracey *et al.*, 1990). The NO level can be calculated by comparing the observed change in absorbance to a

NO/absorbance calibration curve. In this assay, absorbance at 548 nm should increase solely as a function of NO concentration. In view of the short half-life of NO, its major stable metabolite, nitrite, is normally the form measured in biological samples. This technique is less sensitive than chemiluminescence, having a threshold detection level of 500 - 1000 pmol (Green *et al.*, 1982; Tracey *et al.*, 1990).

Fluorometric assay

An alternative means of measuring nitrite, the major breakdown product of NO *in vitro*, involves a fluorometric assay. This is based on the reaction of nitrite with 2,3-diaminonaphthalene (DAN) in acidic solution to form 1-[H]-naphthotriazole, a highly fluorescent compound in alkaline medium (Damiani & Burini, 1986; Misko *et al.*, 1993). The concentration of the sample is measured using an excitation wavelength of 365 nm and read at an emission wavelength of 405 nm. This is intermediate in sensitivity between the chemiluminescence assay and the Griess reaction, having a threshold sensitivity of 120 pmol.

EPR assay

NO is a gaseous, paramagnetic molecule with an unpaired electron in the π orbital (Arroyo & Kohno, 1991). The basic principle of detecting radicals by EPR spectroscopy is that at a discrete energy level (microwave frequency) and magnetic field strength, unpaired electrons are promoted to higher energy levels; relaxation from this state produces a characteristic spectrum. NO and NO_2 cannot be studied by simple EPR as the relaxation time of the stimulated electron to ground state is too rapid to be detected (Maples *et al.*, 1991).

Spin traps are compounds that interact with the unstable radicals, producing a more stable adduct that can be detected by EPR. The spin traps that can be used to detect NO include nitroxide compounds and haemoglobin (Arroyo & Kohno, 1991). Nitroxide traps are stabilised by the resonance of the unpaired electron. There are two types of nitroxide spin trap, the nitrones and the nitroso compounds. The nitroso compounds usually provide the best information about the identity of a radical, as the radical is added directly to the nitroso nitrogen, thereby increasing information in the hyperfine splitting parameters (Bueltner, 1987). However, oxygen-based adducts of the nitroso compounds are unstable and one must usually rely on the nitrones for such studies. The identity of the radical, defined by EPR, is characterised by the magnitude and multiplicity of the hyperfine splitting of the spin adduct's spectra. The amount of radical present is proportional to the magnitude of the signal (Maples *et al.*, 1991; Bueltner, 1987).

A disadvantage of this technique is that EPR spectrometers are not readily available to most biologists. The magnetic field is usually set at or near 330 mT, with a microwave power of 8 - 10 mW, frequency of 9 GHz, and modulation amplitude of 0.1 mT. Experiments need to be performed in darkness to prevent photolysis of the spin traps. When NO interacts with the nitroso compounds (e.g. 2-methyl-2-nitrosopropane and 3,5,-dibromo-4-nitrosobenzene), a triplet signal is formed that can be characterised by its line width and hyperfine coupling constant. This technique has been used for the detection of NO produced by macrophages (Henry *et al.*, 1991).

Methaemoglobin spectrophotometry assay

This technique is based on the rapid oxidation of reduced haemoglobin (Fe^{2+}) to methaemoglobin (Fe^{3+}) by NO rather than on detection of the paramagnetic

NO-Hb complex, which breaks down rapidly in oxygenated solutions (Kelm & Schrader, 1988; Ignarro *et al.*, 1987; Kelm *et al.*, 1988). The reaction is shown below:

$NO + Hb(Fe^{2+})O_2 \rightarrow Hb(Fe^{3+}) + NO_3^-$

The oxidation of reduced haemoglobin by NO occurs in less than 100 ms, suggesting the assay is sufficiently dynamic to study the time course of NO synthesis (Kelm *et al.*, 1988). The threshold for the detection of NO using this assay is excellent at around 1 nM (Kelm *et al.*, 1988), which is within the range of concentrations produced by endothelial cells.

Commercially available haemoglobin is 50 - 95% methaemoglobin and must be reduced with a molar excess of dithionite. NO is detected by observing the characteristic shift in the Soret absorbance peak of haemoglobin from 433 nm to 406 nm (Ignarro *et al.*, 1987; Kelm *et al.*, 1988). The presence of oxyhaemoglobin is not a problem in this assay due to a much higher affinity of haemoglobin for NO than O_2. Furthermore, oxyhaemoglobin can be detected by its Soret peak (416 nm). Conversion of reduced to methaemoglobin can be measured as the excitation difference at 401 and 411 nm (the isobestic point).

Gas chromatography

Although NO can be detected by standard gas chromatography techniques, this assay is much less sensitive than chemiluminescence or EPR assays (Pai *et al.*, 1987). Pai *et al.* (1987) used a Varian gas chromatography with a Porapak Q column and electron capture detector to measure enzymatically generated NO. They attributed the low sensitivity of this assay to the sample processing methodology required for gas chromatography.

Mass spectroscopy

Gustafsson *et al.* (1991) used mass spectroscopy to measure NO in the breath of guinea-pigs. They trapped NO as nitrosothioproline by bubbling the expirate through a degassed aqueous solution of thioproline (15 nM). The nitrosothioproline was extracted with ethyl acetate, and dried and derivatized by N-methyl-N-(tertbutyldimethylsilyl) trifluroacetamide in acetonitrile. Samples were placed on a CP-Sil-5-CB column interfaced with a mass spectrometer operating in the positive chemical ionisation mode with isobutane. The derivatized form of nitrosothioproline had a retention time of 4.76 min (Gustafsson *et al.*, 1991). It is not clear whether this assay is more sensitive than measuring breath chemiluminescence as the readings were in arbitrary units with no calibration with authentic NO. The principle of trapping NO in degassed water for subsequent analysis, regardless of the technique, appears to be a reasonable strategy for measuring exhaled NO.

Microelectrode NO assay

There are currently two amperometric methods available for measuring NO from intact tissues and single cells. Shibuki (1990) recently reported the measurement of NO release in the cerebellum using a microelectrode. He modified a miniature O_2 electrode by sealing its fire-polished 150 - 250 µm tip with a thin chloroprene rubber seal so that only low molecular weight gases could enter. By introducing a platinum cathode within the pipette and holding it at a positive voltage, he was able to detect NO, a readily oxidised gas, by its oxidation at the electrode's surface (Shibuki, 1990). Pipettes were filled with 30 mM NaCl and 0.3 mM HCl (pH 3.5), and a Teflon-coated platinum wire was placed as close as possible to the membrane. A 200 µm silver wire was the anode. The cathode was kept 0.9 V from the anode and the anode was grounded. The principle of the assay is that if the anode is grounded there

should be no current. There was a linear relationship between current and NO concentration *in vivo* over a small range (1 - 3 μM). Using this electrode, Shibuki (1990) was able to measure NO release in rat cerebellar tissue elicited by electrical stimulation of the white matter. The author claims that NO detection limits can easily be improved by signal averaging and that the threshold has now reached nanomolar sensitivity (Shibuki, 1990). The linear concentration range for NO has now been shown to be 0.2 nM - 1 μM (Ichimori *et al.*, 1994).

A second, more sensitive technique is based on the observation that metalloporphyrins catalyse the oxidation of NO, i.e. $NO^{\bullet} - e^{-} \rightarrow NO^{+}$, and in so doing generate electrical current. Malinski and Taha (1992) coated carbon fibres (chosen as a convenient, strong microprobe material) with a thin polymeric porphyrin layer and measured NO as the electrical current produced at 0.63 V. To minimise detection of NO_2^- they coated the carbon fibre with Nafion, a negatively charged material that is highly impermeable to anions. Nafion-coated fibres displayed no change in current when 20-fold excess NO_2^- was added to the sample. This technique permitted measurement of NO production from cultured endothelial and smooth muscle cells with a detection threshold of 10^{-20} M, a linear response to concentrations of up to 300 μM NO, and a response time of 10 ms. This technique offers great promise for the measurement of NO production in single cells, both in culture and *in situ*, once the technology is commercially available. This assay is stated to be quite specific for NO but also detects catecholamines.

Our study

In our study we compared the sensitivity and utility of a number of the above techniques for detection of nitrite and nitrate, the major stable metabolites of NO. Those examined were chemiluminescence using

1,1'-dimethylferrocene/acetonitrile or sodium iodide/glacial acetic acid reflux, the Griess reaction and the fluorometric assay which all measure nitrite. Chemiluminescence using vanadium chloride/hydrochloric acid reflux which measures nitrite and nitrate. We also used the indirect means of assessing NO by measuring the depression of PE-induced contraction of rat aortic rings and measured cyclic GMP content of cultured rat aortic smooth muscle cells.

Chapter 4
Methods

Rat Aortic Rings

Preparation of rat aortic rings

Male Wistar rats (200 - 300 g) were killed by stunning and exsanguination. The thoracic aorta was removed, trimmed of adhering fat and connective tissue and cut into 2.5 mm long transverse rings using a razor blade slicing device. In all experiments the endothelium was removed by placing the aortic ring between two stainless steel hooks under 1 g tension and gently rubbing the intimal surface with a moist match stick for 20 - 30 s. Removal of the endothelium was deemed successful if acetylcholine (ACh; 1 μM) failed to produce relaxation after pre-constriction with phenylephrine (PE ; 0.1 μM).

Tension recording

The aortic rings were mounted on stainless-steel hooks under 1 g resting tension in 12 ml organ baths and bathed at 37°C in Krebs solution aerated with 95% O_2 and 5% CO_2. Tension was recorded isometrically with Grass FTO3C transducers and displayed on a Grass polygraph, model 7. The tissues were allowed to equilibrate for 30 - 45 min before experiments were begun. During this time resting tension was re-adjusted to 1 g, as required and the tissues were washed once. The Grass polygraph and isometric transducers were calibrated at the start of each day and contractions to PE were measured in g tension.

Incubation of endothelium-denuded rings of rat aorta

Previous papers (Rees *et al.*, 1990a; Mitchell *et al.*, 1992; Fleming *et al.*, 1993) have reported that overnight incubation of endothelium-denuded rings of rat aorta at 37°C in Krebs solution leads to the expression of inducible nitric oxide

synthase (iNOS). An attempt was made to determine if this process of induction could be repeated in our laboratory.

Endothelium-denuded rings of rat aorta, prepared as above, were placed in a test tube containing 1 ml of Krebs solution and incubated overnight (18 h) in an incubator (ICN Flow, model 160, UK) at 37°C under an atmosphere of 5% CO_2 in air. On removal from the incubator the rings were suspended for tension recording, as above, and cumulative concentration-response curves to PE (1 nM - 10 μM) were generated. The magnitude of depression of PE-induced contraction was assessed by comparing these responses to those obtained from freshly isolated endothelium-denuded rings. In some experiments the Krebs solution used for the overnight incubation was retained for the measurement of nitrite content using a Dasibi chemiluminescence analyser (model 2107: Quantitech Ltd, Milton Keynes, UK). These measurements were used as an additional means of examining whether there had been expression of iNOS, since nitrite is the major breakdown product of nitric oxide (NO; Wennmalm *et al.,* 1992; Ignarro *et al.,* 1993).

Experimental protocol with incubated rat aortic rings

Drugs added during the incubation period

To examine if the depression of PE-induced contraction was due to the expression of iNOS, drugs known to affect this induction process were added to the Krebs solution for the duration of the overnight incubation (18 h). These include, dexamethasone (1 μM), an inhibitor of the expression of iNOS (Di Rosa *et al.,* 1990; Knowles *et al.,* 1990b; Rees *et al.,* 1990a), and polymixin B (30 μg ml^{-1}), a scavenger of endotoxin (lipopolysaccharide, LPS; Lasfargues *et al.,* 1989), to see if this depression was due to LPS contaminating the Krebs solution. The effects of adding exogenous LPS (100 ng ml^{-1}) from *Salmonella*

typhosa to the Krebs solution were also examined on the magnitude of the depression of PE-induced contraction.

Additional experiments were conducted to examine if the induction was via a direct action of LPS on smooth muscle cells in the arterial wall or via an indirect action where the LPS was stimulating the release of a cytokine from another cell type in the arterial wall which then led to induction in the smooth muscle cells. If the paracrine action of a cytokine was required for induction to occur then antibodies to that cytokine should prevent this process. The antibodies examined were monoclonal antibodies to: murine interferon-γ (anti-m IFN-γ; 10 - 30 $\mu g\ ml^{-1}$); murine interleukin-1β (anti-m IL-1β; 10 - 50 $\mu g\ ml^{-1}$) and human tumour necrosis factor-α (anti-m TNF-α; 10 - 30 $\mu g\ ml^{-1}$). Each of these was present during the overnight incubation (18 h) of endothelium-denuded rings of rat aorta. The effects of pentoxifylline (0.1 - 10 μM), an inhibitor of tumour necrosis factor-α (TNF-α), which inhibits transcription of mRNA for TNF-α (Han *et al.*, 1990; Doherty *et al.*, 1991; Strieter *et al.*, 1988), were also examined on the depression of PE-induced contraction by including it in the Krebs solution for the overnight incubation (18 h).

In all of these experiments cumulative concentration-response curves were prepared to PE (1 nM - 10 μM). Two cumulative curves were prepared, the first to sensitise the tissues to PE and the second curve was taken as the test result.

Drugs added after the incubation period

To examine if the depression of PE-induced contraction was due to the expression of iNOS, drugs which would affect the synthesis or actions of NO were added to the tissue baths after the second cumulative concentration-response curve had been obtained. Also, as an additional control the effects of

all drugs were tested on cumulative concentration-response curves to PE (1 nM - 10 μM) on freshly isolated endothelium-denuded rings of rat aorta. This permitted non-specific effects of drugs to be differentiated from effects on NO synthesis and actions.

The agents which were added to tissues following the overnight incubation were N^G-nitro-L-arginine methyl ester (L-NAME; 100 - 1000 μM), an inhibitor of NOS (Rees *et al.,* 1990b); haemoglobin (10 - 30 μM), which binds and inactivates NO (Gibson & Roughton, 1957; Martin *et al.,* 1985), and methylene blue (10 μM), an inhibitor of soluble guanylate cyclase (Martin *et al.,* 1985). In each case the agent was added to the tissue bath 10 min prior to generating a third cumulative concentration-response curve to PE (1 nM - 10 μM).

An additional series of experiments were conducted in an attempt to determine if the depression of PE-induced contraction resulted from expression of iNOS in the smooth muscle cells or in another cell type in the vessel wall. The approach here was to compare the effects of LY 83583 (6-anilino-5,8-quinolinedione), which generates superoxide anion extracellularly and intracellularly (Mülsch *et al.,* 1988; Mülsch *et al.,* 1989), with the effects of hypoxanthine/xanthine oxidase, which generates superoxide anion only extracellularly. On the basis that NO is destroyed by superoxide anion (Gryglewski *et al.,* 1986), if NO was generated and acted within the same smooth muscle cell then it should be destroyed by LY 83583 but not by hypoxanthine/xanthine oxidase. Alternatively, if NO was produced by another cell type in the vessel wall such as a macrophage and had to diffuse to the smooth muscle cell in order to produce relaxation, then it should be destroyed both by LY 83583 and hypoxanthine/xanthine oxidase. In these experiments LY 83583 (0.1 - 3 μM), was added 10 min prior to generating a third cumulative concentration-response curve to PE (1 nM - 10 μM), and hypoxanthine (100 - 1000 μM)/xanthine oxidase (16 mu ml^{-1}) was added 2 min

prior to generating a third cumulative concentration-response curve to PE (1 nM - 10 μM). The experiments with hypoxanthine/xanthine oxidase were carried out in the presence of catalase (1000 u ml^{-1}) to prevent the accumulation of hydrogen peroxide. The effects of superoxide dismutase (250 u ml^{-1}) which destroys superoxide anion were also examined in some experiments and this was also added 2 min prior to generating a third cumulative concentration-response curve to PE (1 nM - 10 μM).

Rat Aortic Smooth Muscle Cells (RASMC)

Smooth muscle cells from rat aorta were isolated and grown in culture in order to examine the factors which regulate expression of iNOS in this cell type.

Culture of RASMC

Rat aortic smooth muscle cells (RASMC) were cultured by a modification of the explant technique described by Campbell & Campbell (1993).

The aorta was removed from a rat, as above, but placed in sterile saline (0.9%), and all further work was carried out in a laminar flow hood (Gelaire, model TC48) using sterile instruments. A schematic diagram showing a cross section of the aorta is shown in fig. 4.1a. The aorta was trimmed of adhering fat and connective tissue and placed in 5 ml of Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies Ltd, Paisley, UK) containing 0.125% collagenase (type II, Sigma, Poole, UK) and 0.025% elastase (type II, Sigma, Poole, UK) and incubated at 37°C for 5 min. On removal from the incubator, the aorta was cut open longitudinally, laid flat with the intimal surface uppermost and the endothelium removed by scraping with a scalpel blade (fig 4.1b). A transverse incision was made through the medial layer using a scalpel

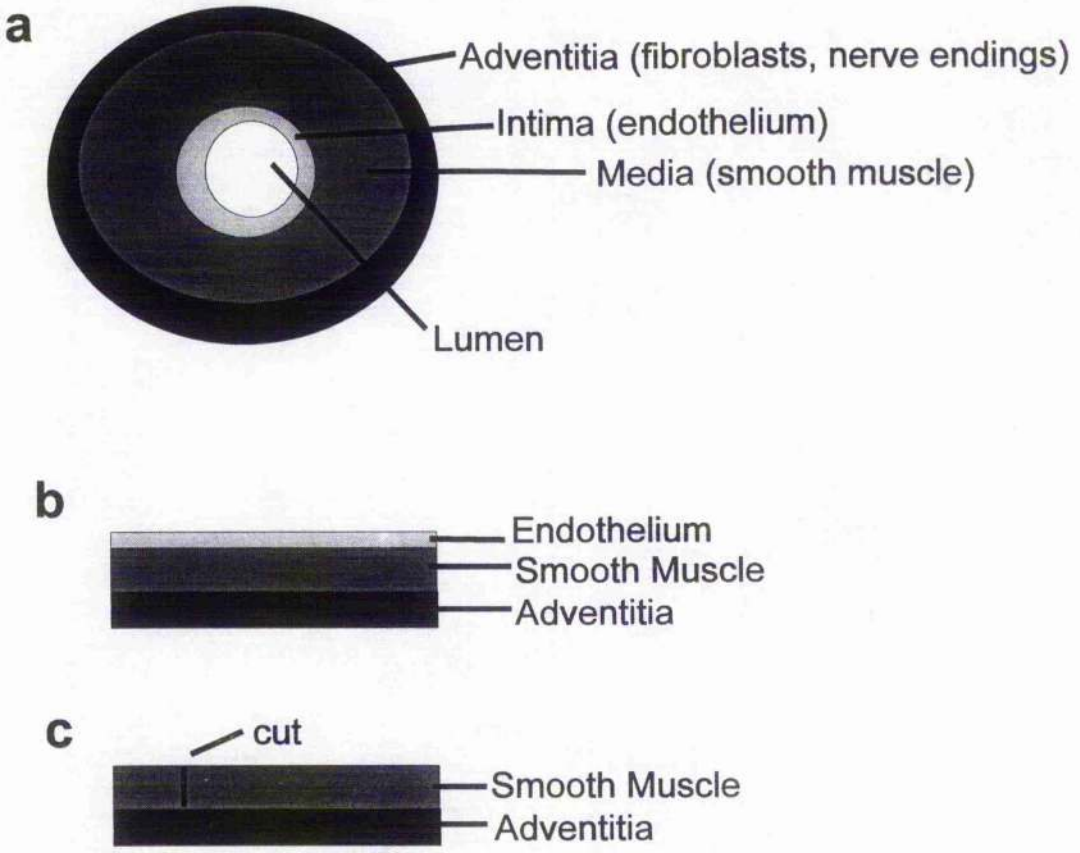


Figure 4.1: Schematic diagram of rat aorta. a) Transverse cross section of rat aorta, b) cross section after opening up the aorta by cutting longitudinally and laying flat and c) longitudinal cross section after removal of the endothelium. The aorta of a rat was removed and placed in sterile saline. It was trimmed of adhering fat and connective tissue and placed in 5 ml of Dulbecco's Modified Eagle's Medium containing 0.125% collagenase (type II) and 0.025% elastase (type II) and incubated for 5 min at 37°C. On removal from the incubator, the aorta was cut open and the endothelium removed by scraping, a transverse incision was made and the smooth muscle was peeled away from the adventitia and cut into 1 mm^2 segments. These segments were then placed on the growth surface of a 25 cm^2 flask which was kept up-ended and had 9 ml of full culture medium added before being placed up-ended in the incubator. After 2 - 4 h the flask was laid flat. The culture medium was replaced after 7 - 10 days and once a few colonies of smooth muscle had grown out of the explants, the cells were subcultured into a new flask.

blade (fig 4.1c), the smooth muscle was peeled away from the adventitia using forceps and cut into 1 mm^2 segments. Twenty to thirty of these segments were placed on the growth surface of a 25 cm^2 flask (Costar Ltd, High Wycombe, UK) and 9 ml of full culture medium was added while holding the flask up-ended. The flask was placed up-ended in an incubator at 37°C in an atmosphere of 5% CO_2 in air for 2 - 4 h to allow the tissues to adhere firmly to the growth surface before laying the flask flat to cover the tissues with full culture medium. After 7 - 10 days the culture medium was removed and the cells were washed twice with 10 ml of sterile saline (0.9%) before fresh full culture medium was added.

Once a few colonies of smooth muscle cells had grown out of the explanted tissues (14 - 20 days), they were subcultured by removing the culture medium, washing three times with sterile saline (0.9%) before adding 3 ml of trypsin (0.05%)/EDTA (0.02%; ICN Biomedicals Ltd, Bucks, UK) and incubating at 37°C for 3 - 5 minutes on an orbital shaker. On removal from the shaker the flask was shaken, the trypsin was inactivated by the addition of 1 - 2 ml of new born serum (NBS; Life Technologies Ltd, Paisley, UK) and the cell suspension was decanted into a sterile tube (Falcon, R & J Wood Laboratory Supplies, Paisley, UK). The flask was then rinsed with 9 ml of full culture medium and this was also decanted into the sterile tube which was then spun at 300 g for 3 min. The supernatant was discarded and the cell pellet was re-suspended in 5 ml of full culture medium before being spun again. The cell pellet was suspended in 20 ml of full culture medium and transferred to a 80 cm^2 flask (Costar LTD, High Wycombe, UK). These spins were to ensure the cells were suspended in fresh full culture medium with no trypsin/EDTA present. The cells were then washed every 2 - 4 days with sterile saline (0.9%) and fresh full culture medium added (20 ml). They were subcultured, as above, when confluent.

Characterisation of RASMC

Characterisation of the cells as smooth muscle cells was confirmed by the fact that they grew in the typical 'hill and valley' pattern, and by previous work in this laboratory where the cells were shown to fluoresce with anti-smooth muscle actin antibodies using the technique of Chamley *et al.* (1977). Fig 4.2 shows these smooth muscle cells in culture, plated at different densities.

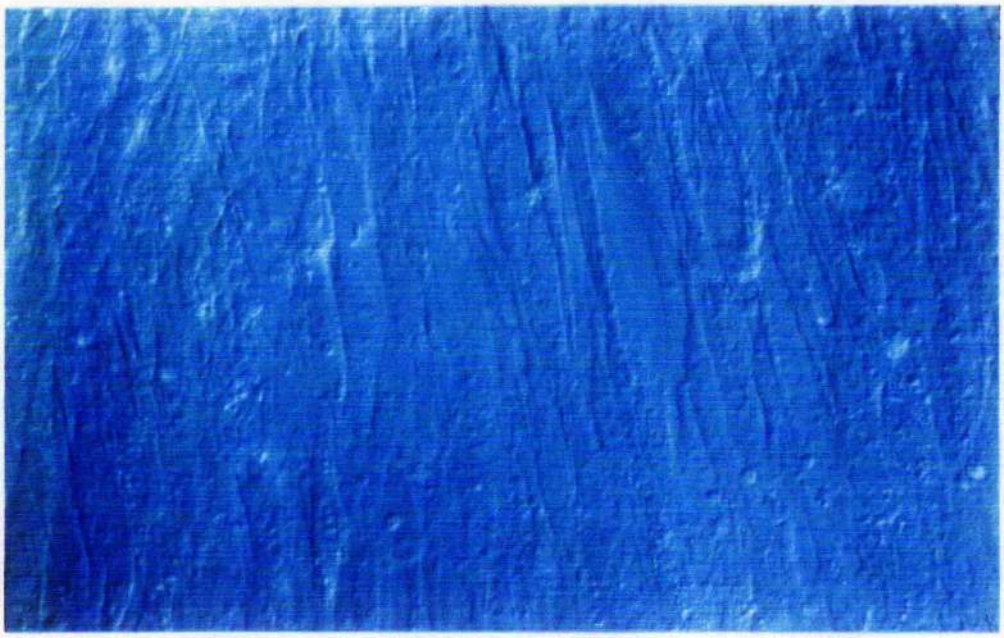
Experimental protocol for nitrite measurement using RASMC in full culture medium

Previous papers (Busse & Mülsch, 1990; Beasley *et al.*, 1991; Kanno *et al.*, 1993; Koide *et al.*, 1994) have indicated that RASMC in culture can express iNOS. We wished to examine the factors controlling this induction process by measuring nitrite, the major breakdown product of NO (Wennmalm *et al.*, 1992; Ignarro *et al.*, 1993), in the medium bathing these cells in culture.

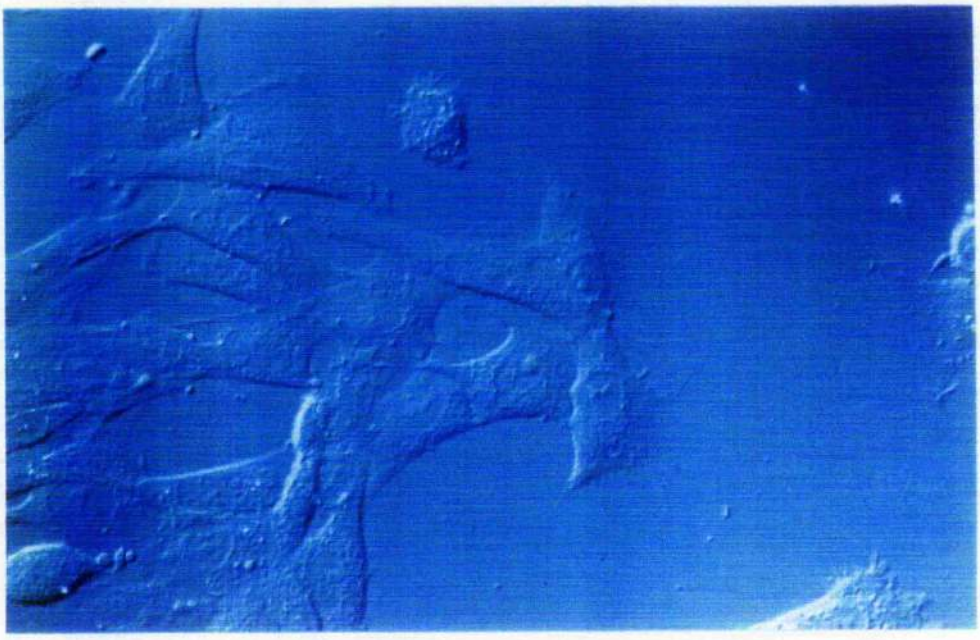
Confluent smooth muscle cells were dispersed as above with trypsin (0.05%)/EDTA (0.02%), re-suspended in 30 ml of full culture medium and cell numbers counted using a haemocytometer. Counting was achieved by placing a coverslip over the haemocytometer and filling the chamber by capillary action from a syringe containing the cell suspension. The cell numbers were counted within three or four of the large squares and the following equation was used to calculate the number of cells:-

$$\text{Total number of cells per ml} = \frac{\text{total number of cells counted} \times \text{dilution} \times 10\,000}{\text{number of squares counted}}$$

The volume of full culture medium was then adjusted to obtain a cell suspension of 3×10^5 cells per ml. One ml of cell suspension was added to



14.75 µm



29.5 µm

Figure 4.2: Photomicrographs of rat aortic smooth muscle cells cultured on glass microscope slides plated at a high seeding density (top) and a low seeding density (bottom).

each well of a 24 multi-well plate (Costar LTD, High Wycombe, UK) and left to reach confluence (1 - 2 days). Upon reaching confluence, the medium was removed from each well, the wells were washed twice with 1 ml of sterile saline (0.9%), 1 ml of fresh full culture medium was added and the cells were then incubated for 6 - 72 h in the absence or presence of drugs (as indicated in the Results). After this period, the medium was removed and nitrite content measured using the Griess reaction. The cells were then dispersed using trypsin (0.05%)/EDTA (0.02%) and counted using a haemocytometer, as above. Nitrite accumulation was expressed as pmol per 10^6 cells.

Experimental protocol for nitrite measurement using RASMC in serum-free culture medium

Our initial attempts to express iNOS in smooth muscle cells in full culture medium were unsuccessful. Since serum contains platelet-derived growth factor$_{AB}$ and $_{BB}$ and transforming growth factor-β which are known to inhibit expression of iNOS in smooth muscle cells (Junquero *et al.*, 1992; Schini *et al.*, 1992; Hirokawa *et al.*, 1994a), a further series of experiments were carried out in serum-free medium.

RASMC were plated into 24 multi-well plates as above. Once they had attained confluence the full culture medium was removed from each well, the wells were washed twice with 1 ml of sterile saline (0.9%) and 1 ml of serum-free culture medium was added. The cells were then incubated for 24 h before the medium was replaced with fresh serum-free culture medium. Drugs were added as indicated in the Results and the plates were incubated for 6 - 72 h. When the effects of dexamethasone (0.1 - 1 μM) or cycloheximide (1 - 100 μg ml^{-1}), inhibitors of the expression of iNOS, or polymixin B (1 - 30 u ml^{-1}), which binds and inactivates LPS, were to be examined, they were added as a 2 h pre-treatment before addition of iNOS-inducing agent; i.e. IFN-γ (30 u ml^{-1}).

After 6 - 72 h, the medium bathing the cells was removed and the nitrite content was measured using the Griess reaction. Cell numbers were counted using a haemocytometer, as above, and nitrite accumulation was expressed as pmol per 10^6 cells.

Experimental protocol for the measurement of cyclic GMP content

Expression of iNOS can also be assessed by measuring the cyclic GMP content of cells, as cyclic GMP is the second messenger produced by NO (Rapoport & Murad, 1983). A series of experiments were therefore conducted in which this second messenger was measured as an additional means of determining if iNOS had been induced in RASMC in culture.

Confluent smooth muscle cells in 80 cm^2 flasks were dispersed using trypsin (0.05%)/EDTA (0.02%) and counted as before. In these experiments the cell suspension was adjusted to give a concentration of 5 x 10^5 cells per ml. Two ml of cell suspension was added to each well of a 6 multi-well plate (Costar LTD, High Wycombe, UK) and the cells were left to reach confluence (1 - 2 days). Upon reaching confluence, the medium was removed, the cells were washed with 1 ml of sterile saline (0.9%) and 2 ml of serum-free culture medium was added. After 24 h this medium was removed, replaced with 2 ml of fresh serum-free culture medium and the cells were incubated in the presence of drugs for 24 h as indicated in the Results. When the effects of cycloheximide (1 μg ml^{-1}) or dexamethasone (1 μM), inhibitors of the expression of iNOS, were to be examined, they were added as a 2 h pre-treatment before addition of iNOS-inducing agents. After 24 hours, the medium bathing the cells was removed and the nitrite content measured using the Griess reaction. Immediately after the medium was removed 1 ml of ice cold ethanol was added to each well. The cells were detached using a 'rubber policeman', harvested into an Eppendorf tube and extracted overnight at 4°C.

Following this, the samples were spun at 13 000 rpm for 2 min to form a cell pellet. The ethanol extract was removed and placed in a Sarstedt (Leicester, UK) 5 ml tube. The ethanol was evaporated by heating at 50°C under a constant stream of air. The dry samples were then stored at 4°C for subsequent cyclic GMP analysis. When required, the extracts were reconstituted in 0.5 ml of 0.05 M sodium acetate buffer, pH 6.2.

Bovine Aortic Endothelial Cells (BAEC)

Experiments were conducted to determine if iNOS could be expressed in bovine aortic endothelial cells (BAEC).

Preparation of BAEC

Bovine aortae were collected from the local abattoir. Immediately on removal from the animals, they were flushed with a sterile saline solution (0.9%) containing penicillin (200 u ml^{-1})/streptomycin (200 µg ml^{-1}). The aortae were ligated at the proximal end using strong string and cannulated at the distal end with an adapter attached to a 60 ml syringe containing the same sterile saline solution (fig 4.3). This solution was injected into the aortae and they were transported to the laboratory, which took approximately 30 min.

At the laboratory all further work was carried out in a laminar flow hood (Gelaire, model TC48). The aortae were filled with 20 ml of fresh sterile saline and the intercostal arteries were ligated using surgical suture (gauge 0). The saline solution was then replaced with 20 ml of 0.1% collagenase (type II) in DMEM and incubated at 37°C for 25 min on an orbital shaker. On removal from the shaker the endothelial cells were harvested by massaging the aorta and by flushing the collagenase solution in and out of the vessel. This cell suspension was spun at 100 g for 4 min and the supernatant was discarded.

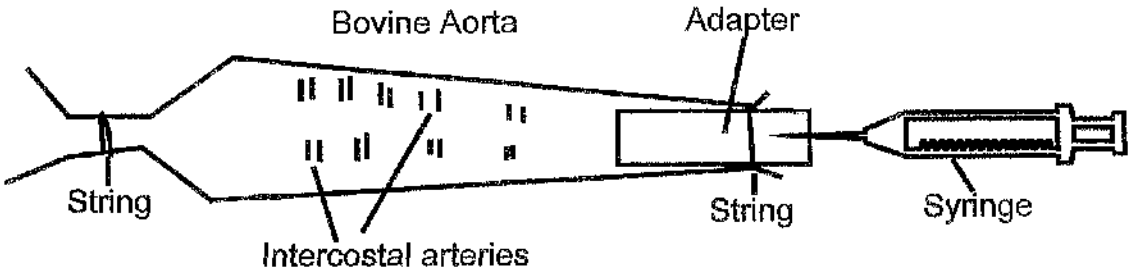


Figure 4.3: Schematic representation of a bovine aorta. Immediately on removal from the animal, the aorta was flushed with a sterile saline solution (0.9%) containing penicillin (200 u ml^{-1})/streptomycin (200 µg ml^{-1}). It was then ligated at the proximal end using strong string and cannulated at the distal end with an adapter attached to a 60 ml syringe containing the same sterile saline solution. At the laboratory the saline solution was removed and replaced with 30 ml of fresh sterile saline (0.9%) and the intercostal arteries were ligated using surgical suture (gauge 0). The saline solution was then replaced with 20 ml of 0.1% collagenase (type II) in Dulbecco's Modified Eagle's Medium and the aorta was incubated at 37°C for 25 min. On removal from the incubator the endothelial cells were harvested and grown in 80 cm^2 tissue culture flasks.

The cell pellet was washed twice, by re-suspension in 10 ml of sterile saline, spinning at 100 g for 4 min and discarding the supernatant, before the cell pellet was finally re-suspended in full culture medium. The endothelial cells were incubated at 37°C in an atmosphere of 5% CO_2 in air and grown to confluence in either 9.5 cm^2 6-well cluster plates (2 ml of cell suspension added) or in 80 cm^2 tissue culture flasks (20 ml of cell suspension added). All cells were washed with sterile saline and fresh full culture medium added every 2 - 3 days.

Characterisation of BAEC

Characterisation of the cells as endothelial cells was confirmed by the fact that they grew in a single layer with a 'cobblestone' morphology and by previous work in this laboratory where they were shown to produce both prostacyclin and EDRF (Martin *et al.*, 1988). Also, no fewer than 98% of cells were observed to fluoresce following incubation with acetylated low density lipoprotein labelled with 1,1'-dioctadecyl-1,3,3',3'-tetramethyl-indocarbocyanine percholate (Voyta *et al.*, 1984; Martin *et al.*, 1988).

Experimental protocol for measurement of nitrite or nitrite/nitrate using BAEC

Previous reports (Radomski *et al.*, 1990b) have suggested that porcine aortic endothelial cells can express iNOS. We wished to examine if BAEC could also express iNOS and the factors controlling this induction by measuring the nitrite or nitrite/nitrate content of the medium bathing these cells in culture.

Once the cells had attained confluence (3 - 5 days) in 80 cm^2 flasks, they were subcultured into 6 multi-well plates for nitrite or nitrite/nitrate analysis. This was achieved by removing the full culture medium from the flask, washing twice

with 20 ml of sterile saline, adding 3 ml of trypsin (0.05%)/EDTA (0.02%) and incubating at 37°C for 3 min. Following this, the flasks were shaken to dislodge the cells, 2 ml of NBS was added to inactivate the trypsin/EDTA, the cell suspension was removed and spun at 100 g for 4 min. The supernatant was discarded and the cell pellet was washed twice with sterile saline (0.9%). The cells were re-suspended in 60 ml of full culture medium and seeded into the 6 multi-well plates with 2 ml of cell suspension added to each well.

When nitrite or nitrite/nitrate accumulation was to be measured, confluent cells in 6 multi-well plates were washed twice with 3 ml of sterile saline and then 2 ml of full culture medium was added to each well. The plates were incubated with drugs, as indicated in the Results, for 24 hours at 37°C in an atmosphere of 5% CO_2 in air. After this incubation, the culture medium was removed and the nitrite or nitrite/nitrate content was measured using a Dasibi chemiluminescence analyser (model 2107, Quantitech Ltd, Milton Keynes, UK).

J774.2 murine macrophage cell line

Experiments were conducted to determine if iNOS could be expressed in the J774.2 murine macrophage cell line.

Preparation of J774.2 cells

The J774.2 murine macrophage cell line was obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). Cells were maintained in siliconised (Replecote, Sigma) Techne stirrer flasks containing 400 ml of full HEPES-buffered culture medium (ICN Biomedicals Ltd, High Wycombe, UK) at 37°C and stirred continuously under an atmosphere of air. The full HEPES-buffered culture medium was changed every 3 - 4 days by spinning the cell

suspension at 100 g for 4 min, then reconstituting the cell pellet in 200 ml of saline before a further spin and reconstitution in 400 ml of fresh full HEPES-buffered culture medium.

Experimental protocol with J774.2 cells

Previous reports (Stuehr & Marletta, 1987a; Stuehr & Marletta, 1987b) have demonstrated expression of iNOS in macrophages following incubation with IFN-γ. We wished to examine this induction by measuring the nitrite or nitrite/nitrate content of the medium bathing the cells.

For experimentation, the cell suspension was removed from the stirrer flask and placed into sterile 50 ml Falcon tubes (R & J Wood Laboratory Supplies, Paisley, UK) and spun at 100 g for 4 min. The supernatant was discarded and the cell pellet was washed twice with 30 ml of sterile saline (0.9%), re-suspended in 50 ml of full HEPES-buffered culture medium and the cell numbers were counted using a haemocytometer, as before. The cells were then spun and reconstituted in an appropriate volume of full HEPES-buffered culture medium to give a final density of 10^6 cells ml^{-1}, and seeded into 24-well cluster plates (Costar, High Wycombe, UK) with 1 ml of cell suspension added to each well. The plates were then incubated in the presence of drugs, as indicated in the Results, for 24 h at 37°C. At the end of this period, the culture medium was removed from the plates, spun in a micro-centrifuge at 13000 rpm for 2 min to remove any dislodged cells. Nitrite content of the supernatant was measured using the Griess reaction or with a Dasibi chemiluminescence analyser (model 2107; Quantitech Ltd, Milton Keynes, UK).

Measurement of nitrite or nitrite/nitrate accumulation

Griess reaction

The accumulation of nitrite, the major breakdown product of NO (Wennmalm *et al.*, 1992; Ignarro *et al.*, 1993), in the medium bathing cells was measured by the formation of a diazo product by a variant of the method of Green *et al.* (1982). This is a two step assay based on the fact that the complex formed between nitroxides and sulfanilic acid reacts with N-(1-naphthyl) ethylenediamine generating a product that is easily measured spectrophotometrically.

The measurement of nitrite involved using 0.4 ml samples from experiments, to which 0.4 ml of 1% (w/v) sulfanilic acid (Sigma) in 2 M HCl and 0.4 ml of 1% (w/v) aqueous N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma) were added, and the absorbance of the purple azo complex determined at 548 nm using a Shimadzu (UV240) recording spectrophotometer. Towards the end of the project, a Dynatech micro plate reader (DS2000) was obtained which permitted 96 samples to be measured simultaneously. Nitrite in these experiments was measured using a 550 nm filter, and the sample volume, as well as the volume of the two reagents, was reduced to 40 µl.

A standard curve was prepared in these experiment using sodium nitrite standards (0 - 9000 pmol) dissolved in the appropriate medium, i.e. full culture medium, serum-free culture medium or full HEPES-buffered culture medium. The Griess reaction could only satisfactorily measure concentrations of 300 pmol and above, and when values between 300 - 9000 pmol were plotted, the resulting line was linear (fig 4.4). A regression line of best fit was used to determine the nitrite content of experimental samples.

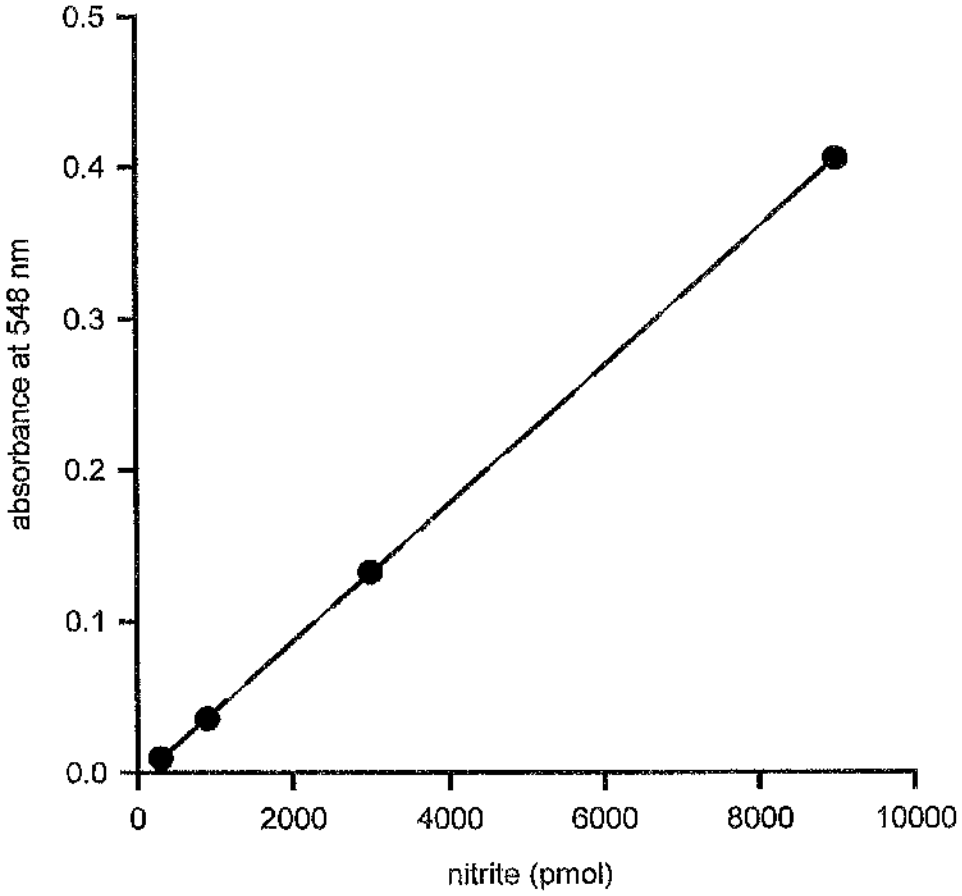


Figure 4.4: Standard curve for sodium nitrite (300 - 9000 pmol) obtained using the Griess reaction by spectrophotometric analysis at 548 nm. Each point is the mean of 4 observations and a regression line of best fit was obtained (broken line).

Chemiluminescence detection of nitrite or nitrite/nitrate

Chemiluminescence has been successfully used for the detection of NO (Palmer *et al.,* 1987). The basis of this method is that NO reacts spontaneously with ozone to emit light as indicated by the following reactions:-

$NO + O_3 \rightarrow NO^{\bullet}_2$

$NO^{\bullet}_2 \rightarrow NO_2 + h\nu$

Native NO has a very short half-life, however, and is not easily measured. Techniques have, therefore, been developed to reduce nitrite and/or nitrate, the more stable metabolites of NO, back to NO (Cox, 1980; Braman & Hendrix, 1989; Termin *et al.,* 1992). Nitrate can be reduced to NO, but this involves the use of strong reducing agents such as titanium trichloride, ammonium molydbate in sulphuric acid (Cox, 1980), or vanadium (III) chloride in hydrochloric acid (Braman & Hendrix, 1989). Nitrite can be reduced to NO in the presence of weaker reducing agents such as sodium iodide in glacial acetic acid (Cox, 1980) or alternatively by 1,1'-dimethylferrocene in acetonitrile (Termin *et al.,* 1992).

The reducing systems used in this study were :-

1,1'-dimethylferrocene/acetonitrile system. This consisted of 900 mg of 1,1'-dimethylferrocene in 90 ml acetonitrile (HPLC grade) to which 1.5 µl of 70% perchloric acid was added. This reducing system was used at room temperature and is reported to reduce nitrite to NO (Termin *et al.,* 1992).

Sodium iodide/glacial acetic acid reflux system. This consisted of 25 ml of a 6% solution of sodium iodide in 75 ml of glacial acetic acid under reflux at 65°C and reduces nitrite to NO (Cox, 1980).

Vanadium chloride/hydrochloric acid reflux system. This consisted of 1.5 g of vanadium chloride in 100 ml of 2 N HCl under reflux at 95°C and reduces both nitrite and nitrate to NO (Braman & Hendrix, 1989).

These reducing systems were used in conjunction with a Dasibi chemiluminescence analyser (model 2107: Quantitech Ltd, Milton Keynes, UK) to measure the nitrite or nitrite/nitrate content of biological samples using standards of sodium nitrite and sodium nitrate. For calibration of this system see Results.

Apparatus for chemiluminescence detection of nitrite or nitrite/nitrate

A schematic diagram of the apparatus used is shown in fig 4.5.

Briefly, this consisted of a 250 ml flask, containing 100 ml of the reducing solution. The 250 ml flask was placed on a heating mantle, which was used when the reducing solution was required to be under reflux (i.e. for acidic vanadium chloride and for sodium iodide/glacial acetic acid). The NO produced by the reducing solution was carried from the flask by a constant flow of oxygen-free nitrogen (British Oxygen Co. Ltd). The flow then passed through a number of traps, to ensure no acid entered the analyser: the first trap was a water-cooled condenser, which was followed by an ice-cooled liquid trap and finally a trap containing sodium hydroxide pellets. The gas flow then entered the analyser. Inside the analyser NO reacted, in the reaction chamber, with ozone produced by a high electrical charge. Ozone and NO react to produce NO^{*}_{2} which decays to NO_2 while emitting light at 650 - 800 nm. The amount of light generated by the system was dependent upon the amount of NO generated from nitrite and/or nitrate and was measured with a photomultiplier tube linked via an integrator to a Linseis recorder and an IBM

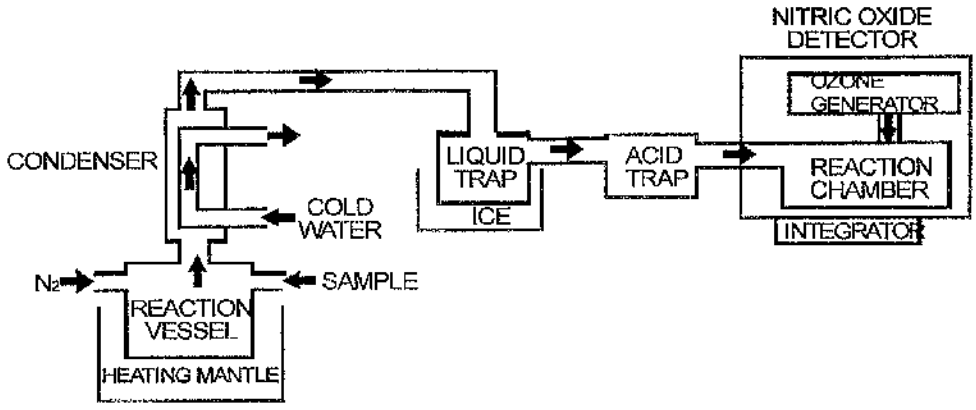


Figure 4.5: A schematic illustration of the chemiluminescence apparatus used to measure nitric oxide (NO) generated from samples containing nitrite and/or nitrate. Samples are injected into the reaction vessel containing the reducing solutions (see Methods), some of which require reflux, and for these the heating mantle was turned on. A constant flow of N_2, was supplied from an oxygen-free nitrogen cylinder. The NO produced from nitrite and/or nitrate is carried in the N_2 stream through the condenser, cooled by cold water direct from a tap, from here the NO passes into a liquid trap, cooled by ice, and then into an acid trap, containing sodium hydroxide pellets to remove any traces of acid, after which the gas then enters the analyser. Inside the analyser the gas reacts with ozone, produced by high voltage, in the reaction chamber to form NO^*_2 which breaks down to emit light. This light is measured by a photomultiplier tube connected through an integrator to a computer.

compatible personal computer. This computer ran a program called 'SCAN' (Dr. Dempster, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, UK) that could measure both the peak of the signal generated in parts per billion (ppb) and the area under the curve in parts per billion times seconds (ppb.s).

NO gas

In the early chemiluminescence experiments known amounts of NO gas were used to calibrate the chemiluminescence NO analyser. NO gas was diluted with oxygen-free nitrogen (British Oxygen Co. Ltd) to pmol levels and injected into the analyser. Specifically, a 2 l volumetric flask was flushed for 5 min with oxygen-free nitrogen and sealed with a rubber subaseal. This subaseal was then punctured with two syringe needles attached via a steel tube to a supply of oxygen-free nitrogen so that the flask could be flushed and the other to allow escape of gas. After flushing for 15 min the flask was re-sealed. A constant flow of NO was established along an open-ended tube from a NO gas cylinder (British Oxygen Co. Ltd). A gas-tight syringe was filled and emptied several times with this NO, before being filled and sealed. Two hundred µl of NO was then injected into the 2 l flask giving a 4.5 µM stock of NO. From this stock, a gas-tight syringe was filled and used to calibrate the analyser. Fresh stock was made once the gas-tight syringe was emptied. The stock was such that a 50 µl sample was equivalent to 225 pmol.

Fluorometric determination of nitrite

In some experiments an alternative fluorometric assay for nitrite was employed. The fluorometric determination of nitrite is based on the reaction of nitrite with 2,3-diaminonaphthalene (DAN) in acidic solution to form

1-[H]-naphthotriazole, a highly fluorescent compound in alkaline medium (Damiani & Burini, 1986; Misko *et al.*, 1993).

The measurement of nitrite using this technique involved using 0.4 ml samples or standards which were mixed with 0.04 ml of DAN solution (0.05 mg ml^{-1} in 0.62 M HCl; pH 1.6) and incubated at 37°C for 10 min. Following incubation the reaction was stopped with 0.01 ml of 5.6 N NaOH (pH 11.6). The 1-[H]-naphthotriazole formed was highly fluorescent and fluorescence intensity of samples was measured using an excitation wavelength of 365 nm and read at an emission wavelength of 405 nm using a Perkin-Elmer LS-3B fluorometer. The 1-[H]-naphthotriazole was stable for approximately 10 min. Validation of this detection system is shown in the Results section.

Measurement of cyclic GMP content

Principle of radioimmunoassay (RIA)

The basic principle of radioimmunoassay (RIA) is the competition between radioactive and non-radioactive antigen for a fixed number of antibody binding sites. This interaction is represented schematically in fig 4.6. If increasing amounts of non-radioactive antigen (i.e. in standard or unknown samples) and a fixed amount of radioactive antigen are allowed to react with a constant amount of antibody, a decreasing amount of radioactive antigen is bound to the antibody. This relationship can be expressed as a standard curve and the amount of unlabelled antigen in a sample determined by interpolation from this curve.

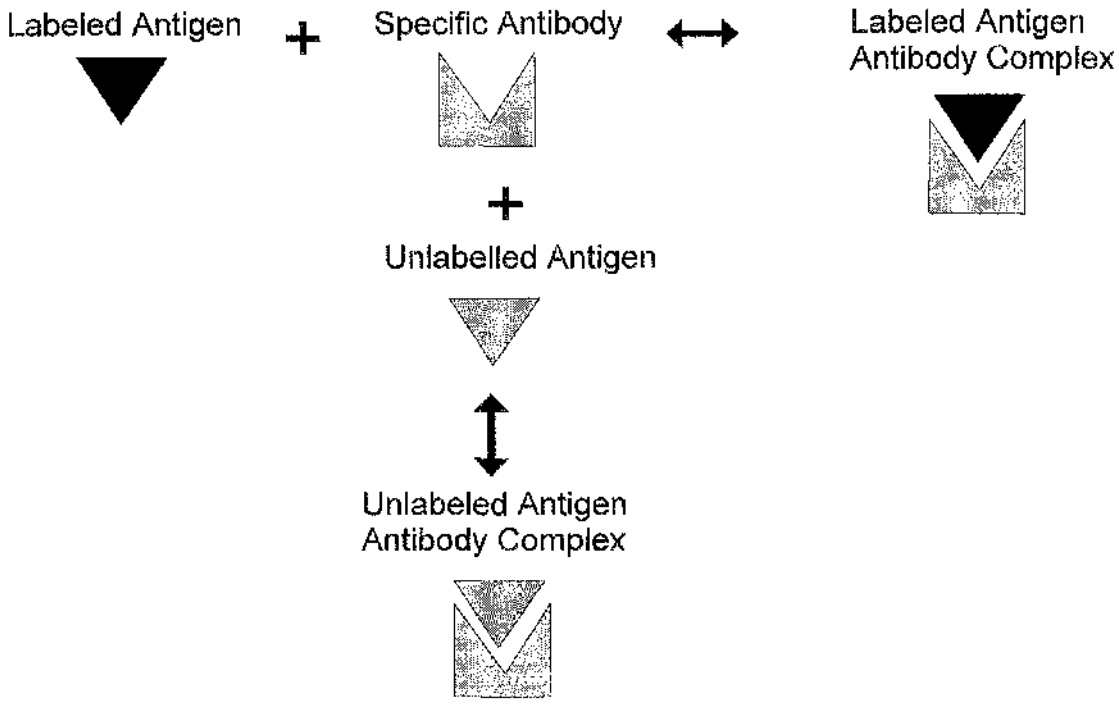


Figure 4.6: A schematic representation of the principle of radioimmunoassy (RIA). RIA is based on the competition between radiolabelled and unlabelled antigen for a fixed number of antibody binding sites. Increasing amounts of unlabelled antigen in the presence of a fixed amount of antibody and radiolabelled antigen, results in decreasing amount of radiolabelled antigen bound to the antibody. This relationship can be expressed as a standard curve after separation of bound from free radiolabelled antigen, and amounts of unlabelled antigen determined by interpolation from the curve.

RIA

Determination of cyclic GMP was adapted from the procedure of Steiner et *al.* (1972), using New England Nuclear RIA kits (Du Pont). Steiner et *al.* (1972) reported that cyclic nucleotides substituted at the 2'-σ-position had a higher affinity for the antibody and thus displace the [^{125}I]-methyl ester derivative better than unsubstituted cyclic nucleotides. Thus, standards and samples were acetylated with acetic anhydride to give 2'-σ-acetyl cyclic GMP, thereby increasing the sensitivity of the assay (Harper & Brooker, 1975).

The labelled antigen was a succinyl tyrosine-[^{125}I]-methyl ester derivative of cyclic GMP. Separation of bound from free antigen was achieved by the use of a pre-reacted primary and secondary antibody complex. The primary antibody was prepared in rabbits against a succinyl cyclic GMP-albumin conjugate, while the second antibody was prepared in sheep against rabbit globulin. With this pre-reacted system, pipetting and incubation times were reduced compared to the usual sequential double antibody assay and no intermittent incubation was required. After the single overnight incubation at 4°C, 0.5 ml of propan-1-ol was added to aid precipitation, the tubes were centrifuged at 2000 g for 15 min, the supernatants discarded, and the radioactivity in the precipitates counted.

Chemicals for RIA

Cyclic GMP antiserum complex

One vial of lyophilised, pre-reacted, first and second antibody was supplied. It was reconstituted with 21 ml of distilled water. The resulting solution, in 0.1 M sodium phosphate buffer (pH 6.2) with 0.05% thimerosal, contained sufficient antibody to bind approximately 40 - 50% of the labelled antigen in the absence

of unlabelled antigen when used as indicated below. The reconstituted antiserum complex was stable for at least 2 months when stored at 4°C.

Succinyl cyclic GMP tyrosine methyl ester-[^{125}I] (Scyclic GMP-TME-[^{125}I])

Two vials of concentrated tracer were supplied. Each vial contained approximately 28 KBq (0.75 µCi) on the calibration date in 1 ml of propan-1-ol:water solution (1:1). The concentrate was stable for at least 2 months when stored at 4°C.

Normal rabbit serum

The normal rabbit serum was used to measure the non-specific binding obtained with non-immune serum. Two vials of lyophilised normal rabbit serum were supplied. Five ml of distilled water was added to each vial of Scyclic GMP-TME-[^{125}I] concentrate, and to each vial of lyophilised normal rabbit serum. The entire contents of the reconstituted normal rabbit serum vial was added to the diluted Scyclic GMP-TME-[^{125}I] vial and the resulting solution mixed. This solution (approximately 11 ml) contained Scyclic GMP-TME-[^{125}I] (0.068 µCi ml^{-1}), 1% normal rabbit serum, and 0.05 M sodium acetate buffer, pH 6.2. The solution was stable for at least 2 weeks when stored at 4°C.

Acetic anhydride and triethylamine

A mixture of these two solutions was used to acetylate cyclic GMP in standards and samples in order to increase the sensitivity of the assay. One vial each of acetic anhydride and triethylamine was supplied. They were allowed to equilibrate to room temperature before use. When protected from moisture these chemicals were stable for at least 2 months. Immediately prior to use, 1 volume of acetic anhydride was mixed with 2 volumes of

triethylamine: the exact volume of each was dependent upon the number of samples to be acetylated.

Cyclic GMP standard

This was the unlabelled antigen. One vial of lyophilised standard was supplied. It was reconstituted with exactly 2 ml of distilled water and contained 2000 pmol ml^{-1} in 0.05 M sodium acetate buffer, pH 6.2. The cyclic GMP standard had been calibrated spectrophotometrically using the molar absorption coefficient for cyclic GMP, ε=13.7 x 10^3 mol^{-1} cm^{-1} at 252 nm, pH 7. The reconstituted standard was stable for at least 2 months when stored at 4°C.

Preparation of standard curve and assay of cell extracts

A series of 5 ml plastic tubes (Sarstedt, Leicester, UK) were numbered for identification and the standard cyclic GMP stock solution (2000 pmol ml^{-1}) was diluted with 0.05 M sodium acetate buffer, pH 6.2, to a concentration of 100 pmol ml^{-1}. Further serial dilutions with sodium acetate buffer were made to prepare the following standards for assay: 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 pmol. ml^{-1} All standards were run in duplicate.

Tubes 1 and 2 were used to measure 'total counts' i.e. the total amount of radioactivity added to each tube, tubes 3 and 4 were 'blanks' and tubes 5 and 6 were used to measure the '0 standard'. One hundred µl volumes of each standard solution (in duplicate) or sample were added to appropriately labelled tube. All tubes except for 'total counts' and 'blanks' received 5 µl of the acetylation mixture, ensuring that the reagents were added directly to the solution and were immediately vortex mixed for 2 s. All tubes received 100 µl of Scyclic GMP-TME-[^{125}I] tracer solution, and all tubes except for 'total counts' and 'blanks' received 100 µl of antiserum complex. All tubes were

vortex mixed for 5 s, covered with aluminium foil and equilibrated overnight (18 h) at 4°C.

Following overnight equilibration at 4°C, 0.5 ml of ice cold propan-1-ol was added to each tube except for 'total counts'. This aided the precipitation of the antigen-antibody complex. The tubes were vortex mixed and centrifuged at 2000 g for 15 min at 4°C (Damon IEC centrifuge). The supernatants were discarded and the pellets together with the 'total counts' tubes were counted for 1 min in a gamma counter (Packard Cobra Auto gamma).

Analysis of radioactivity

Analysis of the radioactivity in the tubes (counting and background reduction) was performed by an IBM-compatible computer with a defined RIA protocol. This allowed the user to define the nature of the samples and the count time. The standard curve was plotted with the characteristics of a Bound Fraction RIA curve with a negative slope (spline-fitted). The content of each unknown sample was calculated by interpolation from the standard curve and the value printed. Table 4.1 and fig 4.7 represent a typical standard curve obtained from an acetylated cyclic GMP RIA.

Drugs

Acetylcholine chloride (ACh), L-arginine hydrocholine, catalase (bovine liver), dexamethasone, hypoxanthine, haemoglobin (bovine; Hb), interferon-γ (murine, recombinant: IFN-γ), interleukin-1α (human; IL1-α), lipopolysaccharide from *Escherichia coli* (prepared by phenol extraction; LPS), lipopolysaccharide from *Salmonella typhosa* (prepared by phenol extraction; LPS), methylene blue, N^G-nitro-L-arginine methyl ester (L-NAME),

	cpm	mean cpm
total counts	6632.9 6337.9	6485.4
blanks	46.9 34.9	40.9
'0' standard	2297.9 2170.9	2234.4
0.00125 pmol	2254.9 2000.9	2127.9
0.0025 pmol	1966.9 2043.9	2005.4
0.005 pmol	1832.9 1819.9	1826.4
0.0125 pmol	1505.9 1493.9	1499.9
0.025 pmol	1154.9 1069.9	1112.4
0.05 pmol	835.9 729.9	782.9
0.125 pmol	422.9 390.9	406.9
0.25 pmol	279.9 273.9	276.9

Table 4.1: Typical data for radioimmunoassay (RIA) of cyclic GMP showing decreased binding of Scyclic GMP-tyrosine methyl ester [^{125}I] (Scyclic GMP-TME-[^{125}I]) tracer to the antibody as the amount of unlabelled cyclic GMP in standards increases. 'Total counts' measures total activity of Scyclic GMP-TME-[^{125}I] tracer, 'blanks' measures non-specific binding, and '0 standard' measures total binding of Scyclic GMP-TME-[^{125}I] tracer to antibody in the absence of any unlabelled antigen.

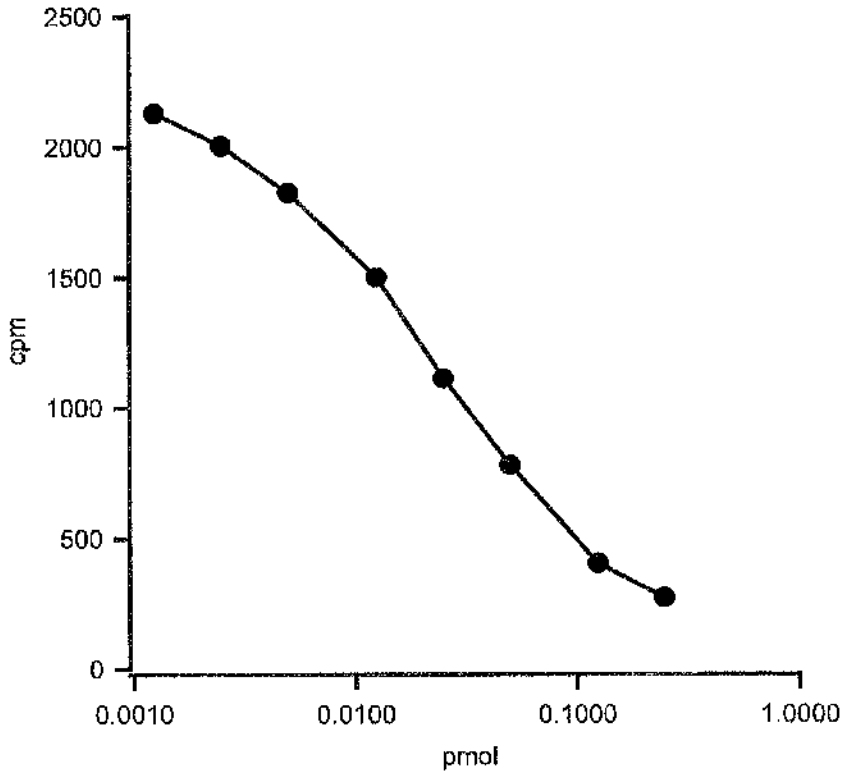


Figure 4.7: Standard curve for cyclic GMP radioimmunoassay using data shown in table 4.1. The amount of cyclic GMP in experimental samples was calculated from this curve by the computer.

pentoxifylline, phenylephrine hydrochloride (PE), polymixin B sulphate, superoxide dismutase (bovine erythrocyte, Cu-Zn-containing) and xanthine oxidase (buttermilk) were obtained from Sigma (Poole, UK). Whilst N^G-monomethyl-L-arginine (L-NMMA) was a gift from Wellcome Laboratories (Beckenham, UK); LY 83583 (6-anilino-5,8-quinolinedione) was obtained from Calbiochem (Nottingham, UK); anti-tumour necrosis factor-α (murine, monoclonal; anti-m TNF-α) and anti-interferon-γ (murine, monoclonal; anti-m IFN-γ) were obtained from Boehringer Mannheim (Lewes, UK) and anti-interleukin-1β (human, monoclonal; anti-m IL-1β) was obtained from TCS Biologicals Ltd (Buckingham, UK). All drugs were dissolved and dilutions made in saline (0.9%), except for hypoxanthine which was dissolved in 0.1% sodium hydroxide and dexamethasone and LY 83583 which were dissolved in 0.1% ethanol with further dilutions in saline. N^G-nitro-L-arginine at 10^{-2} M was soluble only after sonication.

Preparation of Haemoglobin

Bovine haemoglobin (Hb) as supplied by Sigma (Poole, UK) is mainly present in the oxidised state (methaemoglobin, $Fe^{3+}Hb$). A 10 fold molar excess of dithionite was used to reduce methaemoglobin ($Fe^{3+}Hb$) to oxyhaemoglobin ($Fe^{2+}(Hb(O_2)_4$). Briefly, dialysis tubing was boiled in distilled water for 15 min then rinsed with distilled water. Hb 1.29 g was dissolved in 20 ml of distilled water to give a 1 mM solution and 34.82 mg of sodium dithionite was dissolved in 100 µl of distilled water and added immediately to the Hb solution with thorough mixing. This combined solution was then poured into the dialysis tubing expelling all air, this was placed in a flask containing 5 l of distilled water at room temperature for 2 h and shaken frequently. After this time the solution was aliquotted and stored frozen at-20°C.

Culture media and Krebs solutions

The constituents of DMEM (Life Technologies Ltd, Paisley, UK) and HEPES-buffered DMEM (ICN Biomedicals Ltd, High Wycombe, UK) are listed in table 4.2.

Full culture medium

Full culture medium was prepared using DMEM with the addition of foetal calf serum (FCS; 10%), new born calf serum (NBS; 10%), glutamine (4 mM) and penicillin(100 u ml^{-1})/streptomycin (100 µg ml^{-1}). All culture medium reagents obtained from Life Technologies Ltd, Paisley, UK.

Serum-free culture medium

Serum-free culture medium was prepared using DMEM with the addition of bovine serum albumin (0.1%), glutamine (4 mM) and penicillin (100 u ml^{-1})/streptomycin (100 µg ml^{-1}).

Full HEPES-buffered culture medium

HEPES-buffered culture medium was prepared using HEPES-buffered DMEM with the addition of FCS (10%), glutamine (2 mM) and penicillin (100 u ml^{-1})/streptomycin (100 µg ml^{-1}).

Krebs solution

Krebs solution was prepared daily from a 10 times concentrated stock containing NaCl, KCl, $CaCl_2$, $MgSO_4$ and KH_2PO_4. This concentrated stock

Constituents	mg l^{-1} DMEM	mg l^{-1} HEPES-buffered DMEM
L-arginine HCl	84	84
L-cysteine disodium salt	56.78	62.57
L-glutamate	584	584
glycine	30	30
L-histidine HCl H_2O	42	42
L-isoleucine	104.8	104.8
L-leucine	104.8	104.8
L-lysine HCl	146.2	146.2
L-methionine	30	30
L-phenylalanine	66	66
L-serine	42	42
L-threonine	95.2	95.2
L-tryptophan	16	16
L-tyrosine disodium salt	89.5	103.8
L-valine	93.6	94
D-Ca pantothenate	4	4
choline chloride	4	4
folic acid	4	4
i-inositol	7	7.2
nicotinamide	4	4
pyridoxal HCl	4	4
riboflavin	0.4	0.4
thiamin HCl	4	4
$CaCl_2.2H_2O$	264.9	200
$Fe(NO_3)_3.9H_2O$	0.1	0.1
KCl	400	400
$MgSO_4.7H_2O$	200	97.9
NaCl	6400	6400
$NaHCO_3$	3700	------
$NaH_2PO_4.2H_2O$	141.3	125
D-glucose	4500	4500
phenol red sodium salt	15	15
sodium pyruvate	110	110
HEPES	------	4766

Table 4.2: Constituents of Dulbecco's Modified Eagle's Medium (DMEM) and of HEPES-buffered DMEM.

was diluted with distilled water, and glucose and $NaHCO_3$ were added. The final concentration of reagents was: NaCl 118 mM, KCl 4.8 mM, $CaCl_2$ 2.4 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, glucose 11 mM and $NaHCO_3$ 24 mM. This was gassed with 95% O_2 and 5% CO_2.

HEPES-buffered Krebs solution

HEPES-buffered Krebs solution was prepared daily from a 10 times concentrated stock Krebs solution containing NaCl, KCl, $CaCl_2$, $MgSO_4$ and KH_2PO_4. This concentrated stock solution of Krebs was diluted with distilled water, and glucose, HEPES and $NaHCO_3$ were added. The final concentration of reagents was: NaCl 118 mM, KCl 4.8 mM, $CaCl_2$ 2.4 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, glucose 11 mM, $NaHCO_3$ 2.4 mM and HEPES 5 mM. This solution was not gassed.

Statistical Analysis

Results are expressed as means ± s.e. mean and were compared using one-way analysis of variance followed by Fisher's test. A probability of less than 0.05 was taken as significant.

Chapter 5
Validation of chemiluminescence and fluorometric detection systems

Nitric oxide (NO) gas

The first set of experiments conducted using the chemiluminescence analyser involved calibration with authentic nitric oxide (NO) gas. NO (200 - 1600 pmol) produced chemiluminescence signals that were proportional to the amount added (fig 5.1a). When the amount of NO (pmol) was plotted against the area under the curve (ppb.s) for each signal, a direct linear relationship was found (fig 5.1b). The peak chemiluminescence signal also increased with the amount of NO (200 - 1600 pmol) added, but the relationship was not strictly linear (fig 5.1c). Also shown on fig 5.1b & c are second standard curves obtained 90 min after preparing the gas and these show that the NO had decayed during this time despite being in a sealed flask.

Conversion of nitrite or nitrite/nitrate to NO

Before measuring nitrite or nitrite/nitrate content of biological samples it was first necessary to develop a suitable reducing system to convert these to NO. Three different systems were evaluated:-

1: Reduction using 1,1'-dimethylferrocene/acetonitrile system

The reducing system using 1,1'-dimethylferrocene/acetonitrile (Termin *et al.*, 1992) was attractive since it operated at room temperature and did not involve the use of boiling acid. However, when samples of sodium nitrite (500 - 1000 pmol) were reduced using this system, the chemiluminescence signals generated were small and difficult to discern from the background noise (fig 5.2). Furthermore, they were long in duration (approximately 10 min) suggesting a slow rate of reduction of nitrite to NO. Since only small, slow signals were generated by large amounts of sodium nitrite, the use of this reducing system was abandoned.

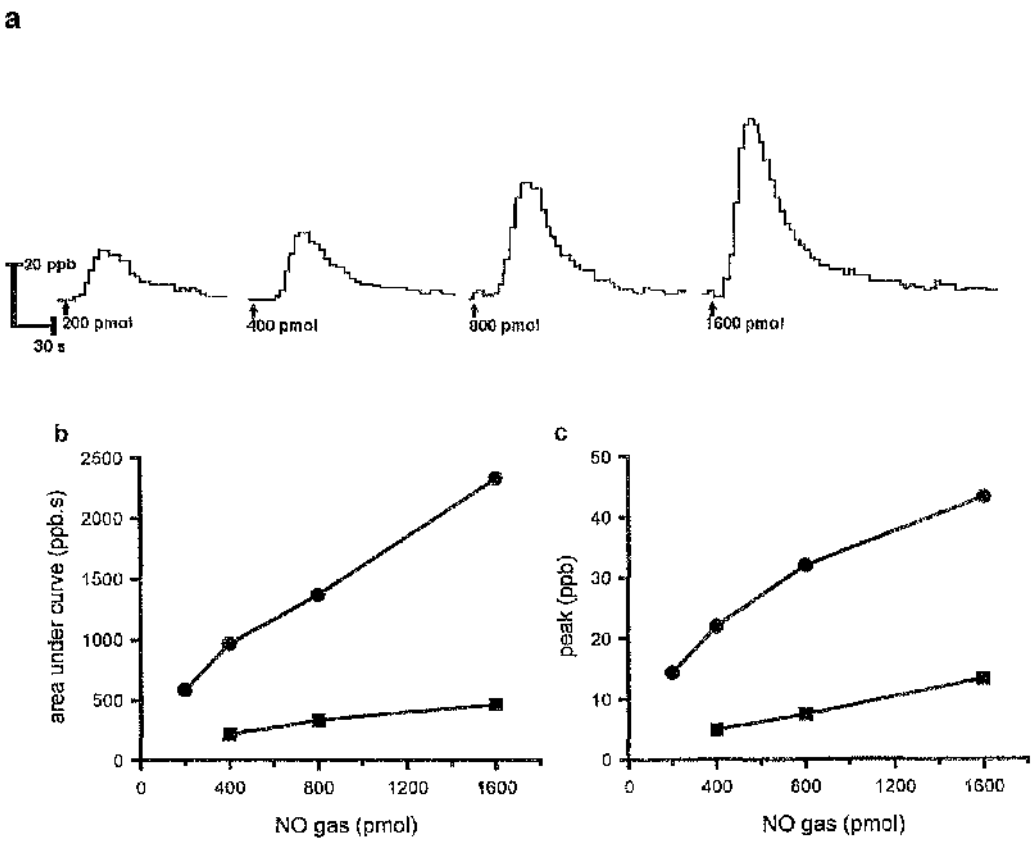


Figure 5.1: Standard curve to nitric oxide (NO) gas (200 - 1600 pmol) obtained from chemiluminescence: **(a)** chemiluminescence signals plotted as ppb versus time (s); **(b)** plotted as the area under the curve of the signal in ppb.s and **(c)** plotted as the peak signal in ppb. NO gas tested immediately after preparation (●) and NO gas tested 90 min after preparation (■) showing the decay of NO even in a sealed flask. Each point is the mean of 2 observations.

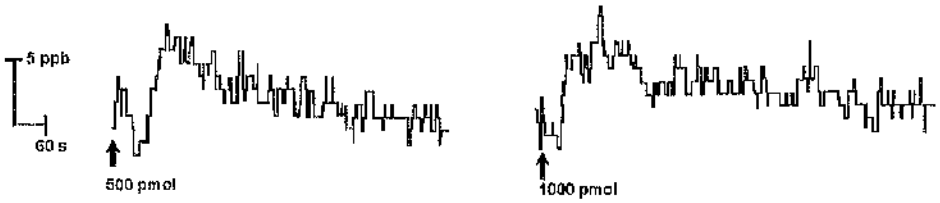


Figure 5.2: Chemiluminescence signals produced by sodium nitrite standards (500 and 1000 pmol) using the 1,1'-dimethylferrocene/acetonitrile reducing system plotted as ppb versus time (s).

2: Reduction using sodium iodide/glacial acetic acid reflux system

The reducing system using sodium iodide/glacial acetic acid is well established (Cox, 1980; Palmer *et al.,* 1987), but involves a reflux procedure at high temperature (65°C). When samples of sodium nitrite (200 - 1000 pmol) were reduced using this system, the chemiluminescence signals were much larger and easier to distinguish from the background noise (fig 5.3a) than those produced using the 1,1'-dimethylferrocene/acetonitrile system (fig 5.2). Furthermore, the signals were complete within approximately 5 min suggesting a rapid reduction of nitrite to NO. A standard curve (100 - 1000 pmol) was then prepared and the amount of sodium nitrite was plotted against the area under the curve (ppb.s) for each signal. A direct linear relationship was found (fig 5.3b) and the minimum detection level for nitrite measured using this chemiluminescence technique was 100 pmol. The peak chemiluminescence signal also increased with the amount of sodium nitrite (100 - 1000 pmol) added, but the relationship was not strictly linear (fig 5.3c). To calculate the nitrite content in experimental samples a regression line of best fit for the area under the graph was used (fig 5.3b).

In order to validate the selectivity of the chemiluminescence assay for the detection of nitrite, certain compounds containing nitrogen which were subsequently used in experiments were examined using the sodium iodide/glacial acetic acid reflux system to see if they generated false 'nitrite' signals. Sodium nitrate (1000 pmol) was injected into the reducing system and this produced no signal (fig 5.4). L-Arginine (1000 pmol), the endogenous substrate for nitric oxide synthase (NOS)(Palmer *et al.,* 1988), and analogues of L-arginine, which inhibit NOS (Moore *et al.,* 1990; Rees *et al.,* 1990b), i.e. N^G-nitro-L-arginine (L-NOARG; 1000 pmol) and N^G-monomethyl-L-arginine (L-NMMA; 1000 pmol), also produced no signal (fig 5.4).

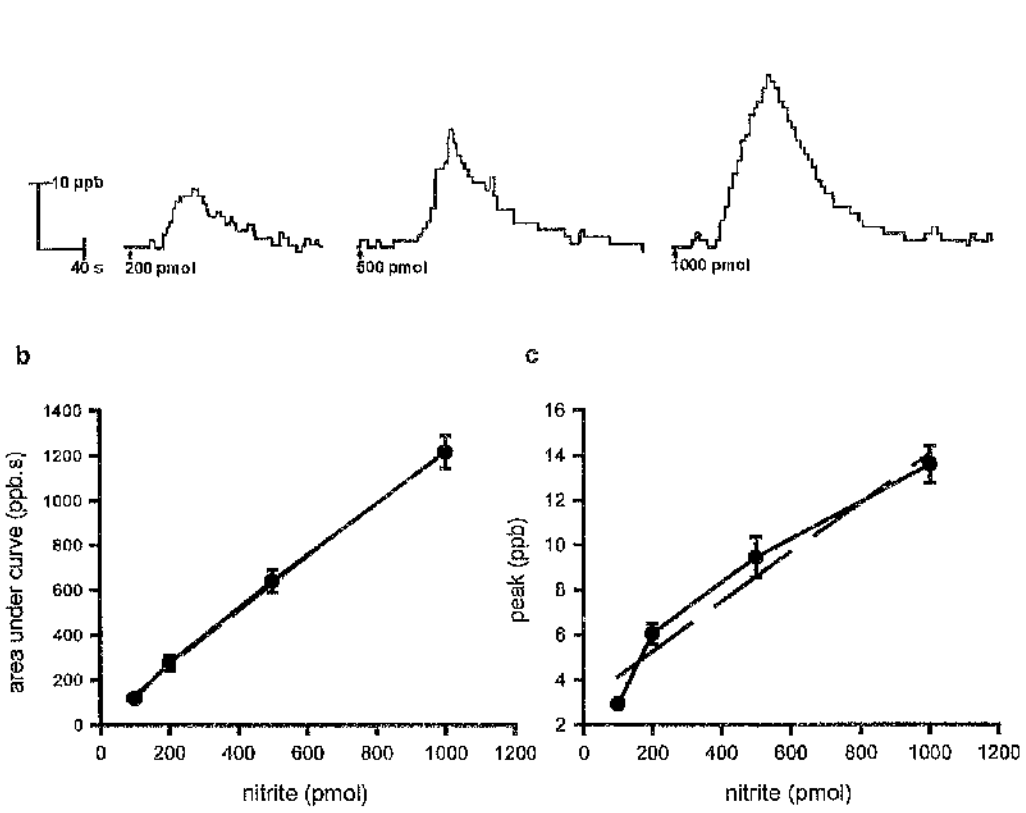


Figure 5.3: Standard curve to sodium nitrite (100 - 1000 pmol) obtained using the sodium iodide/glacial acetic acid reflux system: **(a)** chemiluminescence signals plotted as ppb versus time (s); **(b)** plotted as the area under the curve of the signal in ppb.s and **(c)** plotted as the peak signal in ppb. A continuous line joins the points and the broken line indicates a regression line of best fit. Each point is the mean of 10 - 34 observations with vertical bars representing the s.e. mean.

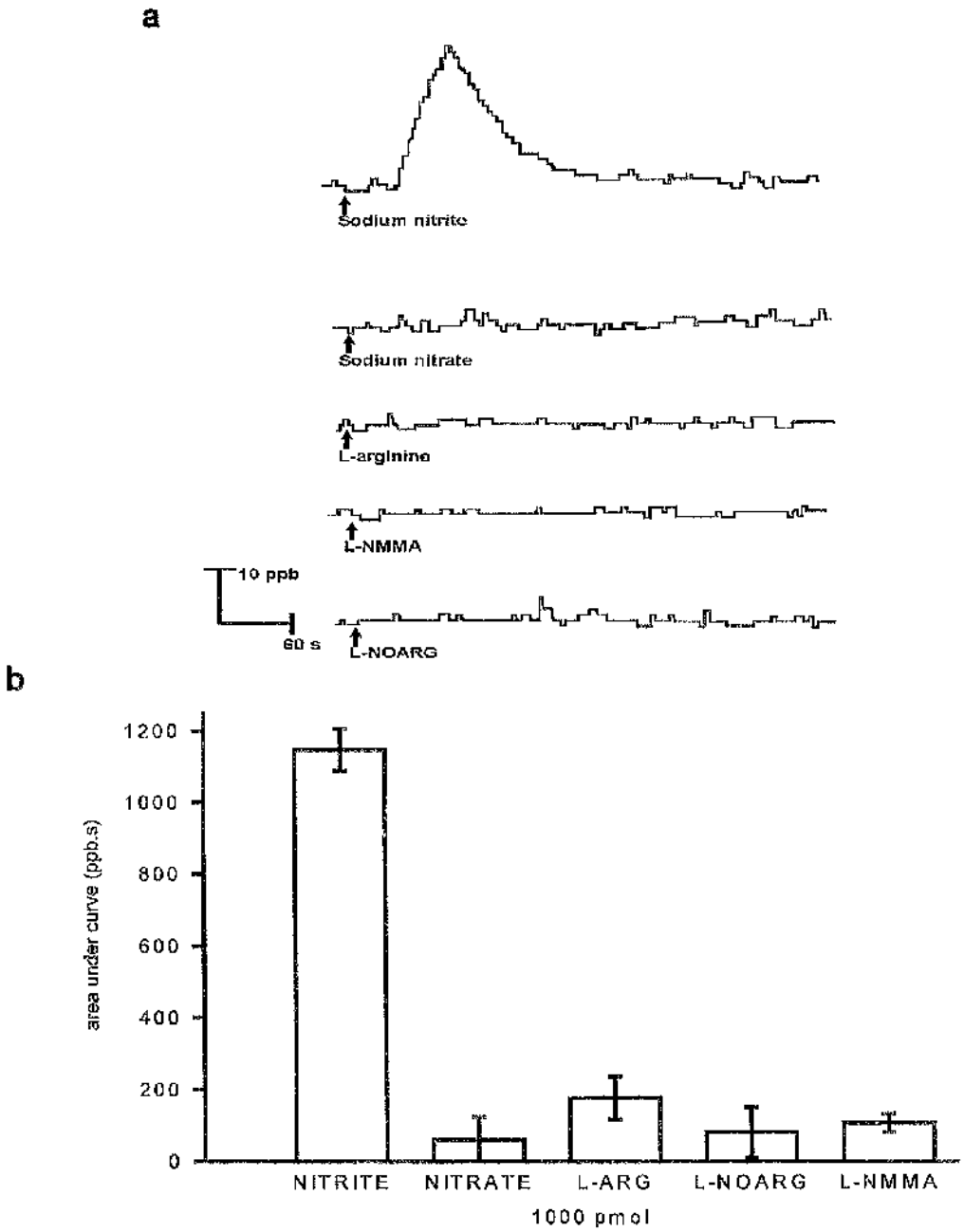


Figure 5.4: Chemiluminescence signals detected following injection of sodium nitrite, sodium nitrate, L-arginine (L-ARG), N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (L-NOARG), all at 1000 pmol, into the sodium iodide/glacial acetic acid reflux system: **(a)** plotted as ppb versus time (s) and **(b)** plotted as the area under the curve in ppb.s. Each column is the mean of 3 - 15 observations with vertical bars representing the s.e. mean.

Culture media and Krebs solutions which were subsequently used in experiments were also examined to see if they generated false 'nitrite' signals (fig 5.5). One ml volumes of bicarbonate-buffered Krebs or HEPES-buffered Krebs produced no signal, nor did 100 µl of foetal calf serum (FCS), but 100 µl of new born serum (NBS) produced a small signal (220 ± 33 ppb.s, n=4). One ml of bicarbonate-buffered Dulbecco's Modified Eagles Medium (DMEM) produced a signal equivalent to 723 ± 165 ppb.s (n=4), and when FCS (10%), NBS (10%), penicillin (100 u ml^{-1})/streptomycin (100 µg ml^{-1}) and glutamate (4 mM) were added, to give full culture medium, there was no significant difference in the signal obtained (888 ± 114 ppb.s, n=8). Similarly, 1 ml of HEPES-buffered DMEM produced a signal (830 ± 105 ppb.s, n=5) that was not significantly different from bicarbonate-buffered DMEM, and when FCS (10%), penicillin (100 u ml^{-1})/streptomycin (100 µg ml^{-1}) and glutamate (2 mM) were added, to give full HEPES-buffered culture medium, there was no significant difference in the signal obtained (632 ± 89 ppb.s, n=8). The constituents of DMEM producing the chemiluminescence signals are unknown but a full list is given in the Methods (table 4.2). In experiments, the area under the curve of the signals obtained from culture media was subtracted from experimental samples so that cellular production of nitrite could be assessed. Since, Krebs produced no signal by itself, background subtractions were not required when this was used in experiments.

3: Reduction using vanadium chloride/hydrochloric acid reflux system

The reducing system using vanadium chloride/hydrochloric acid reflux is also well established (Braman & Hendrix, 1989; Bush *et al.*, 1992), but involves a reflux procedure at high temperature (95°C). This reducing system was expected to be a more sensitive index of NO production by cells than the sodium iodide/glacial acetic acid reflux system as it had the additional

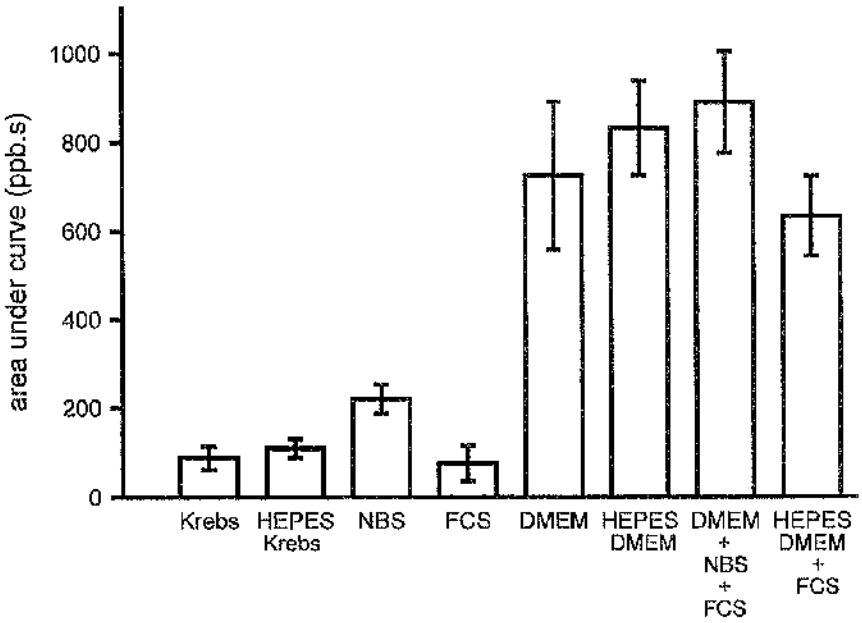


Figure 5.5: Chemiluminescence signals detected following injection of culture media or Krebs solutions into the sodium iodide/glacial acetic acid reflux system. New born serum (NBS) and foetal calf serum (FCS) were injected in 100 µl volumes, all other samples were in 1 ml volumes. Values are plotted as area under the curve in ppb.s. Samples tested were: Krebs; HEPES-buffered Krebs; NBS; FCS; Dulbecco's Modified Eagle's Medium (DMEM); HEPES-buffered DMEM; DMEM containing NBS (10%), FCS (10%), penicillin (100 u ml^{-1})/streptomycin (100 µg ml^{-1}) and glutamate (4 mM) (full culture medium) and HEPES-buffered DMEM containing 10% FCS, penicillin (100 u ml^{-1})/streptomycin (100 µg ml^{-1}) and glutamate (2 mM) (full HEPES-buffered culture medium). Each column is the mean of 3 - 10 observations with vertical bars representing the s.e. mean.

advantage of reducing both of NO's major metabolites, nitrite and nitrate, back to NO. When samples of sodium nitrite (200 - 1000 pmol) or sodium nitrate (200 - 1000 pmol) were reduced using this system, the chemiluminescence signals were large and easy to distinguish from the background noise (fig 5.6). Furthermore, the signals were complete within approximately 5 min, suggesting a rapid reduction of nitrite and nitrate to NO. Standard curves for sodium nitrite (200 - 1000 pmol) and sodium nitrate (200 - 1000 pmol) were prepared and when the amount added was plotted against the area under the curve (ppb.s) for each signal a direct linear relationship was found (fig 5.7a). Furthermore, at each concentration tested, the signals for nitrite and nitrate were not significantly different. The peak chemiluminescence signals also increased with the amount of sodium nitrite or sodium nitrate added, but the relationship was not strictly linear (fig 5.7b). Since biological samples would be expected to contain a mixture of nitrite and nitrate, and as the two standard curves for the area under the curve were similar, a combined standard curve (nitrite/nitrate) was produced (fig 5.7c) and this too gave a linear relationship. A regression line of best fit for these combined data was used to calculate the total oxides of nitrogen in experimental samples (fig 5.7c). The minimum detection limit for nitrite/nitrate using this chemiluminescence technique was lower (200 pmol) than for the sodium iodide/glacial acetic acid reflux system.

In order to validate the selectivity of the chemiluminescence assay for the detection of nitrite/nitrate, certain compounds containing nitrogen which were subsequently used in experiments were examined using the vanadium chloride/hydrochloric acid reflux system to see if they generated false 'nitrite/nitrate' signals (fig 5.8). Sodium nitrite (1000 pmol) and sodium nitrate (1000 pmol) gave signals (1493 ± 112 ppb.s n=10 and 1562 ± 126 ppb.s n=21, respectively; fig 5.8) that were not significantly different from each other. L-Arginine (1000 pmol), the endogenous substrate of NOS (Palmer *et al.*,

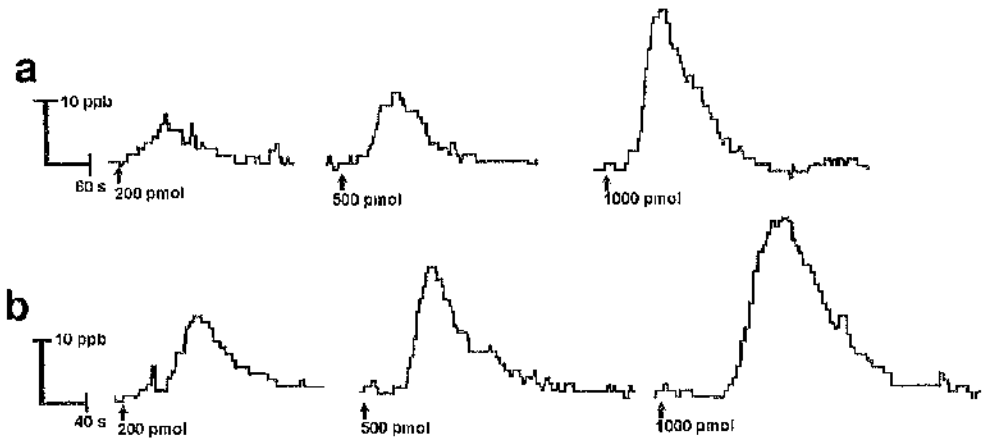


Figure 5.6: Chemiluminescence signals produced by **(a)** sodium nitrite and **(b)** sodium nitrate using the vanadium chloride/hydrochloric acid reflux system and plotted as ppb versus time (s).

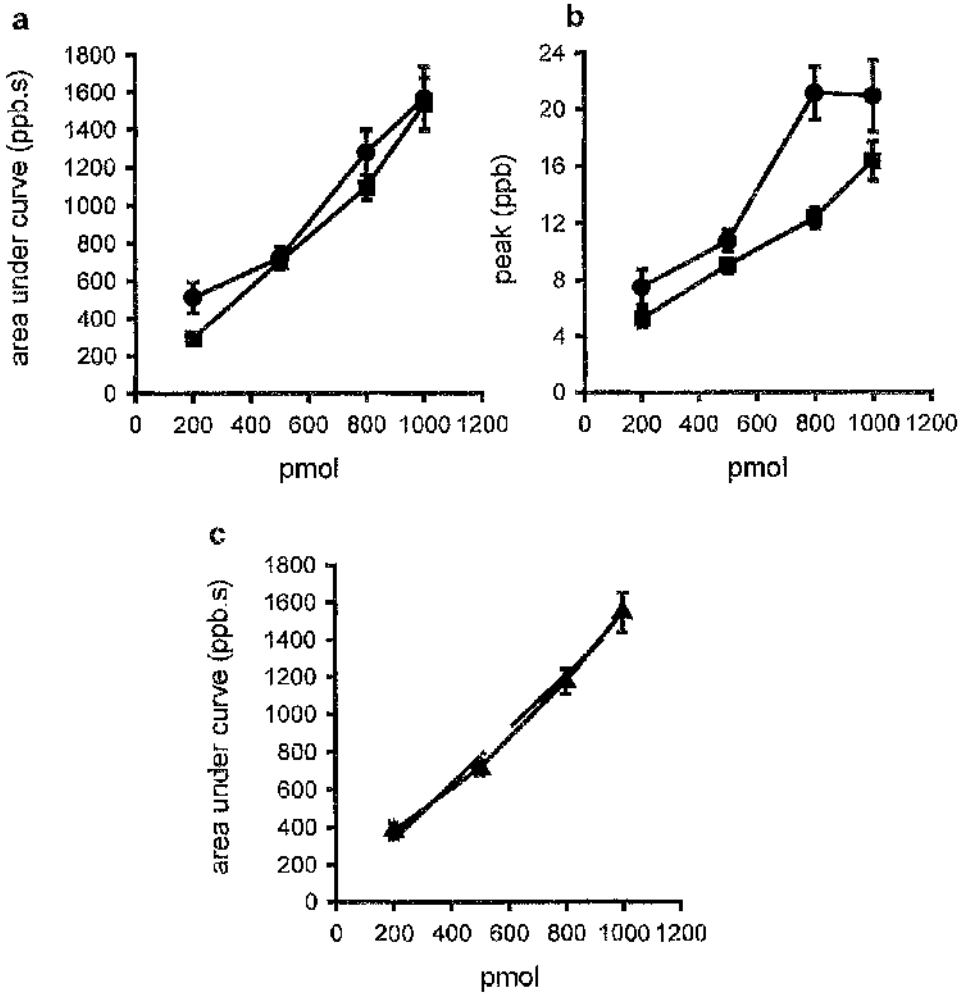


Figure 5.7: Standard curves to sodium nitrite (200 - 1000 pmol; ●), sodium nitrate (200 - 1000 pmol; ■) and total oxides of nitrogen (nitrite/nitrate 200-1000 pmol; ▲) obtained using the vanadium chloride/hydrochloric acid reflux system: **(a & c)** plotted as the area under the curve of the signal in ppb.s and **(b)** plotted as the peak signal in ppb. In **(c)** the continous line joins the points and the broken line indicates a regression line of best fit. Each point is the mean of 5 - 44 observations with vertical bars representing the s.e. mean.

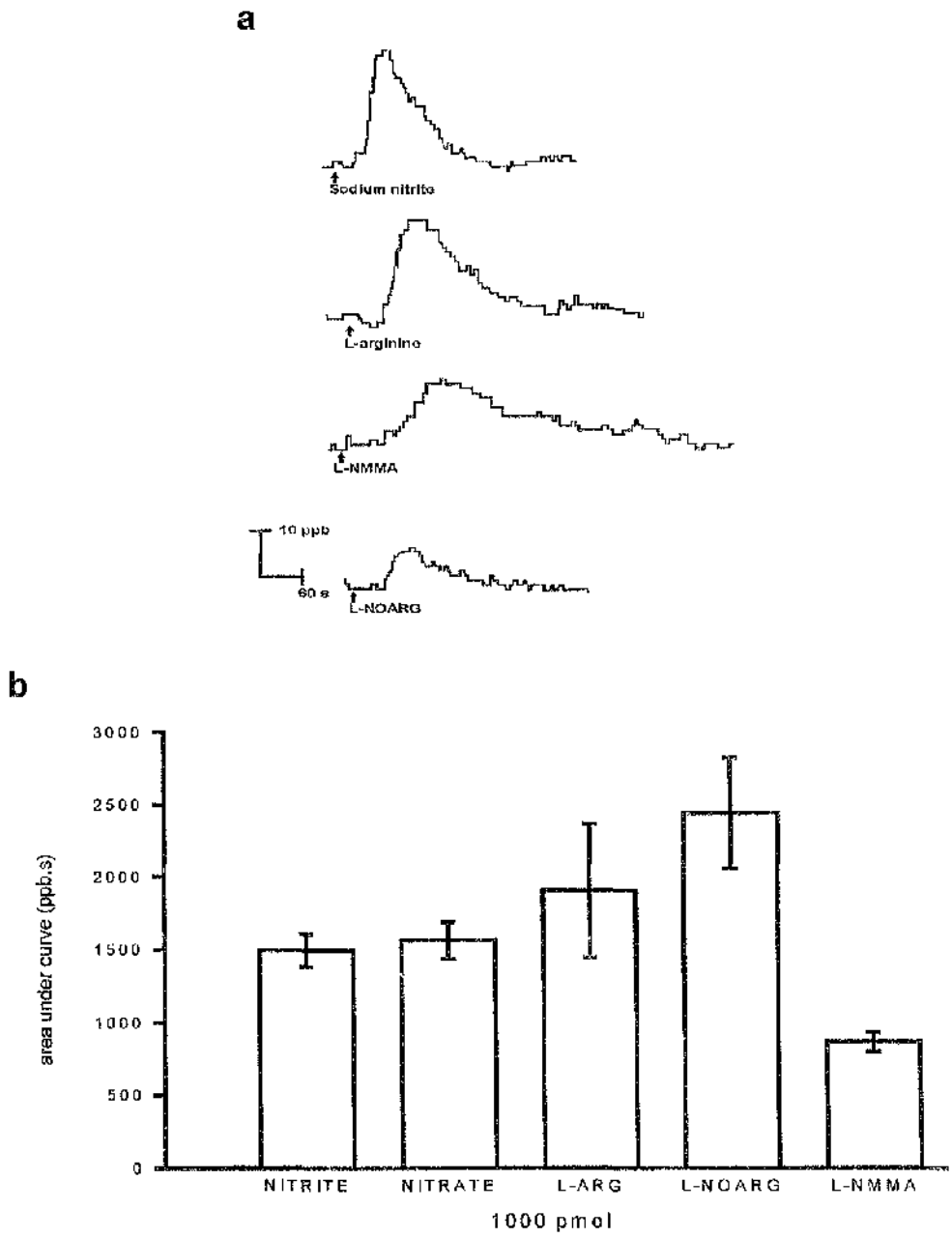


Figure 5.8: Chemiluminescence signals detected following the injection of sodium nitrite, sodium nitrate, L-arginine (L-ARG), N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (L-NOARG), all at 1000 pmol, into the vanadium chloride/hydrochloric acid reflux system, and **(a)** plotted as ppb versus time (s) and **(b)** plotted as the area under the curve of the signal in ppb.s. Each column is the mean of 3 - 21 observations with vertical bars representing the s.e. mean.

1988), produced a detectable signal (1908 ± 460 ppb.s, n=5) and analogues of L-arginine, which inhibit NOS (Moore *et al.*, 1990; Rees *et al.*, 1990b), i.e. L-NOARG (1000 pmol) and L-NMMA (1000 pmol), also produced detectable signals (2441 ± 381 ppb.s, n=8 and 867.8 ± 68.2 ppb.s, n=3, respectively). The large chemiluminescence signals produced by L-arginine and the L-arginine analogues would thus pose problems when attempting to estimate the nitrite/nitrate content of experimental samples if these compounds were present.

Culture media and Krebs solutions which were subsequently used in experiments were also examined to see if they generated false 'nitrite/nitrate' signals (fig 5.9). One ml of bicarbonate-buffered Krebs gave no signal. One hundred µl of NBS or FCS produced substantial signals equivalent to 2582 ± 157 ppb.s (n=6) and 4987 ± 515 ppb.s (n=4), respectively. One ml of bicarbonate-buffered DMEM alone produced a signal (692 ± 52 ppb.s, n=4) but when FCS (10%), NBS (10%), penicillin (100 u ml^{-1})/streptomycin (100 µg ml^{-1}) and glutamate (4 mM) were added, to give full culture medium, the signal was substantially larger (11373 ± 854 ppb.s, n=8). HEPES-buffered DMEM produced a signal (5508 ± 2000 ppb.s, n=3), which was substantially larger when FCS (10%), penicillin (100 u ml^{-1})/streptomycin (100 µg ml^{-1}) and glutamate (2 mM) were added (11477 ± 2177 ppb.s, n=7). Apart from the presence of nitrate ($Fe(NO_3)_3.9H_2O$; 0.1 mg ml^{-1}), the constituents of DMEM responsible for the chemiluminescence signals are unknown but a full list is given in the Methods (table 4.2). Clearly, the tissue culture media employed gave much larger signals using the vanadium chloride/hydrochloric acid reflux system compared with the sodium iodide/glacial acetic acid reflux system. In experiments, the area under the curve of the signals obtained from culture media alone were subtracted from experimental samples so that cellular

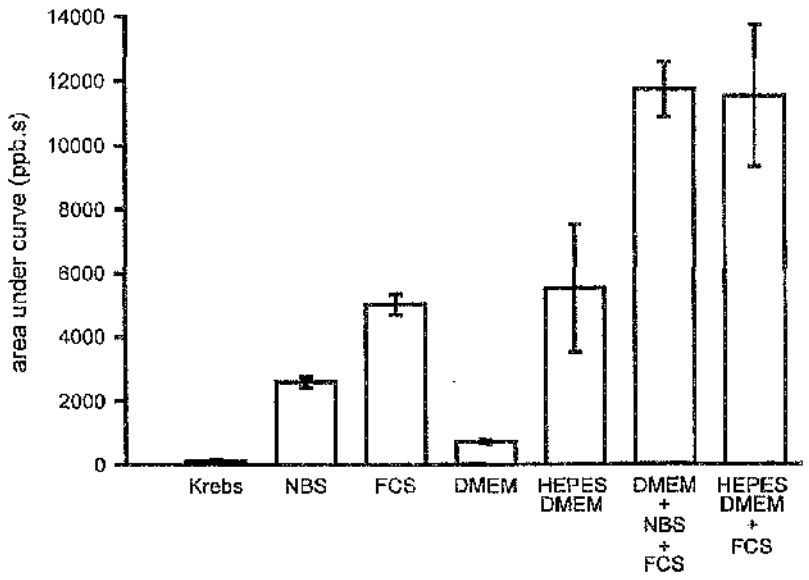


Figure 5.9: Chemiluminescence signals detected following the injection of culture media and Krebs solutions into the vanadium chloride/hydrochloric acid reflux system. New born serum (NBS) and foetal calf serum (FCS) were injected in 100 µl volumes, all other samples were in 1 ml volumes. Values are plotted as the area under the curve in ppb.s. Samples tested were: Krebs; NBS; FCS; Dulbecco's Modified Eagle's Medium (DMEM); HEPES-buffered DMEM; DMEM containing NBS (10%), FCS(10%), penicillin (100 u ml^{-1})/streptomycin (100 µg ml^{-1}) and glutamate (4 mM) (full culture medium) and HEPES-buffered DMEM containing 10% FCS penicillin (100 u ml^{-1})/streptomycin (100 µg ml^{-1}) and glutamate (2 mM) (full HEPES-buffered culture medium). Each column is the mean of 3 - 8 observations with vertical bars representing the s.e. mean.

production of nitrite/nitrate could be measured. Since, Krebs produced no signal by itself, background subtractions were not required when this was used in experiments.

Nitrite or nitrite/nitrate content of experimental samples measured using chemiluminescence

Having established that the sodium iodide/glacial acetic acid reflux system could measure nitrite content and that the vanadium chloride/hydrochloric acid reflux system could measure nitrite/nitrate content, we then went on to examine the utility of these methods for assessing cellular production of NO. The values obtained using these techniques were compared with those measured using the more established Griess reaction. As indicated in the Methods the threshold sensitivity of the Griess reaction for nitrite was 300 pmol.

Expression of iNOS in J774.2 murine macrophages

We first wished to examine J774.2 murine macrophages since these cells are known to produce large amounts of NO following expression of inducible nitric oxide synthase (iNOS) (Stuehr & Marletta, 1985; Stuehr & Marletta, 1987a). To do this we examined basal accumulation of nitrite and nitrite/nitrate into the medium bathing unstimulated cells then examined accumulation following expression of iNOS stimulated by endotoxin (lipopolysaccharide; LPS) and/or interferon-γ (IFN-γ). A comparison was made of the measurements of accumulation of nitrite and/or nitrite/nitrate into the bathing media using the Griess reaction and both chemiluminescence techniques (sodium iodide/glacial acetic acid reflux system and vanadium chloride/hydrochloric acid reflux system).

Basal production of nitrite into the medium bathing J774.2 murine macrophages incubated for 24 h at 37°C was similar when measured using the Griess reaction and the sodium iodide/glacial acetic acid reflux system (5.3 ± 0.3 nmol, n=10 and 4.5 ± 0.5 nmol, n=3 respectively: fig 5.10a & b). When basal production of nitrite/nitrate into the medium bathing the cells was measured using the vanadium chloride/hydrochloric acid reflux system the value was similar to the value measured by the other two systems (5.3 ± 4.7 nmol, n=4; fig 5.10c). IFN-γ (1 - 100 u ml^{-1}) produced a concentration-dependent stimulation of nitrite accumulation into the medium bathing J774.2 murine macrophages following a 24 h incubation (fig 5.10a). Maximal nitrite accumulation at IFN-γ, 100 u ml^{-1} following subtraction of basal production, as measured by the Griess reaction (44.4 ± 0.6 nmol, n=3), was slightly lower than that measured using the sodium iodide/glacial acetic acid reflux system (54.5 ± 3.5 nmol, n=4; fig 5.10b). Maximal accumulation of nitrite/nitrate measured using the vanadium chloride/hydrochloric acid reflux system (fig 5.10c), was between the values obtained using the other two systems (52.5 ± 7.3 nmol, n=4). The major NO-derived metabolite produced by unstimulated and stimulated J774.2 murine macrophages was therefore nitrite.

LPS from *Salmonella typhosa* (1 - 1000 ng ml^{-1}) also stimulated the production of nitrite by J774.2 murine macrophages in a concentration-dependent manner (fig 5.11a and b). Maximal nitrite accumulation into the medium bathing cells stimulated by LPS (1000 ng ml^{-1}), following subtraction of basal nitrite production, was lower when measured using the Griess reaction (7.5 ± 0.9 nmol, n=4; fig 5.11a) than that obtained using the sodium iodide/glacial acetic acid reflux system (10.6 ± 0.7 nmol, n=4; fig 5.11b).

An examination was conducted to see if following a 24 h incubation of J774.2 murine macrophages a combined stimulus of LPS and IFN-γ could further

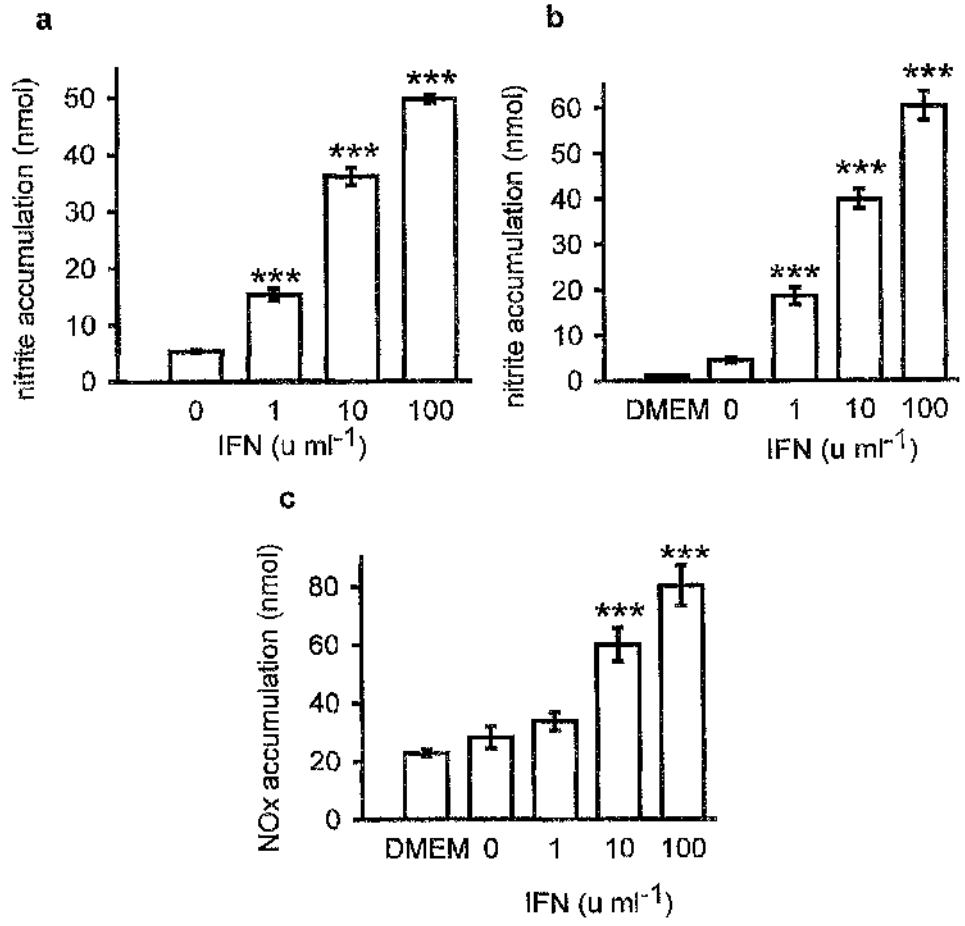


Figure 5.10: Effects of 24 h incubation with interferon-γ (IFN; 0 - 100 u ml^{-1}) on nitrite or nitrite/nitrate production by J774.2 murine macrophages measured by **(a)** the Griess reaction **(b)** the sodium iodide/glacial acetic acid reflux system and **(c)** the vanadium/chloride hydrochloric acid reflux system. Values are given as either nitrite accumulation (nmol per 10^6 cells) or nitrite/nitrate (NO_x) accumulation (nmol per 10^6 cells). Also shown on **(b)** and **(c)** are the background readings obtained from full HEPES-buffered culture medium (DMEM). Each column is the mean of 3 - 10 observations with vertical bars representing the s.e. mean. *** $P<0.005$, indicates a significant difference from cells receiving no IFN.

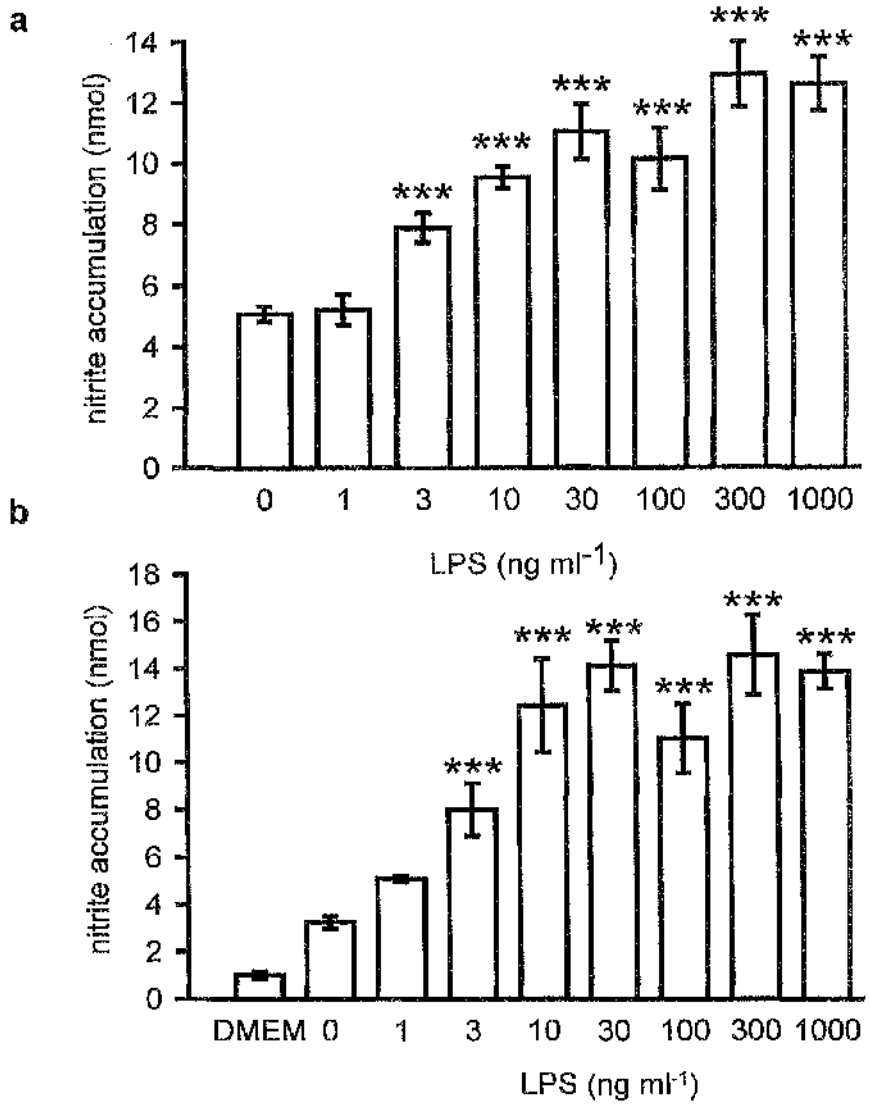


Figure 5.11: Effects of 24 h incubation with endotoxin from *Salmonella typhosa* (LPS; 0 - 1000 ng ml^{-1}) on nitrite production by J774.2 murine macrophages measured by **(a)** the Griess reaction and **(b)** the sodium iodide/glacial acetic acid reflux system. Values are given as nitrite accumulation (nmol per 10^6 cells). Also shown in **(b)** is the background reading obtained from full HEPES-buffered culture medium (DMEM). Each column is the mean of 3 - 8 observations with vertical bars representing the s.e. mean. *** $P<0.005$, indicates a significant difference from cells receiving no LPS.

induce nitrite production over that produced by either stimulus alone. The LPS concentration of 100 ng ml^{-1} was chosen as this alone gave a near maximal increase in nitrite accumulation, when measured using the Griess reaction (8.4 ± 0.9 nmol, n=12; fig 5.12a) or the sodium iodide/glacial acetic acid reflux system (5.5 ± 1.5 nmol, n=4; fig 5.12b). The concentration of IFN-γ was varied (1 - 100 u ml^{-1}) but each concentration itself produced a significant increase in nitrite accumulation. When measured using the Griess reaction and the sodium iodide/glacial acetic acid reflux system the combination of LPS (100 ng ml^{-1}) with IFN-γ at all concentrations tested produced levels of nitrite accumulation that were significantly greater than those induced by IFN-γ or LPS alone (fig 5.12).

Attempted expression of iNOS in BAEC

We wished to examine if expression of iNOS could be stimulated in bovine aortic endothelial cells (BAEC). Accumulation of nitrite or nitrite/nitrate into the bathing media were measured using both chemiluminescence techniques (sodium iodide/glacial acetic acid reflux system and vanadium chloride/hydrochloric acid reflux system).

The full culture medium bathing the BAEC during a 24 h incubation was taken for analysis of nitrite accumulation using the sodium iodide/glacial acetic acid reflux system. Accumulation of nitrite into the medium bathing unstimulated BAEC was 27.8 ± 4 nmol and this was unaffected by treatment for 24 h with LPS from *Salmonella typhosa* (1 - 1000 ng ml^{-1}, n=3; fig 5.13a). When, for comparison, the nitrite/nitrate content of unstimulated BAEC were measured using the vanadium chloride/hydrochloride acid reflux system, following subtraction of background levels for full culture medium, almost identical values were obtained (31.2 ± 4 nmol, n=3) and again no stimulation of production was observed in response to LPS (1 - 1000 ng ml^{-1}; fig 5.13b).

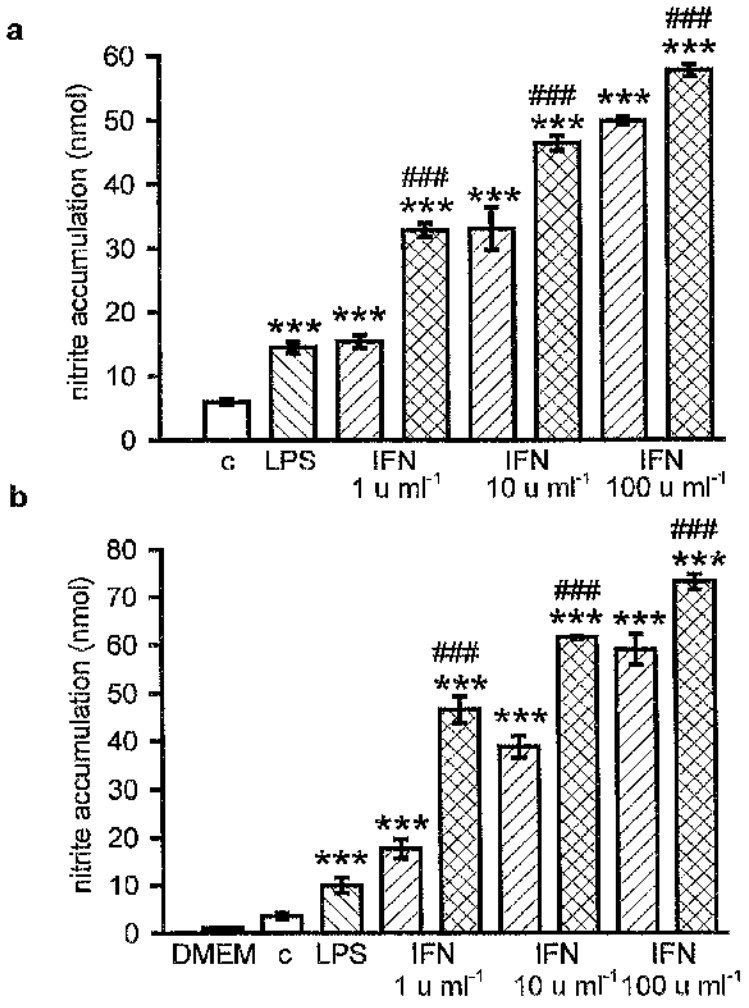


Figure 5.12: Effects of 24 h incubation with interferon-γ (IFN; 0 - 100 u ml^{-1}; ▨); endotoxin from *Salmonella typhosa* (LPS; 100 ng ml^{-1}; ▨) or a combination of IFN (1, 10 and 100 u ml^{-1}) with a fixed concentration of LPS (100 ng ml^{-1}; ▨) on nitrite production by J744.2 murine macrophages measured by **(a)** the Griess reaction and **(b)** the sodium iodide/glacial acetic acid reflux system. Values are given as nitrite accumulation (nmol per 10^6 cells). Also shown on **(b)** is the background reading obtained from full HEPES-buffered culture medium (DMEM). Each column is the mean of 3 - 16 observations with vertical bars representing the s.e. mean. ** P<0.01 and *** P<0.005, indicate a significant difference from control (c; ▭). ### P<0.005, indicates a significant difference from either drug alone.

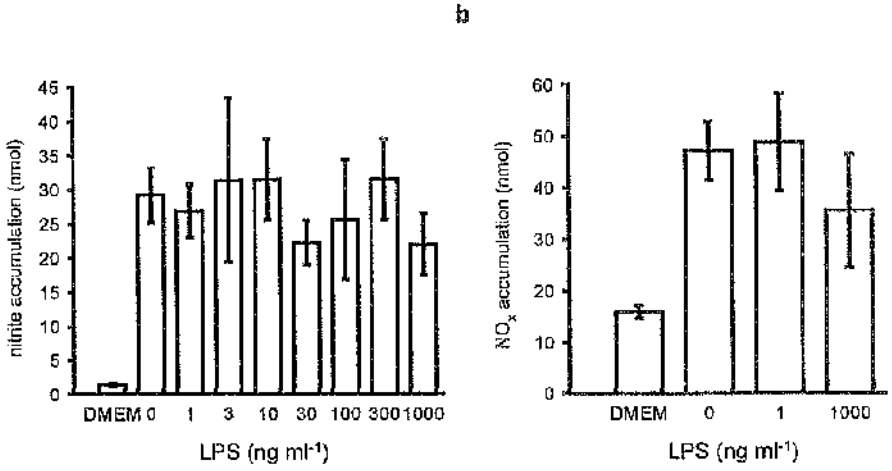


Figure 5.13: Effects of 24 h incubation with endotoxin from *Salmonella typhosa* (LPS; 1 - 1000 ng ml^{-1}) on **(a)** nitrite accumulation and **(b)** nitrite/nitrate accumulation into the medium bathing bovine aortic endothelial cells measured by **(a)** the sodium iodide/glacial acetic acid reflux system and **(b)** the vanadium chloride/hydrochloric acid reflux system. Values are given as nitrite accumulation (nmol per 10^6 cells) or nitrite/nitrate (NO_x) accumulation (nmol per 10^6 cells). Also shown are the background signals obtained from full culture medium (DMEM). Each column is the mean of 3 - 23 observations with vertical bars representing the s.e. mean.

Experiments were then conducted to determine if the basal production of nitrite by BAEC was derived from the L-arginine/NO system. These experiments involved examining the effects on basal levels of nitrite of two inhibitors of NOS, L-NMMA and N^G-nitro-L-arginine methyl ester (L-NAME)(Moore *et al.*, 1990; Rees *et al.*, 1990a), as well as dexamethasone, which inhibits the expression of iNOS (Di Rosa *et al.*, 1990; Knowles *et al.*, 1990b; Rees *et al.*, 1990a). Neither L-NMMA (100 μM) nor L-NAME (100 μM) when present throughout the 24 h incubation significantly affected basal production of nitrite, as measured using the sodium iodide/glacial acetic acid reflux system (fig 5.14a). Furthermore, dexamethasone (1 μM), when present throughout the 24 h incubation also failed to affect basal production of nitrite, measured using the same assay (fig 5.14b).

Validation of a novel fluorometric method for determination of nitrite

Towards the end of these experiments an apparently attractive fluorometric method for the determination of nitrite in biological samples was published (Damiani & Burini, 1986; Misko *et al.*, 1993). This was based upon the reaction of nitrite with 2,3-diaminonaphthalene (DAN) in acidic solutions to form 1-[H]-naphthotriazone, a highly fluorescent compound in alkaline medium. An attempt was made to compare the utility and sensitivity of this new technique with the Griess reaction and the chemiluminescence techniques.

Following reaction with DAN at 37°C for 10 min a standard solution containing sodium nitrite (1200 pmol) in Krebs solution was excited at a wavelength of 365 nm and the emission spectrum measured (fig 5.15). The greatest difference in the emission spectrum from that of the blank (Krebs solution reacted in the same way as the nitrite standard) occurred at a wavelength of approximately 405 nm. This emission wavelength of 405 nm was then used to

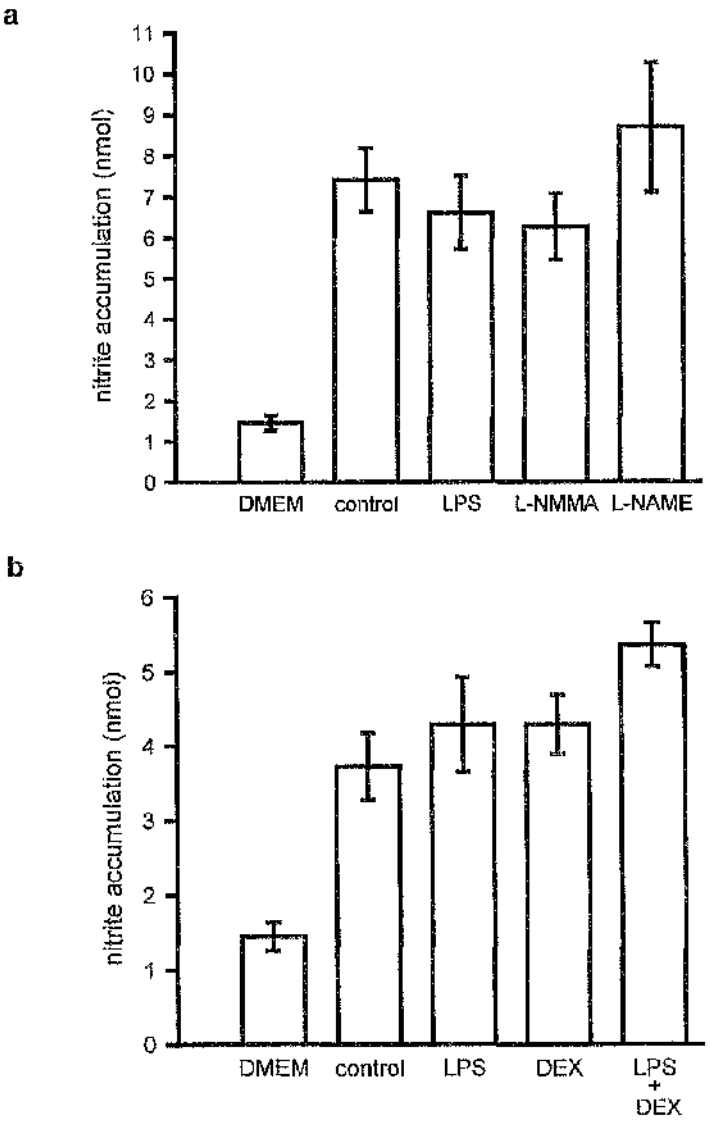


Figure 5.14: Effects of 24 h incubation with **(a)** endotoxin from *Salmonella typhosa* (LPS; 100 ng ml^{-1}), N^G-monomethyl-L-arginine (L-NMMA 100 μM) and N^G-nitro-L-arginine methyl ester (L-NAME 100 μM) and **(b)** LPS (100 ng ml^{-1}) and dexamethasone (DEX; 1 μM), alone and in combination on nitrite accumulation into the medium bathing bovine aortic endothelial cells measured by the sodium iodide/glacial acetic acid reflux system. Values are given as nitrite accumulation (nmol per 10^6 cells). Also shown are the background readings obtained from full culture medium (DMEM). Each column is the mean of 3 - 10 observations with vertical bars representing the s.e. mean.

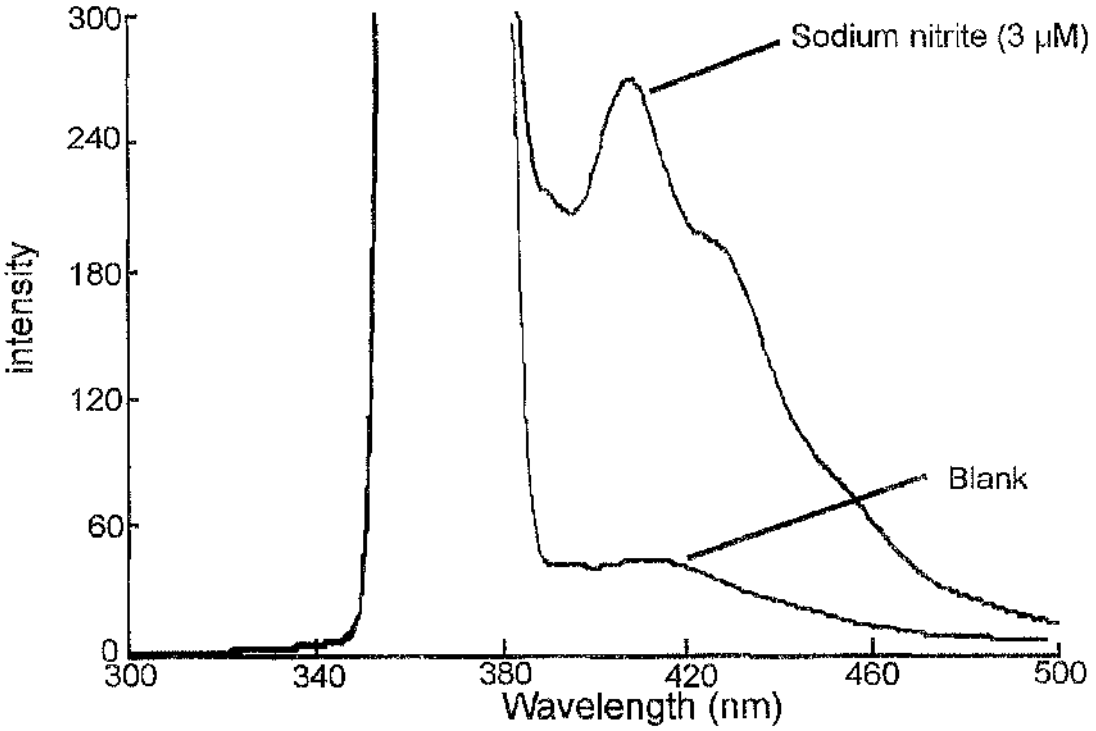


Figure 5.15: Emission spectra of the fluorescence intensity of Krebs (blank) and sodium nitrite (3 µM; 1200 pmol) dissolved in Krebs solution following reaction with 2,3-diaminonaphthalene at 37°C for 10 min measured at an excitation wavelength of 367 nm.

examine the excitation spectrum (fig 5.16). The greatest difference in the excitation spectrum from that of the blank occurred at a wavelength of 365 nm. A standard curve was then prepared using sodium nitrite (4 - 1200 pmol) dissolved in Krebs solution and following reaction with DAN at 37°C for 10 min, the relative fluorescent intensities were measured at 405 nm, with excitation at 365 nm (fig 5.17). The threshold sensitivity was around 120 pmol and the fluorescent intensity increased in a reasonably linear manner up to values of 1200 pmol. A second standard curve was prepared to sodium nitrite (0 - 4000 pmol) dissolved in full tissue culture medium (fig 5.18). Again, the threshold sensitivity was 120 pmol and the fluorescent intensity increased in a linear manner up to values of 4000 pmol.

Comparison of measuring techniques

A comparison of the standard curves for each of the techniques for measurement of nitrite and nitrite/nitrate used in this study can be made from fig 5.19.

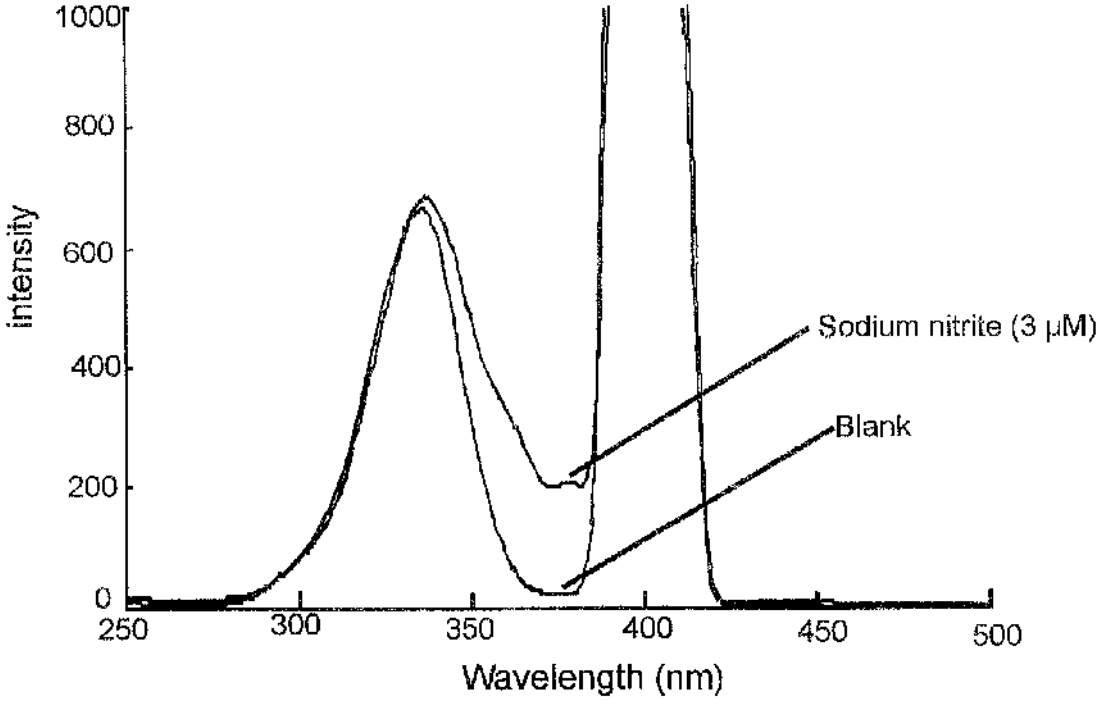


Figure 5.16: Excitation spectra of the fluorescence intensity of Krebs (blank) and sodium nitrite (3 µM; 1200 pmol) dissolved in Krebs solution following reaction with 2,3-diaminonaphthalene at 37°C for 10 min measured at an emission wavelength of 405 nm.

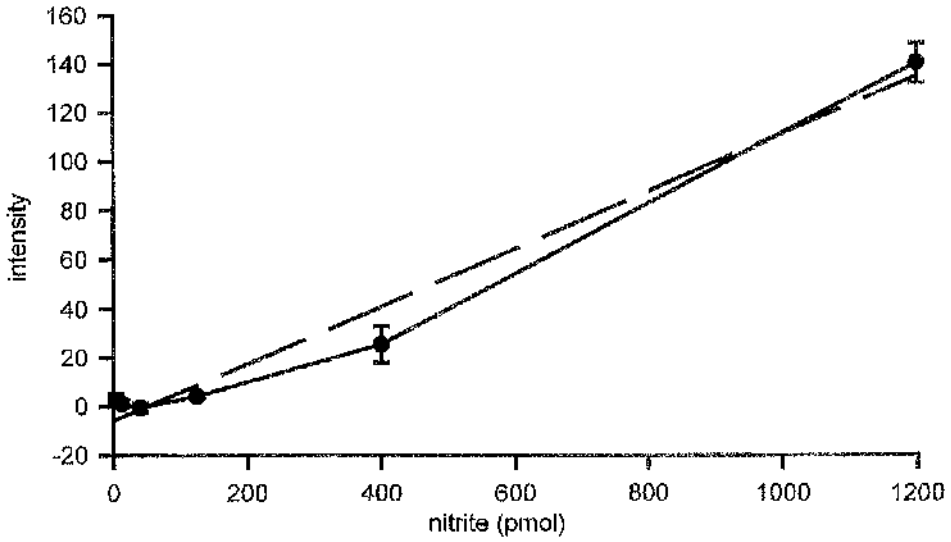


Figure 5.17: Standard curve to sodium nitrite (4 - 1200 pmol) prepared in Krebs solution obtained by measuring the fluorescent intensity at 405 nm of the product obtained following the reaction with 2,3-diaminonapthalene when excited at 365 nm. The continous line joins the points and the broken line represents the regression line of best fit. Each point is the mean of 4 observations with vertical bars representing the s.e. mean.

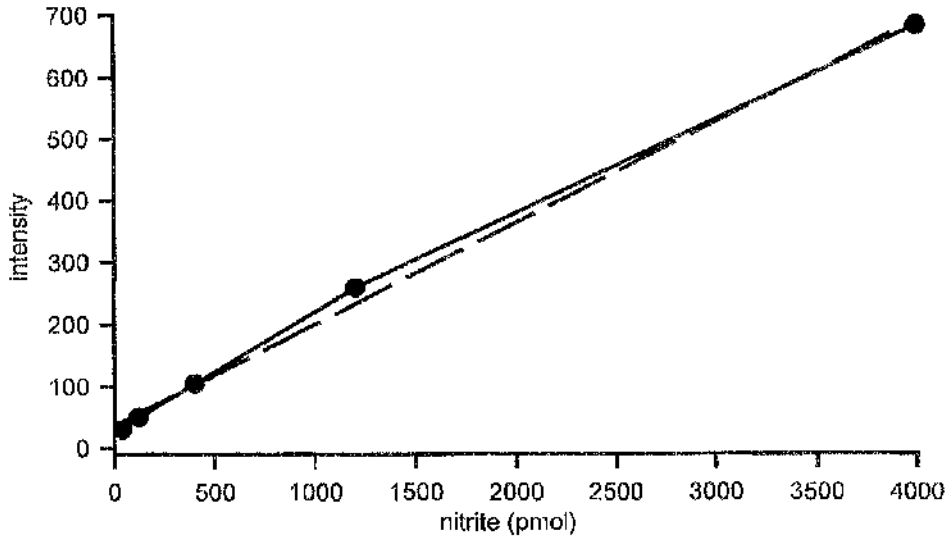


Figure 5.18: Standard curve to sodium nitrite (40 - 4000 pmol) prepared in full culture medium obtained by measuring the fluorescent intensity at 405 nm of the product obtained following the reaction with 2,3-diaminonapthalene when excited at 365 nm. The continous line joins the points and the broken line represents the regression line of best fit. Each point is the mean of 2 observations.

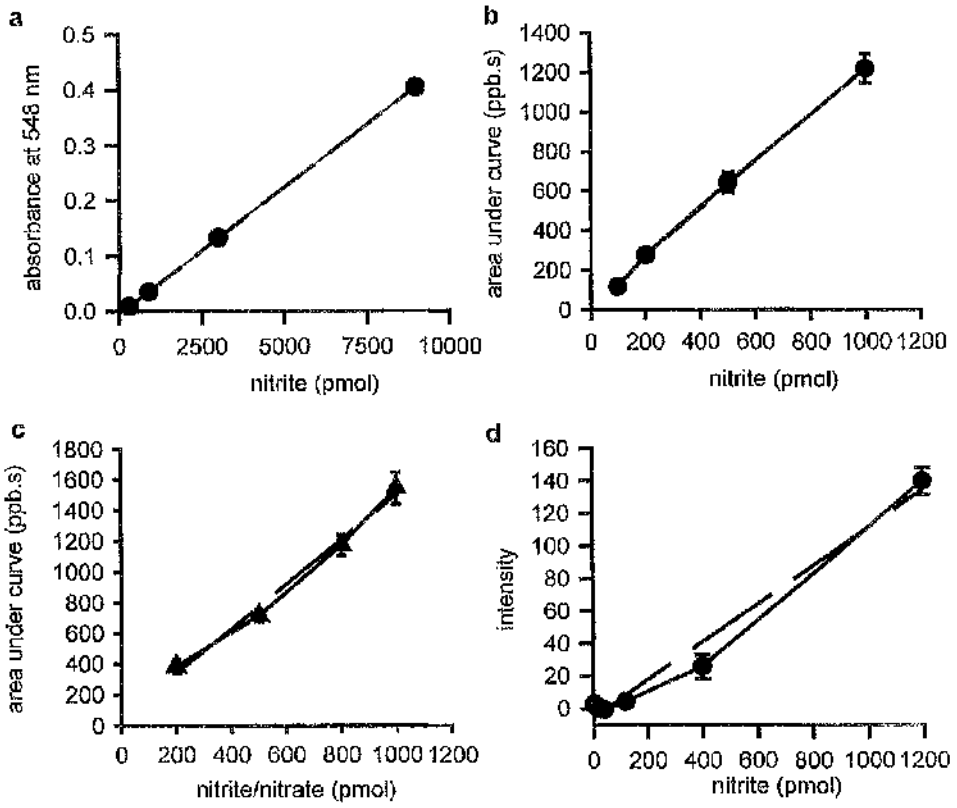


Fig 5.19: Standard curves to sodium nitrite (4 - 9000 pmol; ●) and to sodium nitrite/nitrate (200 - 1000 pmol; ▲) obtained using **(a)** the Griess reaction; **(b)** the sodium iodide/glacial acetic acid reflux system; **(c)** the vanadium chloride/hydrochloric acid reflux system and **(d)** the fluorometric assay. The continous lines join the points and the broken lines indicate regression lines of best fit. Each point is the mean of 4 - 30 observations with vertical bars representing the s.e. mean.

Chapter 6

Expression of inducible nitric oxide synthase in isolated endothelium-denuded rings of rat aorta

Expression of iNOS in endothelium-denuded rings of rat aorta assessed by nitrite accumulation into the Krebs solution

Previous reports described expression of inducible nitric oxide synthase (iNOS) in isolated arterial rings following overnight incubation, as indicated by depression of vasoconstriction (Wakabayashi *et al.*, 1987; Fleming *et al.*, 1991b; Szabó *et al.*, 1992). An attempt was made to determine if expression of iNOS could be assessed by measuring nitrite accumulation into the Krebs solution bathing endothelium-denuded rings of rat aorta using the sodium iodide/glacial acetic acid reflux system. All rings were denuded of endothelium and incubated overnight (18 h) in Krebs solution in the absence and presence of drugs as indicated below.

At the end of the overnight (18 h) incubation of rat aortic rings in Krebs solution, nitrite accumulation reached 680 ± 90 pmol, n=8 and this was unaffected if L-arginine (1 mM), the endogenous substrate for NOS (Palmer *et al.*, 1987), was included (fig 6.1a). In addition, if the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 1 mM)(Rees *et al.*, 1990b; McCall *et al.*, 1993), was included with L-arginine in the Krebs solution for the overnight (18 h) incubation this also had no effect on nitrite accumulation (fig 6.1a). Furthermore, including endotoxin from *Salmonella typhosa* (LPS; 100 ng ml^{-1}) or polymixin B (30 µg ml^{-1}), a scavenger of lipopolysaccharide (LPS) (Lasfargues *et al.*, 1989), during the overnight (18h) incubation also failed to influence the accumulation of nitrite into the Krebs bathing the aortic rings (fig 6.1b).

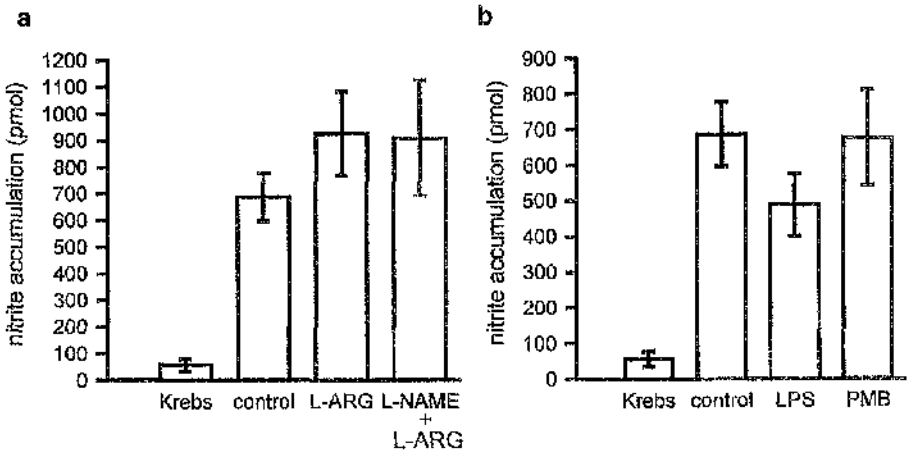


Figure 6.1: Effects of overnight (18 h) incubation with **(a)** L-arginine (L-ARG; 1 mM) alone and in combination with N^G-nitro-L-arginine methyl ester (L-NAME; 1mM) and **(b)** endotoxin from *Salmonella typhosa* (LPS; 100 ng ml^{-1}) and polymixin B (PMB; 30 µg ml^{-1}) on nitrite accumulation into the medium bathing endothelium-denuded rings of rat aorta measured by the sodium iodide/glacial acetic acid reflux system. Values are given as nitrite accumulation (pmol). Also shown are the levels attained in the absence of drugs (control) as well as the background signals obtained from Krebs solution. Each column is the mean of 4 - 13 observations with vertical bars representing the s.e. mean.

Expression of iNOS in endothelium-denuded rings of rat aorta assessed by the depression of PE-induced contraction

In the previous section nitrite accumulation into the Krebs bathing endothelium-denuded rings of rat aorta incubated at 37°C for 18 h was measured. Although nitrite did accumulate into the Krebs solution, there was no evidence that it was derived from the L-arginine/NO system. It was therefore not possible to conclude if iNOS had indeed been expressed. Consequently, a further set of experiments were conducted to determine if expression of iNOS could be assessed by measuring the depression in phenylephrine (PE)-induced contraction following incubation of endothelium-denuded rings of rat aorta.

Depression of PE-induced contraction

Freshly isolated endothelium-denuded rings of rat aorta contracted in a concentration-dependent manner to PE (1 nM - 10 μM; fig 6.2 and 6.3). The sensitivity and the maximal contraction to PE declined following incubation in Krebs solution at 37°C for 18 h.

Attempts were made to determine if this depression of PE-induced contraction was due to the production of NO following expression of iNOS in rat aortic rings. These experiments involved using drugs known to inhibit the synthesis, actions of NO and the expression of iNOS.

Reversal of depression of PE-induced contraction by L-NAME

The concentration-dependent contraction to PE (1 nM - 10 μM) in freshly isolated endothelium-denuded rings of rat aorta was unaltered by the addition

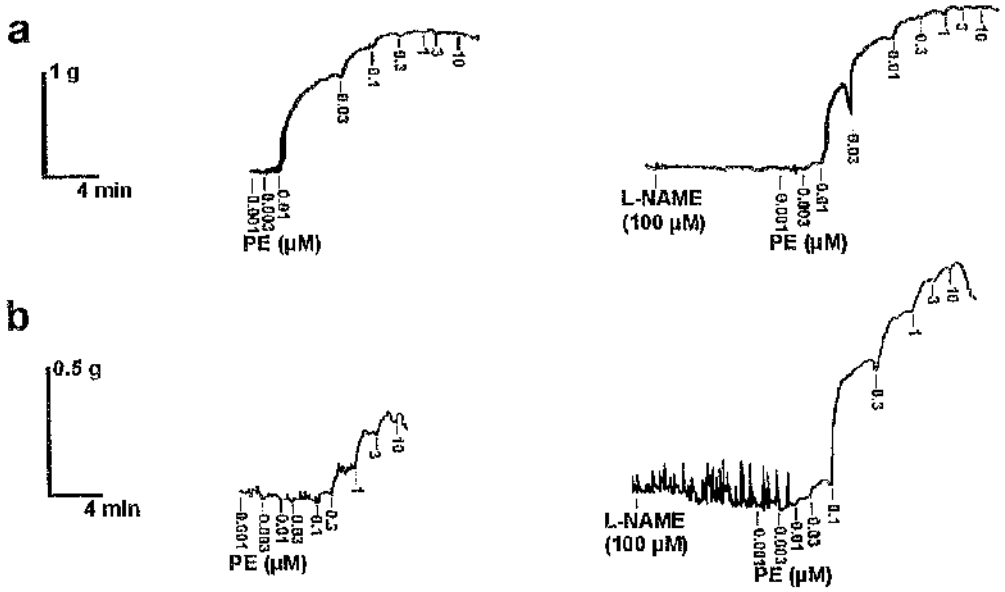


Figure 6.2: Individual traces showing contraction to phenylephrine (PE; 1 nM - 10 μM) in endothelium-denuded rings of rat aorta and the effects of L-NAME (100 μM) on these responses. **(a)** Freshly isolated rings and **(b)** rings incubated at 37°C for 18 h in Krebs solution.

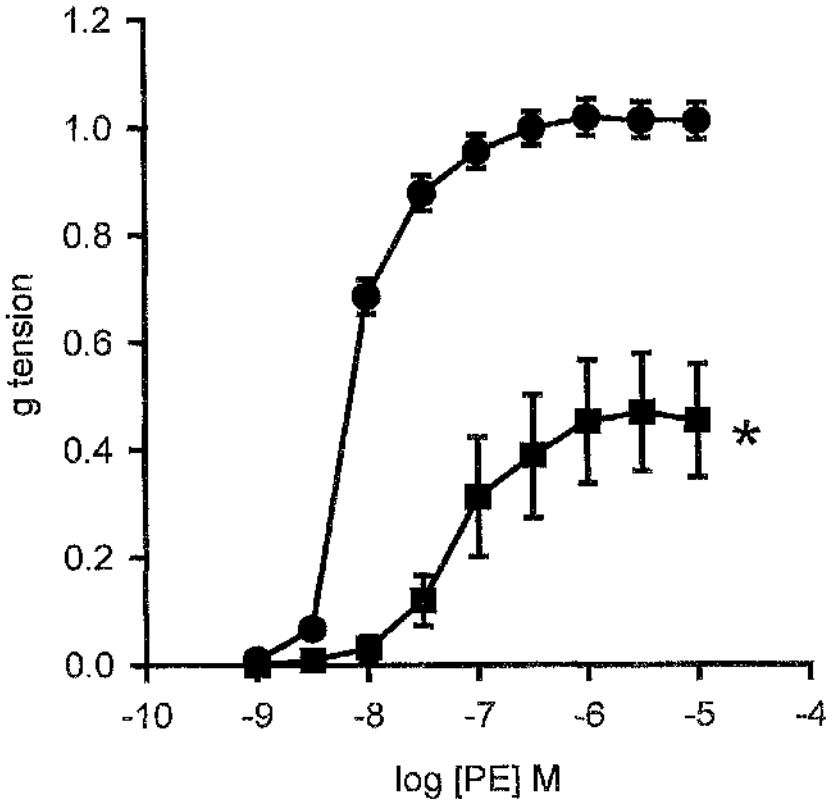


Figure 6.3: Concentration-response curves showing the contractile effects of phenylephrine (PE; 1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●) and rings incubated at 37°C for 18 h in Krebs solution (■). Each point is the mean of 6 - 10 observations with vertical bars representing the s.e. mean. * $P<0.005$, indicates a significant difference from freshly isolated rings.

of L-NAME (100 μM), an inhibitor of NOS (McCall *et al.,* 1993; Rees *et al.,* 1990b), to the tissue baths 10 min prior to the addition of PE (fig 6.2 and 6.4). However, following an 18 h incubation of endothelium-denuded rings of rat aorta, the addition of L-NAME (100 - 1000 μM) to the tissue baths 10 min prior to PE resulted in a concentration-dependent reversal of the depression of PE (1 nM - 30 μM)-induced contraction (fig 6.2 and 6.4). The depression of PE-induced contraction therefore appeared to be due to production of NO from L-arginine.

Reversal of depression of PE-induced contraction by methylene blue

Methylene blue (10 μM), an inhibitor of soluble guanylate cyclase (Martin *et al.,* 1985), when added to the tissue baths had no effect on the concentration-dependent contraction to PE (1 nM - 10 μM) in freshly isolated endothelium-denuded rings of rat aorta (fig 6.5). However, methylene blue (10 μM) produced a substantial reversal of the depression of PE (1 nM - 10 μM)-induced contraction seen in endothelium-denuded rings of rat aorta following incubation at 37°C for 18 h (fig 6.5). The depression of PE-induced contraction therefore appeared to be due to activation of soluble guanylate cyclase.

Reversal of depression of PE-induced contraction by haemoglobin

Haemoglobin (30 μM), which binds and inactivates NO (Gibson & Roughton, 1957; Martin *et al.,* 1985), when added to the tissue baths had no effect on the concentration-dependent contraction to PE (1 nM - 10 μM) in freshly isolated endothelium-denuded rings of rat aorta (fig 6.6a). However, haemoglobin (10 and 30 μM) produced a concentration-dependent reversal of the depression of PE (1 nM - 10 μM)-induced contraction seen in endothelium-denuded rings of rat aorta following incubation at 37°C for 18 h (fig 6.6b). The depression of PE-induced contraction therefore appeared to be due to the actions of NO.

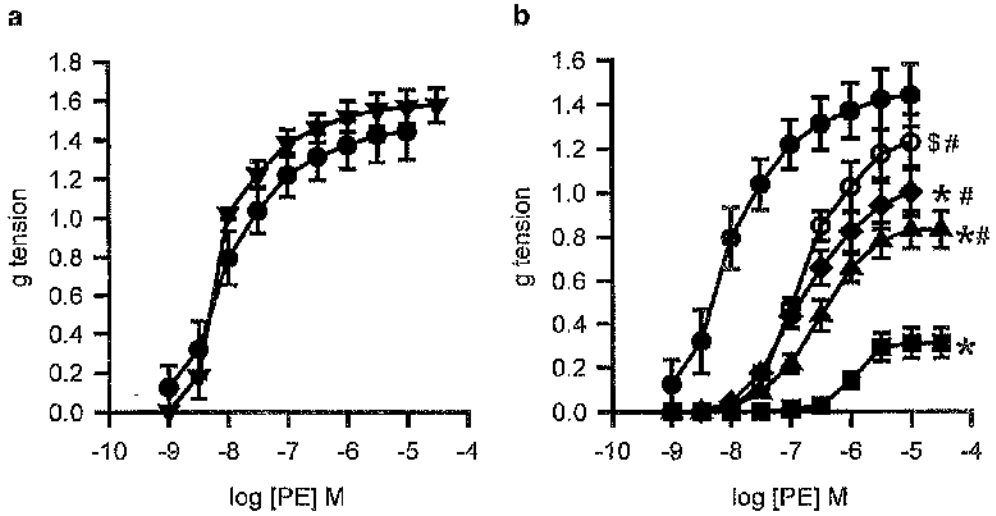


Figure 6.4: Concentration-response curves showing the contractile effects of phenylephrine (PE; 1 nM - 30 μM) on endothelium-denuded rings of rat aorta: control freshly isolated rings (●); **(a)** freshly isolated rings treated by the addition of L-NAME (100 μM) to the tissue baths (▼) and **(b)** rings incubated at 37°C for 18 h in Krebs solution (■) and rings incubated at 37°C for 18 h followed by the addition of L-NAME (100 μM, ▲; 300 μM, ◆ and 1000 μM, ○) to the tissue baths. Each point is the mean of 4 - 16 observations with vertical bars representing the s.e. mean. * and # $P<0.005$, indicate a significant difference from freshly isolated rings and from rings incubated at 37°C for 18 h, respectively. $ $P<0.005$, indicates a significant difference from freshly isolated rings contracted with PE (1 nM - 1 μM).

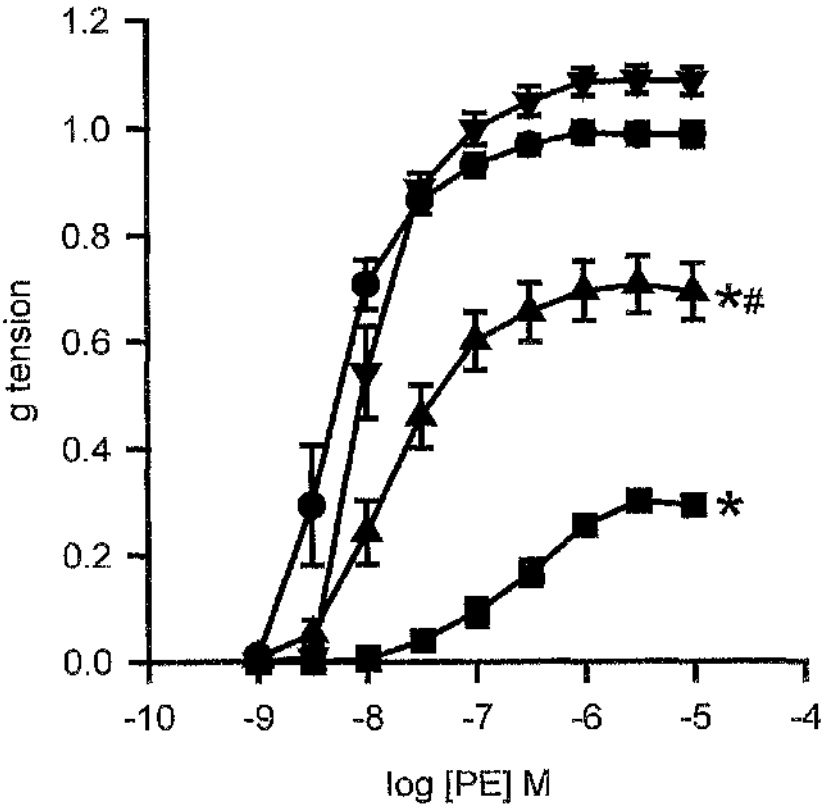


Figure 6.5: Concentration-response curves showing the contractile effects of phenylephrine (PE; 1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); freshly isolated rings following the addition of methylene blue (10 μM) to the tissue baths (▼); rings incubated at 37°C for 18 h in Krebs solution (■) and rings incubated for 18 h followed by the addition of methylene blue (10 μM) to the tissue baths (▲). Each point is the mean of 8 - 12 observations with vertical bars representing the s.e. mean. * and # $P<0.005$, indicate a significant difference from freshly isolated rings and from rings incubated at 37°C for 18 h, respectively.

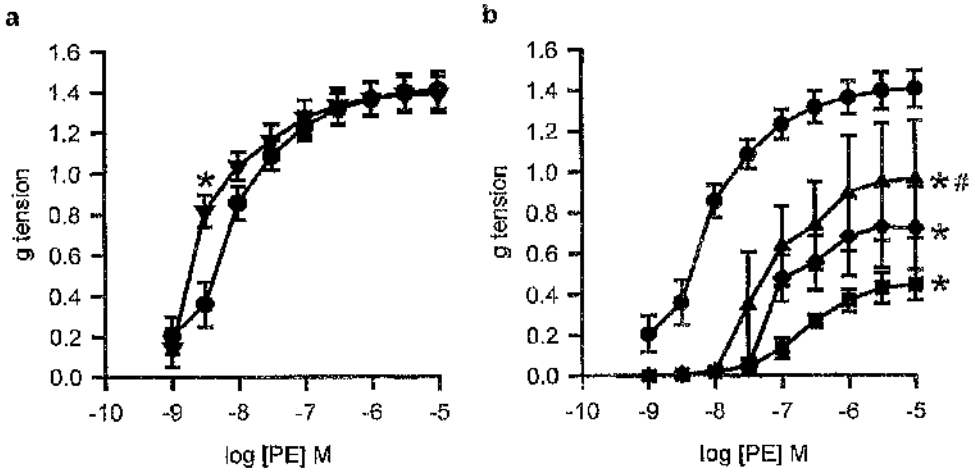


Figure 6.6: Concentration-response curves showing the contractile effects of phenylephrine (PE; 1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); **(a)** freshly isolated rings following the addition of haemoglobin (30 μM) to the tissue baths (▼) and **(b)** rings incubated at 37°C for 18 h in Krebs solution (■) and rings incubated for 18 h followed by the addition of haemoglobin (10 μM, ◆ and 30 μM, ▲) to the tissue baths. Each point is the mean of 3 - 15 observations with vertical bars representing the s.e. mean. * $P<0.005$, indicates a significant difference from freshly isolated rings and # $P<0.05$, indicates a significant difference from rings incubated at 37°C for 18 h.

Inability of dexamethasone to reverse the depression of PE-induced contraction

When dexamethasone (1 μM), which is reported to inhibit the expression of iNOS (Di Rosa *et al.*, 1990; Knowles *et al.*, 1990b; Rees *et al.*, 1990a), was present during the 18 h incubation, the depression of PE (1 nM - 10 μM)-induced contraction was unaffected (fig 6.7). Furthermore, the reversal of the depression of PE (1 nM - 10 μM)-induced contraction produced by the addition of L-NAME (1 mM) to the tissue baths was not enhanced if dexamethasone (1 μM) had been present during the 18 h incubation (fig 6.7).

Reversal of depression of PE-induced contraction by polymixin B

Polymixin B, which binds and inactivates LPS (Lasfargues *et al.*, 1989), was used to determine if the depression of PE (1 nM - 10 μM)-induced contraction in aortic rings after overnight incubation resulted from contamination of the Krebs solution with bacterial endotoxin (LPS). When polymixin B (30 μg ml^{-1}) was present during the 18 h incubation it produced a substantial reversal of the depression of PE-induced contraction (fig 6.8a). The magnitude of this reversal was similar to that obtained when the NOS inhibitor, L-NAME (1 mM), was added to rings following overnight incubation. However, no further reversal of depression was seen when L-NAME (1 mM) was added to rings incubated in the presence of polymixin B (30 μg ml^{-1}; fig 6.8b). It was likely therefore that contamination by bacterial endotoxin could account for the major part of the depression of PE-induced contraction following overnight incubation.

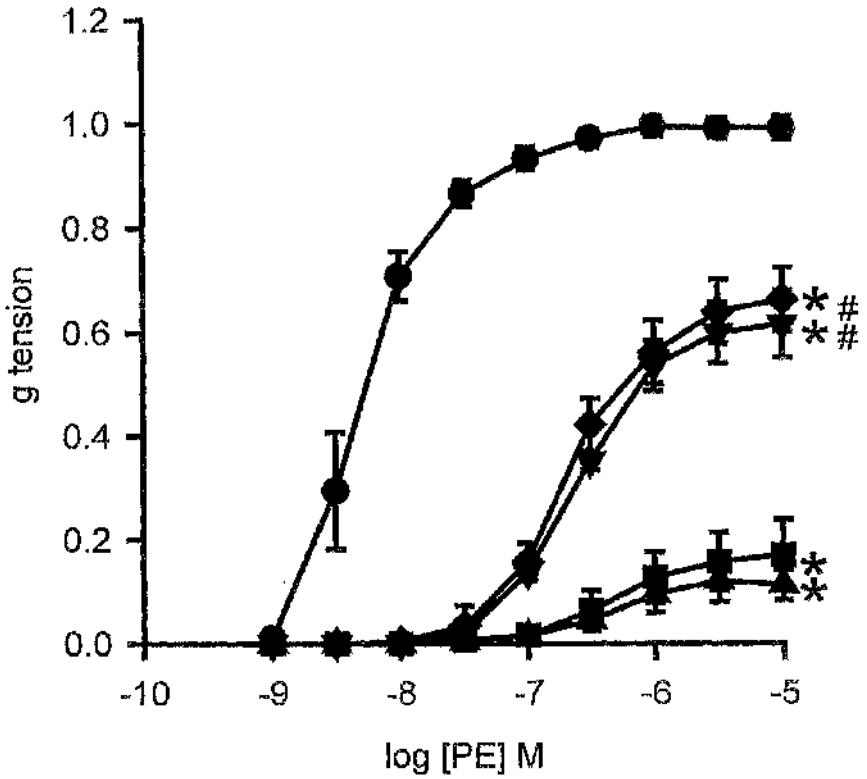


Figure 6.7: Concentration-response curves showing the contractile effects of phenylephrine (PE; 1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); rings incubated at 37°C for 18 h in Krebs solution (■); rings incubated overnight in Krebs solution containing dexamethasone (1 μM; ▲); rings incubated overnight followed by the addition of L-NAME (1 mM) to the tissue baths (▼) and rings incubated overnight with dexamethasone followed by the addition of L-NAME (1 mM) to the tissue baths (♦). Each point is the mean of 3 - 8 observations with vertical bars representing the s.e. mean. * and # $P<0.005$, indicate a significant difference from freshly isolated rings and from rings incubated at 37°C for 18 h, respectively.

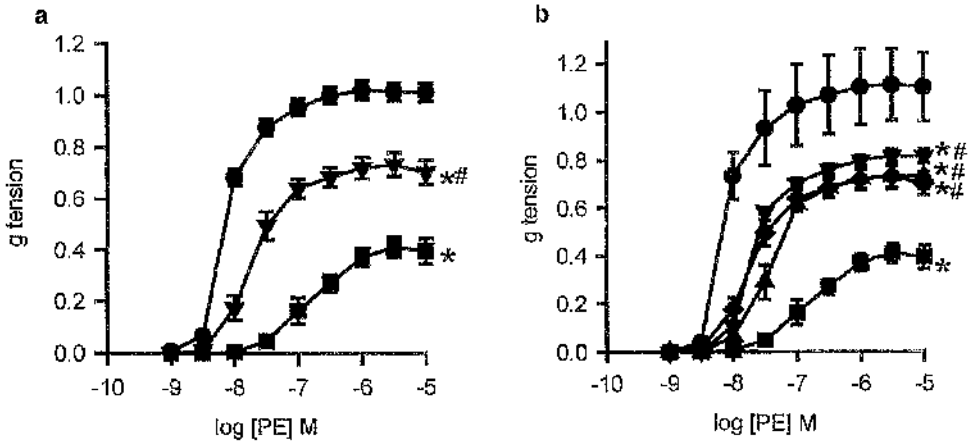


Figure 6.8: Concentration-response curves showing the contractile effects of phenylephrine (PE; 1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); rings incubated at 37°C for 18 h in Krebs solution (■) and rings incubated in Krebs solution containing polymixin B (30 μg ml^{-1}; ▼) and **(b)** rings incubated in Krebs solution followed by the addition of L-NAME (1 mM) to the tissue bath (◆) and rings incubated in Krebs solution containing polymixin B (30 μg ml^{-1}) followed by the addition of L-NAME (1 mM, ▲) to the tissue bath. Each point is the mean of 6 - 10 observations with vertical bars representing the s.e. mean. * and # $P<0.005$, indicate a significant difference from freshly isolated rings and from rings incubated at 37°C for 18 h, respectively.

Effects of LPS on the depression of PE-induced contraction

Having established that contamination by LPS appeared to be responsible for the depression of PE-induced contraction in endothelium-denuded rings of rat aorta following overnight (18 h) incubation, an attempt was made to determine if this depression could be potentiated by adding further exogenous LPS to the Krebs solution.

The depression of PE-induced contraction was not enhanced if LPS from *S. typhosa* (100 ng ml^{-1}) was present throughout the 18 h incubation (fig 6.9). This depression was partially prevented if polymixin B (30 µg ml^{-1}) was present together with LPS (100 ng ml^{-1}) during the 18 h incubation (fig 6.9).

Interactions of superoxide anion with NO produced by iNOS in endothelium-denuded rings of rat aorta

The free radical superoxide anion destroys the vascular activity of NO (Gryglewski *et al.,* 1986). We made use of superoxide anion in an attempt to determine the locus within the arterial wall where NO was being produced following overnight (18 h) incubation of endothelium-denuded rings of rat aorta.

One possibility was that expression of iNOS had taken place in non-smooth muscle cells in the arterial wall and that the NO released from this cell type diffused to the smooth muscle cells producing relaxation. If this hypothesis was correct then superoxide anion generated extracellularly would interact with NO and inactivate it. Furthermore, superoxide dismutase either endogenously present or added exogenously to the extracellular space would be expected to destroy superoxide anion thus protecting NO.

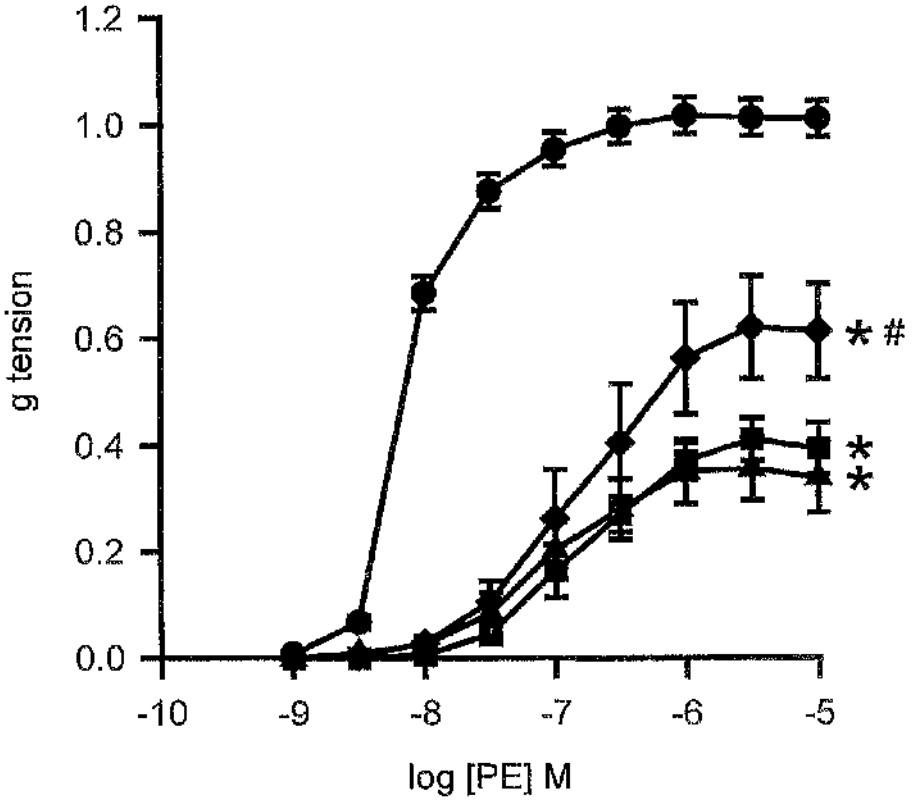


Figure 6.9: Concentration-response curves showing the contractile effects of phenylephrine (PE; 1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); rings incubated at 37°C for 18 h in Krebs solution (■); rings incubated for 18 h in Krebs solution containing endotoxin (LPS; 100 ng ml^{-1}; ▲) and rings incubated for 18 h in Krebs solution containing LPS (100 ng ml^{-1}) and polymixin B (30 μg ml^{-1}; ♦). Each point is the mean of 6 - 10 observations with vertical bars representing the s.e. mean. * and # $P<0.005$, indicate a significant difference from freshly isolated rings and from rings incubated at 37°C for 18 h, respectively.

An alternative possibility was that expression of iNOS had taken place in the smooth muscle cells themselves. In this particular case, superoxide anion generated only intracellularly would have the opportunity of destroying NO. Furthermore, only endogenous, intracellularly located superoxide dismutase would have the opportunity of protecting NO and any enzyme in the extracellular space would have no effect.

To test which of these two possibilities was correct, we used two generators of superoxide anion; hypoxanthine/xanthine oxidase, which generates the free radical only extracellularly (McCord & Fridovich, 1968), and LY 83583, which generates the free radical intracellularly (Mülsch *et al.*, 1988; Mülsch *et al.*, 1989). Bovine erythrocyte superoxide dismutase was also used as a tool to destroy superoxide anion in the extracellular space.

Effects of hypoxanthine/xanthine oxidase on PE-induced contraction

In all experiments involving the use of hypoxanthine/xanthine oxidase, catalase (1000 u ml^{-1}) was present in order to remove any hydrogen peroxide generated from superoxide anion. The concentration-dependent contraction to PE (1 nM - 10 μM) in freshly isolated endothelium-denuded rings of rat aorta was unaltered by the addition of hypoxanthine (100 μM)/xanthine oxidase (16 mu ml^{-1}) to the tissue baths (fig 6.10a). Following incubation of endothelium-denuded rings of rat aorta for 18 h at 37°C, the addition of hypoxanthine (100 μM or 1000 μM)/xanthine oxidase (16 mu ml^{-1}) to the tissue baths had no effect on the depression of PE (1 nM - 10 μM)-induced contraction (fig 6.10a and b). Thus, generation of superoxide anion extracellularly failed to destroy the depressant action of NO on PE-induced contraction.

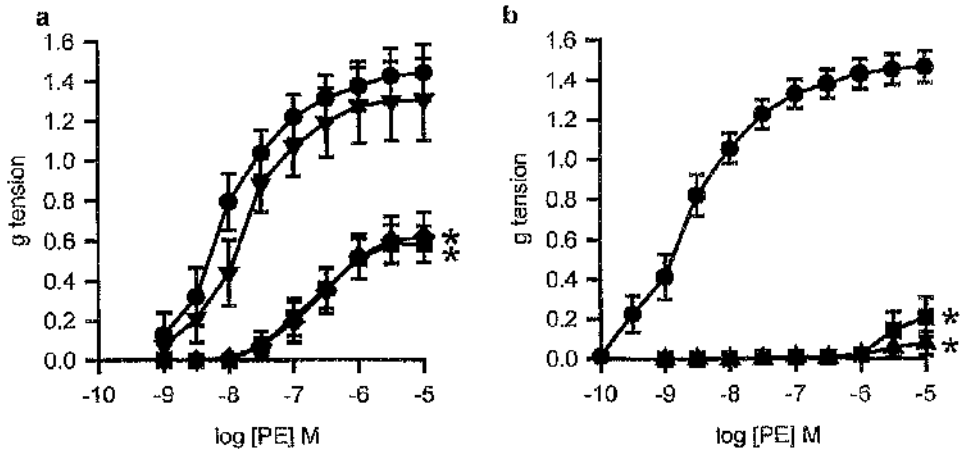


Figure 6.10: Concentration-response curves showing the contractile effects of phenylephrine (PE; 1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●) and rings incubated at 37°C for 18 h in Krebs solution (■); **(a)** freshly isolated rings with the addition of hypoxanthine (100 μM)/xanthine oxidase (16 mu ml^{-1}) to the tissue baths (▼) and rings incubated for 18 h in Krebs solution followed by the addition of hypoxanthine (100 μM)/xanthine oxidase (16 mu ml^{-1}) to the tissue baths (♦) and **(b)** rings incubated for 18 h in Krebs solution followed by the addition of hypoxanthine (1000 μM)/xanthine oxidase (16 mu ml^{-1}) to the tissue baths (▲). Catalase (1000 u ml^{-1}) was present in all experiments to remove any hydrogen peroxide generated. Each point is the mean of 5 - 17 observations with vertical bars representing the s.e. mean. * $P<0.005$, indicates a significant difference from freshly isolated rings.

Reversal of depression of PE-induced contraction by LY 83583

The concentration-dependent contraction to PE (0.1 nM - 10 μM) in freshly isolated endothelium-denuded rings of rat aorta was unaltered by the addition of LY 83583 (1 μM) to the tissue baths (fig 6.11a). In contrast, the addition of LY 83583 (0.1 - 1 μM) to the tissue baths following incubation of rings for 18 h at 37°C produced a concentration-dependent reversal of the depression of PE (0.1 nM - 10 μM)-induced contraction (fig 6.11b). Thus, taken together with the findings using hypoxanthine/xanthine oxidase, generation of superoxide anion intracellularly was necessary to destroy the depressant action of NO on PE-induced contraction. It was therefore likely, that NO was being produced and acting within the same smooth muscle cells.

Effects of superoxide dismutase on the reversal of depression of PE-induced contraction by LY 83583

If LY 83583 does indeed destroy NO through the intracellular generation of superoxide anion then addition of exogenous superoxide dismutase, which would be restricted to the extracellular space, should have no effect on this action.

Overnight (18 h) incubation of endothelium-denuded rings of rat aorta resulted in the depression of PE (0.1 nM - 30 μM)-induced contraction and this was unaltered if superoxide dismutase (250 u ml^{-1}) was added to the tissue bath prior to PE contraction (fig 6.12).

As before LY 83583 (1 μM), produced a significant reversal of the depression of PE (0.1 nM - 10 μM)-induced contraction when added to the tissue baths

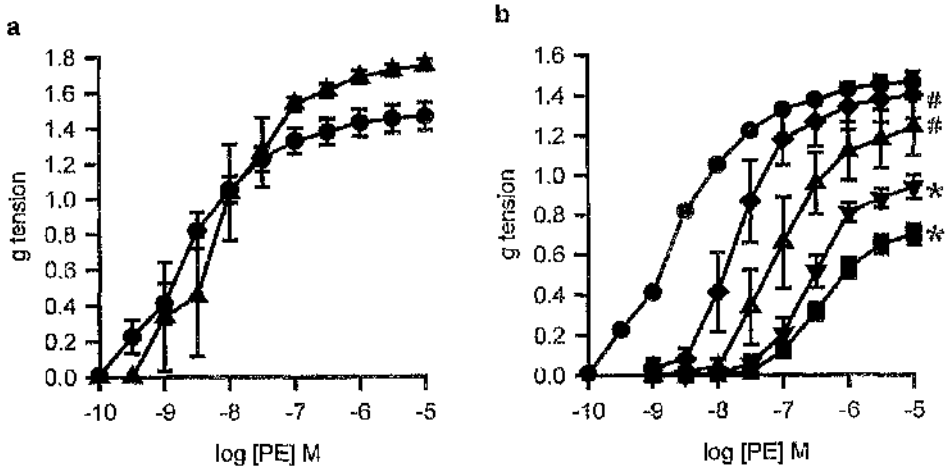


Figure 6.11: Concentration-response curves showing the contractile effects of phenylephrine (PE; 0.1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); **(a)** freshly isolated rings treated with LY 83583 (1 μM; ▲) and **(b)** rings incubated at 37°C for 18 h in Krebs solution (■); rings incubated for 18 h in Krebs solution followed by the addition of LY 83583 (0.1 μM, ▼; 0.3 μM, Δ and 1 μM, ♦) to the tissue baths. Each point is the mean of 3 - 16 observations with vertical bars representing the s.e. mean. * and # $P<0.05$, indicate a significant difference from freshly isolated rings and from rings incubated at 37°C for 18 h, respectively.

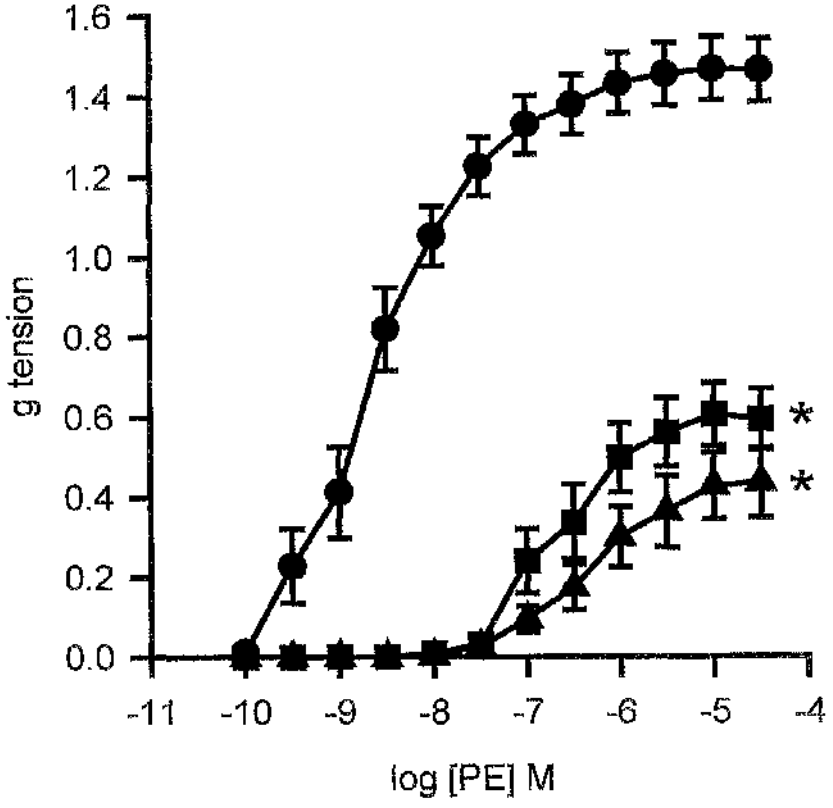


Figure 6.12: Concentration-response curves showing the contractile effects of phenylephrine (PE; 0.1 nM - 30 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); rings incubated at 37°C for 18 h in Krebs solution (■) and rings incubated overnight in Krebs solution followed by the addition of superoxide dismutase (250 u ml^{-1}) to the tissue baths (▲). Each point is the mean of 7 - 16 observations with vertical bars representing the s.e. mean. * $P<0.005$, indicates a significant difference from freshly isolated rings.

(fig 6.13). This reversal of depression was completely unaffected by the addition of superoxide dismutase (250 u ml^{-1}). Again suggesting that NO was being produced and acting within the same smooth muscle cells.

Effects of catalase on the reversal of PE-induced contraction by LY 83583

Although LY 83583 (1 μM) produced a concentration dependent reversal of the depression of PE (1 nM - 10 μM)-induced contraction seen in rings of rat aorta following an overnight (18 h) incubation, we found that surprisingly a concentration of 3 μM produced a lesser degree of reversal than 1 μM (fig 6.14a). We investigated if this smaller degree of reversal was a result of the accumulation of the relaxant, hydrogen peroxide, by examining the effects of catalase. As can be seen in fig 6.14b. However, the addition of catalase (1000 u ml^{-1}) to the tissue baths had no effect on the ability of LY 83583 (3 μM) to reverse the depression of PE (1 nM - 10 μM)-induced contraction.

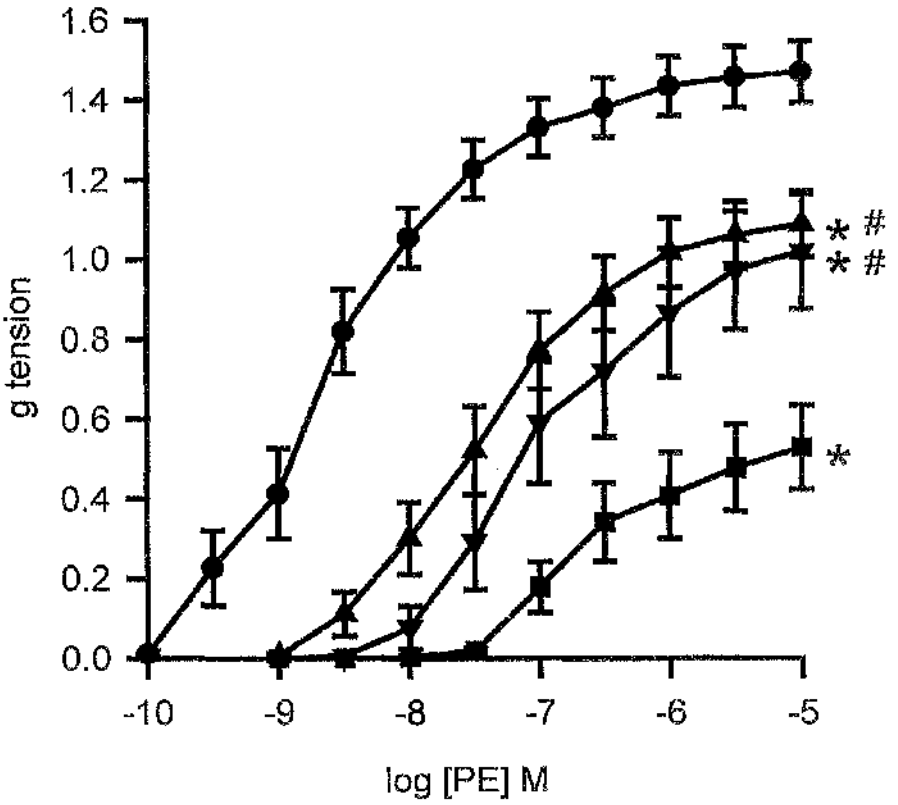


Figure 6.13: Concentration-response curves showing the contractile effects of phenylephrine (PE; 0.1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); rings incubated at 37°C for 18 h in Krebs solution (■); rings incubated overnight in Krebs solution followed by the addition of LY 83583 (1 μM) to the tissue baths (▲) and rings incubated overnight in Krebs solution followed by the addition of superoxide dismutase (250 u ml^{-1}) 10 min prior to the addition of LY 83583 (1 μM) to the tissue baths (▼). Each point is the mean of 4 - 16 observations with vertical bars representing the s.e. mean. * and # $P<0.005$, indicate a significant difference from freshly isolated rings and from rings incubated at 37°C for 18 h, respectively.

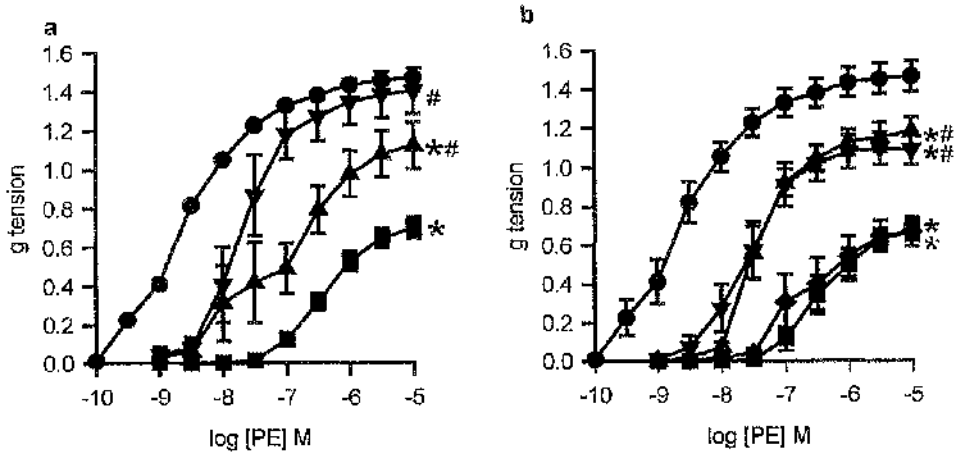


Figure 6.14: Concentration-response curves showing the contractile effects of phenylephrine (PE; 0.1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); rings incubated at 37°C for 18 h in Krebs solution (■); rings incubated for 18 h in Krebs solution followed by the addition of LY 83583 (3 μM) to the tissue baths (▲); **(a)** rings incubated for 18 h in Krebs solution followed by the addition of LY 83583 (1 μM) to the tissue baths (▼) and **(b)** rings incubated overnight followed by the addition of catalase (1000 u ml^{-1}; ♦) or catalase (1000 u ml^{-1}) and LY 83583 (1 μM; ▲) to the tissue baths. Each point is the mean of 3 - 16 observations with vertical bars representing the s.e. mean. * and # $P<0.05$, indicate a significant difference from freshly isolated rings and from rings incubated at 37°C for 18 h, respectively.

Chapter 7

Assessment of inducible nitric oxide synthase expression by measuring nitrite accumulation into the medium bathing rat aortic smooth muscle cells

In the previous chapter it was suggested that inducible nitric oxide synthase (iNOS) could be expressed in rat aortic rings. In this section a more detailed investigation of the factors regulating expression of iNOS was made using rat aortic smooth muscle cells (RASMC) in culture. In order to assess the expression of iNOS, we measured the accumulation of nitrite, the major stable breakdown product of NO (Wennmalm *et al.*, 1992; Ignarro *et al.*, 1993), into the medium bathing the cells. All measurements were made using the Griess reaction and are expressed as nmol per 10^6 cells.

Induction of nitrite accumulation into full culture medium bathing RASMC

All experiments in this section were conducted on RASMC grown in full culture medium, i.e. Dulbecco's Modified Eagle's Medium (DMEM) containing foetal calf serum (FCS; 10%), new born serum (NBS; 10%), glutamine (4 mM) and penicillin (100 u ml^{-1})/streptomycin (100 µg ml^{-1}). Basal accumulation of nitrite into the full culture medium bathing RASMC was remarkably similar between experiments (4 - 6.5 nmol, Fig 7.1 - 7.6). Nevertheless, all experiments contained internal controls of untreated cells.

Effects of LPS on nitrite accumulation into full culture medium bathing RASMC

Incubation for 24 h with endotoxin (lipopolysaccharide, LPS) from *Salmonella typhosa* at 1000 ng ml^{-1} had no effect on nitrite accumulation into the full culture medium bathing RASMC (fig 7.1).

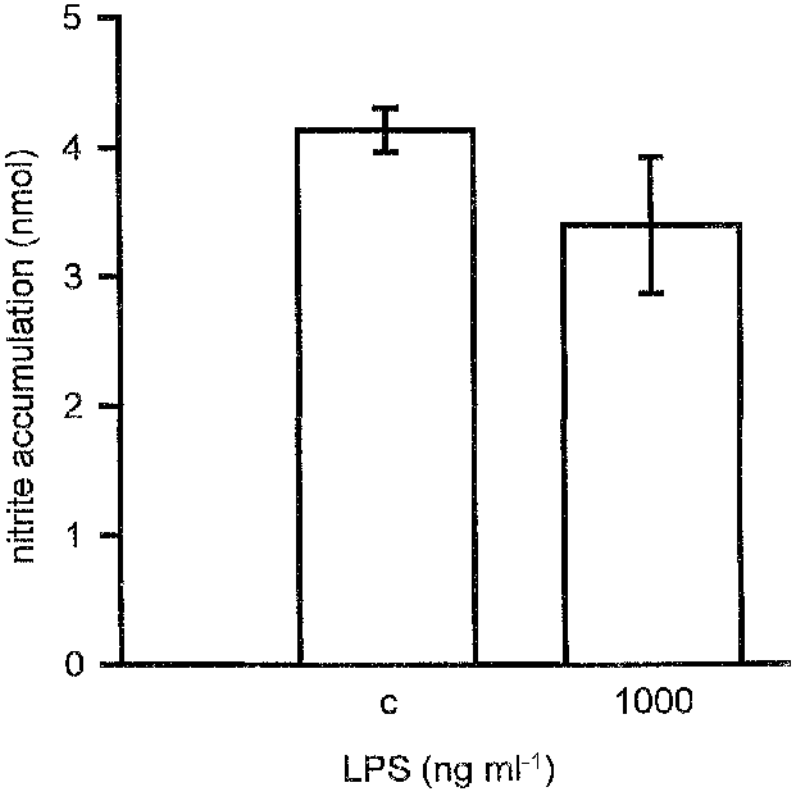


Figure 7.1: Nitrite accumulation (nmol per 10^6 cells) into the full culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with lipopolysaccharide from *Salmonella typhosa* (LPS; 1000 ng ml^{-1}). Each value is the mean of 6 observations with vertical bars representing the s.e. mean.

Effects of IFN-γ on nitrite accumulation into the full culture medium bathing RASMC

Incubation for 24 h with the cytokine, murine interferon-γ (IFN-γ), in the concentration range 1 - 100 u ml^{-1}, produced a fall in nitrite accumulation into the full culture medium bathing RASMC (fig 7.2).

Effects of IL-1α on nitrite accumulation into the full culture medium bathing RASMC

Incubation for 24 h with a second cytokine, human interleukin-1α (IL-1α) at 10 and 40 u ml^{-1} produced a significant decrease in nitrite accumulation into the full culture medium bathing RASMC (fig 7.3).

Combined effects of IFN-γ and LPS on nitrite accumulation into the full culture medium bathing RASMC

In order to investigate potential interactions between IFN-γ and LPS on nitrite production by RASMC in culture, additional experiments were conducted in which cells were exposed to both stimuli.

As previously stated, incubation for 24 h with LPS (1000 ng ml^{-1}) alone had no effect on nitrite accumulation into the full culture medium bathing RASMC and IFN-γ (10 u ml^{-1}) alone produced a slight decrease (fig 7.4). When RASMC were treated with both of these stimuli for 24 h no effect was seen on nitrite accumulation (fig 7.4).

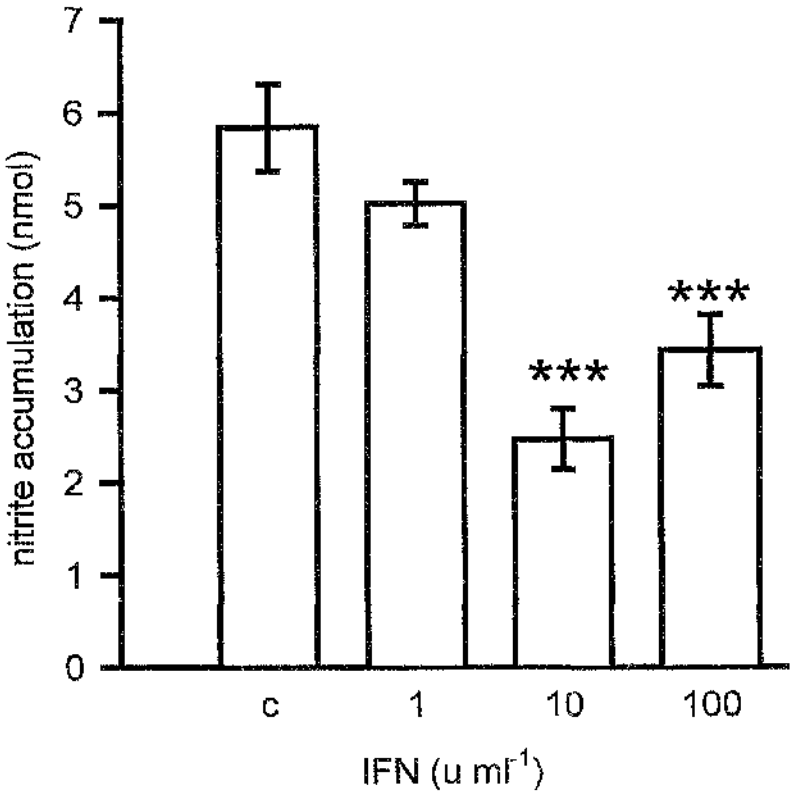


Figure 7.2: Nitrite accumulation (nmol per 10^6 cells) into the full culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with interferon-γ (IFN; 1 - 100 u ml^{-1}). Each value is the mean of 6 - 12 observations with vertical bars representing the s.e. mean. *** $P<0.005$, indicates a significant difference from control (c).

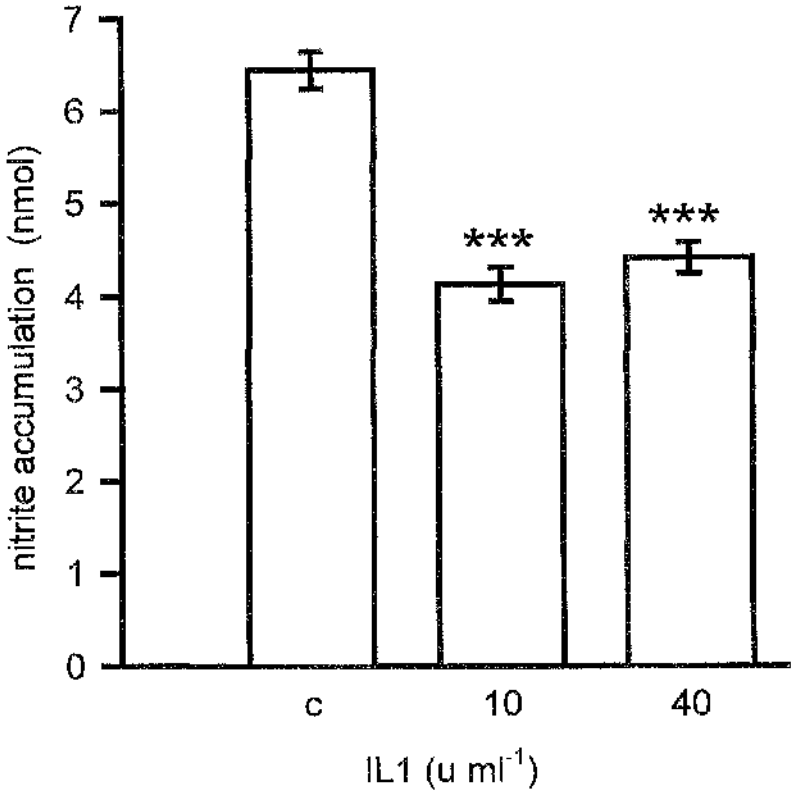


Figure 7.3: Nitrite accumulation (nmol per 10^6 cells) into the full culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with interleukin-1α (IL1; 10 and 40 u ml^{-1}). Each value is the mean of 6 observations with vertical bars representing the s.e. mean. *** $P<0.005$, indicates a significant difference from control (c).

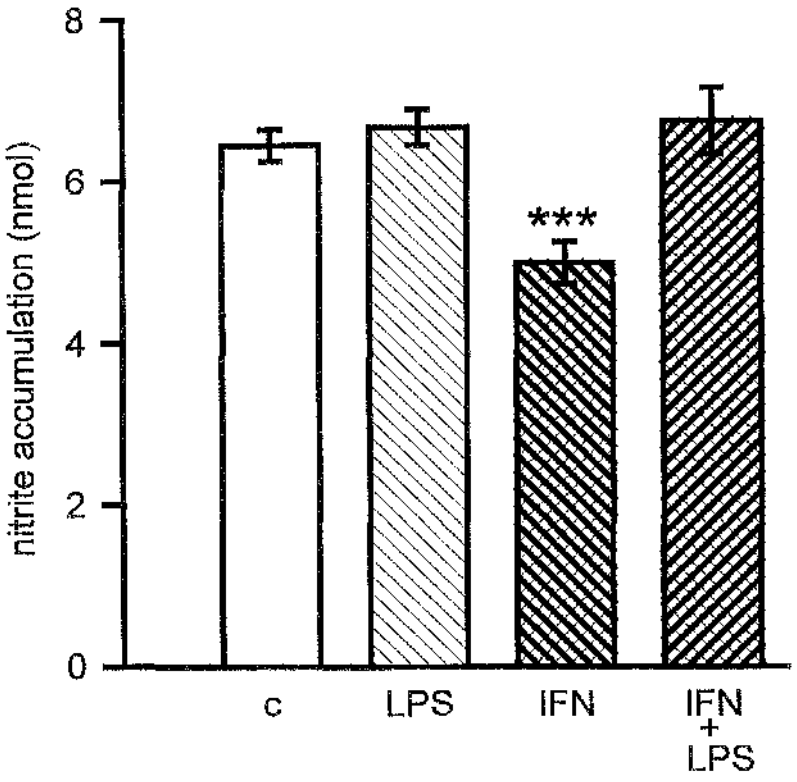


Figure 7.4: Nitrite accumulation (nmol per 10^6 cells) into the full culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with lipopolysaccharide (LPS; 1000 ng ml^{-1}) and interferon-γ (IFN; 10 u ml^{-1}) alone and in combination. Each value is the mean of 6 observations with vertical bars representing the s.e. mean. *** $P<0.005$, indicates a significant difference from control (c).

Combined effects of IFN-γ and IL-1α on nitrite accumulation into the full culture medium bathing RASMC

In order to investigate potential interactions between IFN-γ and IL-1α on nitrite production by RASMC in culture, additional experiments were conducted in which cells were exposed to both stimuli.

As previously stated, incubation for 24 h with IFN-γ (10 u ml^{-1}) alone or with IL-1α (10 u ml^{-1}) alone resulted in decreases in nitrite accumulation into the full culture medium bathing RASMC. When cells were treated with both of these stimuli, the level of nitrite accumulation was similar to that of untreated cells (fig 7.5a). However, an increase in nitrite accumulation was produced by the combination of IFN-γ (10 u ml^{-1}) and IL-1α (40 u ml^{-1}), although both stimuli on their own decreased nitrite accumulation (fig 7.5b).

Combined effects of LPS and IL-1α on nitrite accumulation into the full culture medium bathing RASMC

In order to investigate potential interactions between LPS and IL-1α on nitrite production by RASMC in culture, additional experiments were conducted in which cells were exposed to both stimuli.

As previously stated, incubation for 24 h with IL-1α (10 u ml^{-1}) alone decreased nitrite accumulation into the full culture medium bathing RASMC and LPS (100 ng ml^{-1}) alone had no effect. When RASMC were treated with both of these stimuli, the level of nitrite accumulation was similar to that of untreated cells (fig 7.6).

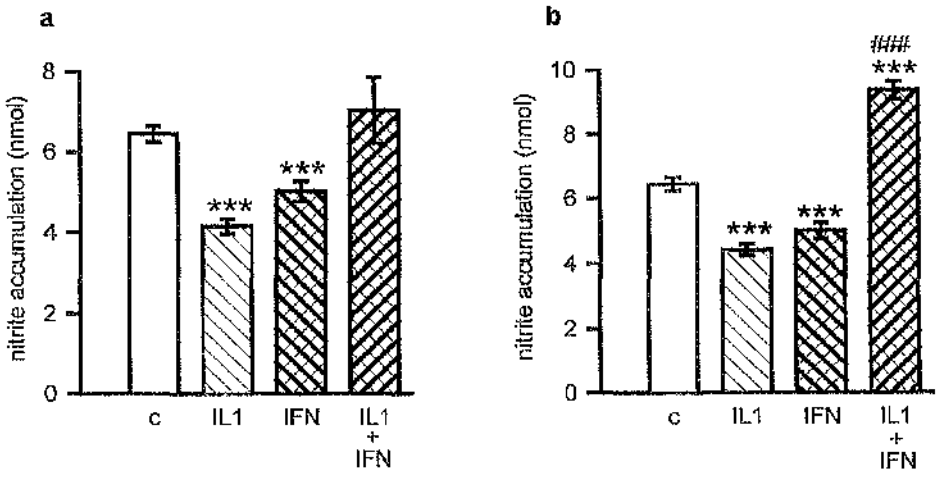


Figure 7.5: Nitrite accumulation (nmol per 10^6 cells) into the full culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with **(a)** interleukin-1α (IL1; 10 u ml^{-1}) and interferon-γ (IFN; 10 u ml^{-1}) alone and in combination and **(b)** IL1 (40 u ml^{-1}) and IFN (10 u ml^{-1}) alone and in combination. Each value is the mean of 6 observations with vertical bars representing the s.e. mean. *** $P<0.005$ and ### $P<0.005$, indicate a significant difference from control (c) and from either drug alone, respectively.

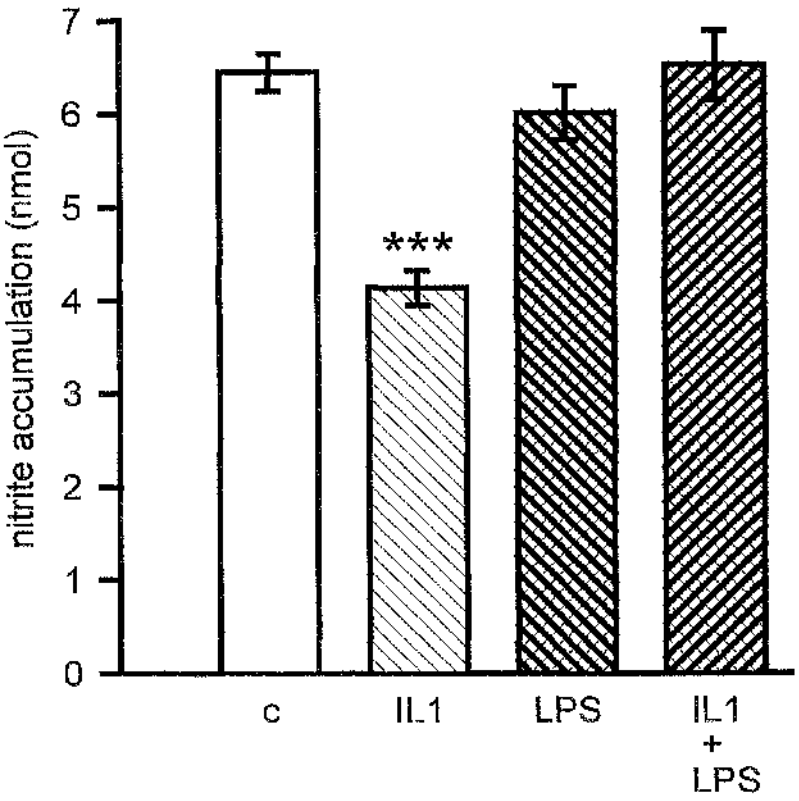


Figure 7.6: Nitrite accumulation (nmol per 10^6 cells) into the full culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with interleukin-1α (IL1; 10 u ml^{-1}) and lipopolysaccharide (LPS; 100 ng ml^{-1}) alone and in combination. Each value is the mean of 6 observations with vertical bars representing the s.e. mean. *** $P<0.005$, indicates a significant difference from control (c).

Effects of serum on nitrite accumulation into the medium bathing RASMC

The above results suggested that agents, i.e. IFN-γ, IL-1α and LPS, known to stimulate expression of iNOS in other cell types failed to do so in RASMC (Stuehr & Marletta, 1987a; Geller *et al.*, 1993b). When a recent report (Schini *et al.*, 1992) was published showing that serum and two of its constituents, platelet-derived growth factor$_{AB}$ and $_{BB}$ and transforming growth factor β1, could inhibit the induction process, experiments were conducted to determine if this could explain our inability to promote nitrite production by RASMC in full culture medium. In order to test this, a series of experiments was conducted on RASMC that had been grown in serum-free culture medium (DMEM containing bovine serum albumin (0.1%), glutamate (4 mM) and penicillin (100 u ml^{-1})/streptomycin (100 μg ml^{-1}) for 24 h prior to experimentation. After that time they were washed and the experiment continued in fresh serum-free culture medium.

The basal accumulation of nitrite into the serum-free culture medium during a 24 h incubation was highly variable amongst different batches of cells (4 - 40 nmol, fig 7.8 - 7.21). There was, however, little variability within a given batch of cells. Consequently, all experiments were conducted with their own internal untreated controls.

In contrast to its effect in full culture medium, IFN-γ (10 u ml^{-1}) produced a significant increase in nitrite accumulation into the serum-free culture medium bathing RASMC following a 48 h incubation (fig 7.7). The presence of FCS (10%) or NBS (10%) had no effect on basal nitrite accumulation into the medium bathing RASMC during the 48 h incubation, but both FCS and NBS significantly inhibited the increase in nitrite accumulation stimulated by

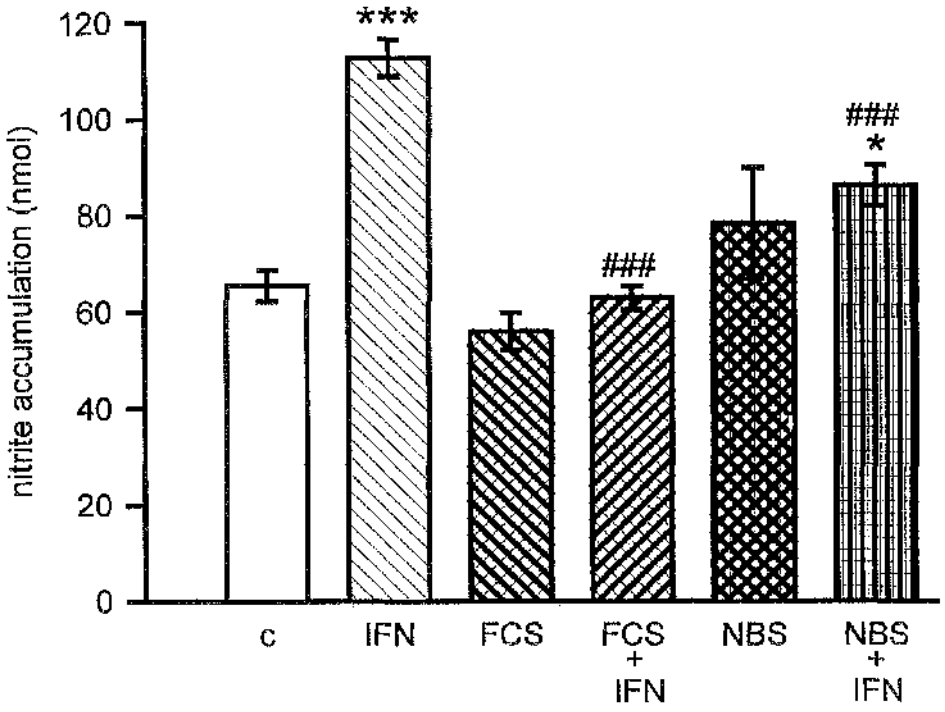


Figure 7.7: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 48 h incubation with interferon-γ (IFN; 10 u ml^{-1}), foetal calf serum (FCS; 10%) alone and in combination with IFN, and new born calf serum (NBS; 10%) alone and in combination with IFN. Each value is the mean of 6 observations with vertical bars representing the s.e. mean. *** $P<0.005$ and * $P<0.05$, indicate a significant difference from control (c) and ### $P<0.005$, indicates a significant difference from IFN alone.

IFN-γ (10 u ml^{-1}). Thus, the presence of serum was likely to have accounted for the inability to stimulate expression of iNOS in RASMC grown in full culture medium.

Induction of nitrite accumulation into serum-free culture medium bathing RASMC

As a result of our observation that the presence of serum in the culture medium inhibits IFN-γ-induced increases in nitrite accumulation into the medium bathing RASMC, all further experiments were carried out in serum-free culture medium.

Effects of LPS on nitrite accumulation into serum-free culture medium bathing RASMC

Following a 24 h incubation with LPS from *Salmonella typhosa*, in the concentration range 1 - 1000 ng ml^{-1}, the level of nitrite accumulation into the serum-free culture medium bathing RASMC was no different from control unstimulated cells (fig 7.8). Higher concentrations of LPS (1 - 100 µg ml^{-1}) also had no effect on nitrite accumulation (fig 7.9). Extremely high concentrations of LPS (300 µg ml^{-1} and 1000 µg ml^{-1}) did significantly increase nitrite accumulation into the serum-free culture medium bathing RASMC but these concentrations clearly produced toxic actions, resulting in detachment of cells from the growth surface. The effects of a second type of LPS, i.e. from *Esherichia coli,* were examined and compared with those of *S. typhosa*. Incubation for 24 h with LPS from *E. coli* in the concentration range 0.1 - 1000 ng ml^{-1} had no consistent effect on nitrite accumulation into the serum-free culture medium bathing RASMC, but statistically the concentrations of 10 ng ml^{-1} and 1000 ng ml^{-1} did produce small but significant increases (fig 7.10).

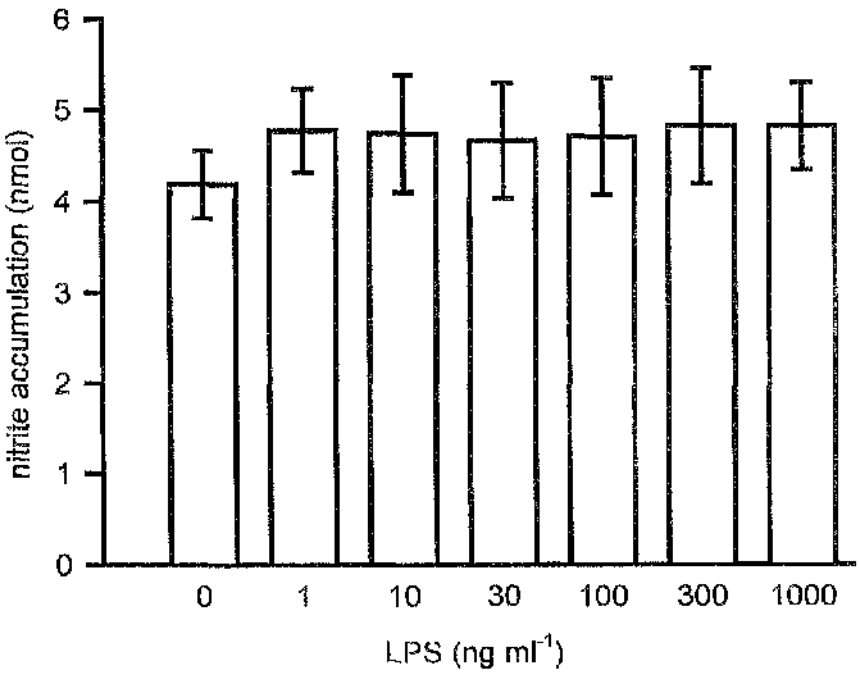


Figure 7.8: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with lipopolysaccharide from *Salmonella typhosa* (LPS; 1 - 1000 ng ml^{-1}). Each value is the mean of 6 observations with vertical bars representing the s.e. mean.

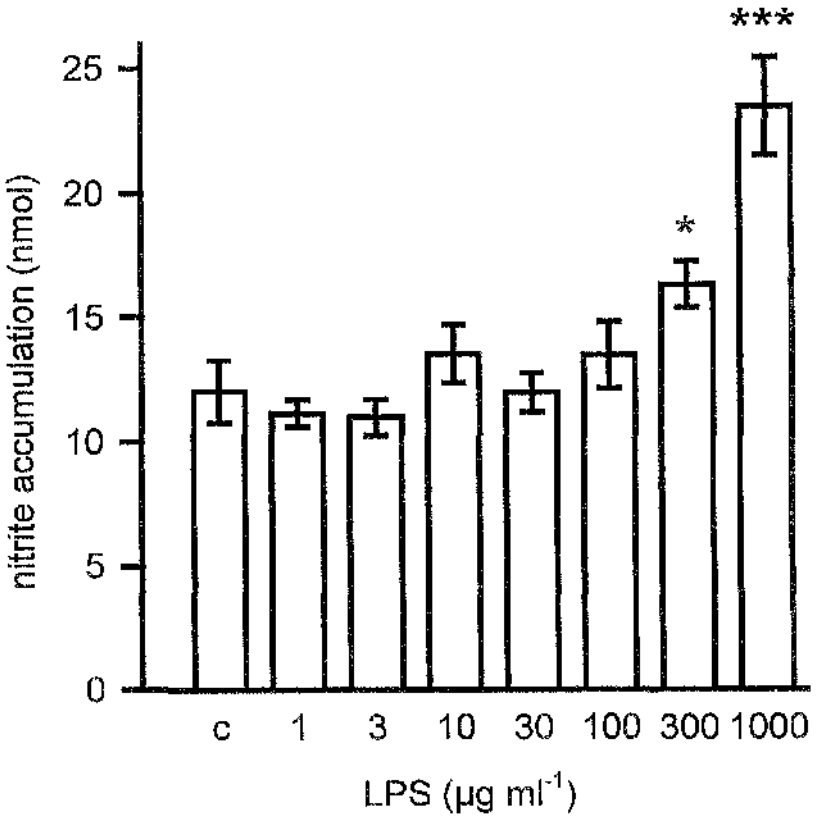


Figure 7.9: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with lipopolysaccharide from *Salmonella typhosa* (LPS; 1 - 1000 µg ml^{-1}). Each value is the mean of 6 observations with vertical bars representing the s.e. mean. * $P<0.05$ and *** $P<0.005$, indicate a significant difference from control (c), but these concentrations of LPS resulted in detachment of cells indicating a toxic action.

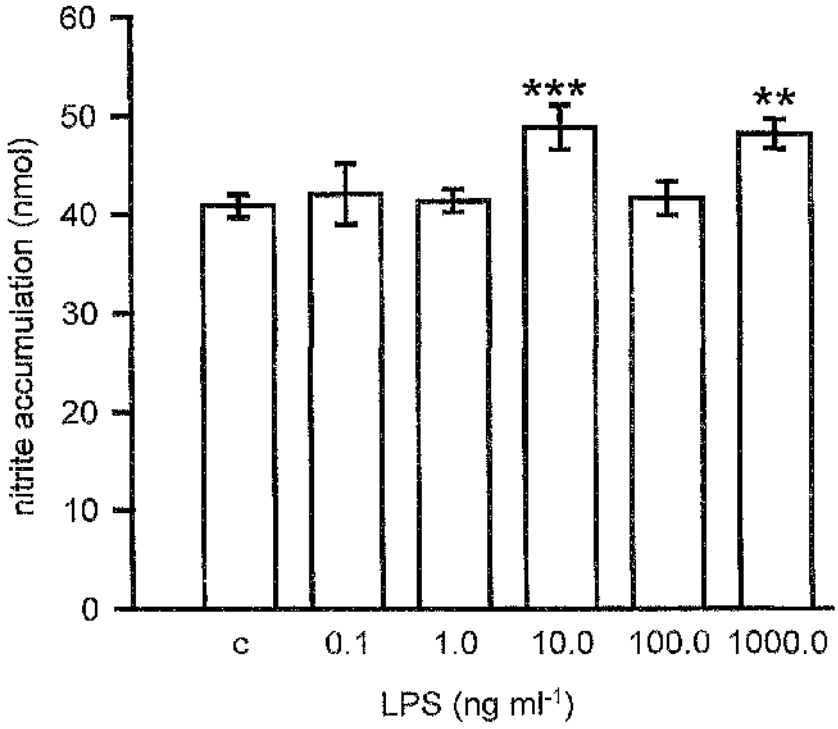


Figure 7.10: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with lipopolysaccharide from *Escherichia coli* (LPS; 0.1 - 1000 ng ml^{-1}). Each value is the mean of 6 observations with vertical bars representing the s.e. mean. ** $P<0.01$ and *** $P<0.005$, indicate a significant difference from control (c).

Effects of IFN-γ on nitrite accumulation into the serum-free culture medium bathing RASMC

Incubation for 24 h with IFN-γ in the concentration range 1 - 100 u ml^{-1} produced a concentration-dependent increase in nitrite accumulation into the serum-free culture medium bathing RASMC (fig 7.11).

Time course of the effects of IFN-γ on nitrite accumulation into the serum-free culture medium bathing RASMC

A separate experiment was conducted to determine the time course of the increase in nitrite production stimulated by IFN-γ. The level of nitrite accumulation into the serum-free culture medium bathing RASMC produced by IFN-γ became significantly greater than for unstimulated cells at 9 h and levels continued rising faster than for control cells until at least 74 h (fig 7.12). For convenience, a time point of 24 h was adopted for all further experiments, since at this time a significant increase in nitrite accumulation was obtained.

Effects of IL-1α on nitrite accumulation into serum-free culture medium bathing RASMC

Incubation for 24 h with a different cytokine, IL-1α, in the concentration range 1 - 10 u ml^{-1}, produced a concentration-dependent increase in nitrite accumulation into the serum-free culture medium bathing RASMC (fig 7.13).

Having established that LPS had no consistent effect on nitrite accumulation into the serum-free culture medium bathing RASMC, and that IFN-γ and IL-1α each stimulated an increase, a new series of experiments were conducted to determine the effects of combinations of these stimuli.

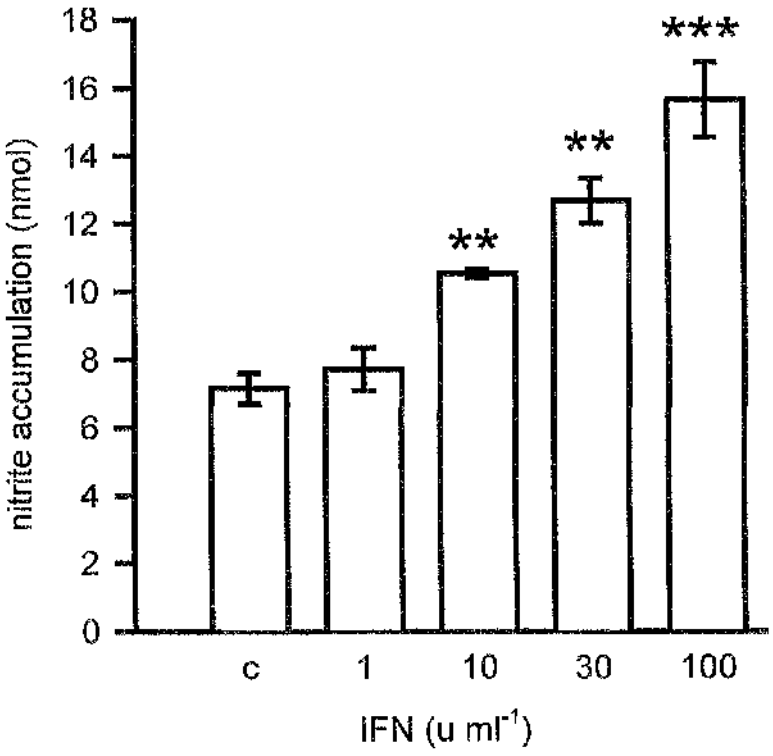


Figure 7.11: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with interferon-γ (IFN; 1 - 100 u ml^{-1}). Each value is the mean of 6 observations with vertical bars representing the s.e. mean. ** $P<0.01$ and *** $P<0.005$, indicate a significant difference from control (c).

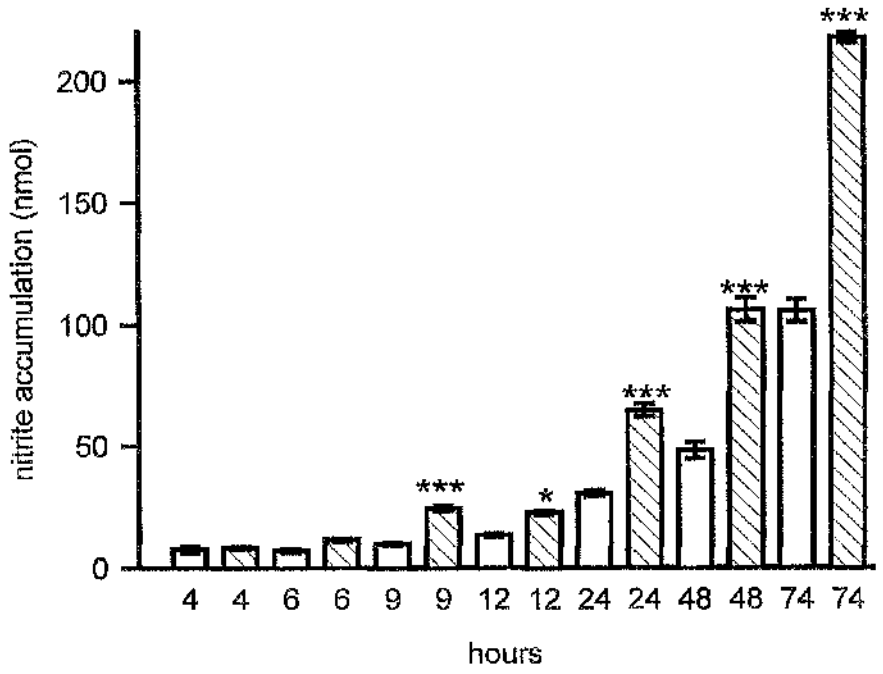


Figure 7.12: Time course of nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells, incubated in the absence (control; ▭) or presence of interferon-γ (IFN; 30 u ml^{-1}; ▨). Each value is the mean of 6 observations with vertical bars representing the s.e. mean. * $P<0.05$ and *** $P<0.005$, indicate a significant difference from control cells at same time point.

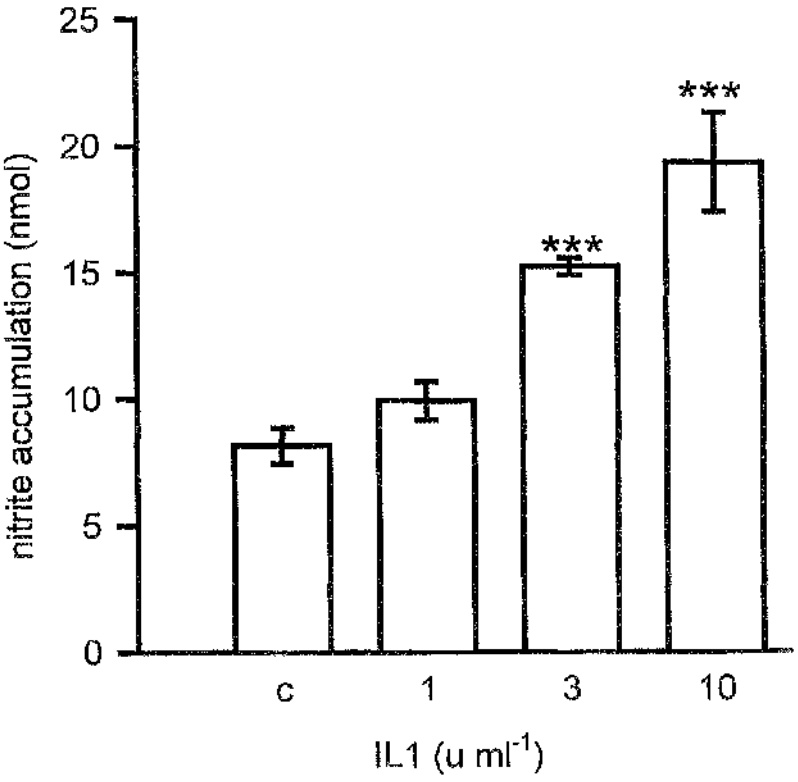


Figure 7.13: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with interleukin-1α (IL1; 1 - 10 u ml^{-1}). Each value is the mean of 6 observations with vertical bars representing the s.e. mean. *** $P<0.005$, indicates a significant difference from control (c).

Combined effects of LPS and IFN-γ on nitrite accumulation into the serum-free culture medium bathing RASMC

Incubation for 24 h with the combination of a low concentration of IFN-γ (10 u ml^{-1}), which itself had no effect on nitrite accumulation, and LPS (100 ng ml^{-1}), which in this case significantly decreased nitrite accumulation, produced a significant increase in nitrite accumulation into the serum-free culture medium bathing RASMC (fig 7.14a). In contrast, if a higher concentration of IFN-γ (30 u ml^{-1}), which itself significantly increased nitrite accumulation, was used in combination with LPS (100 ng ml^{-1}), there was no further increase in nitrite accumulation over that seen with IFN-γ alone (fig 7.14b). Thus, additivity was seen between the effects of LPS and IFN-γ but only when sub-threshold concentrations of the agents were used.

Combined effects of IFN-γ and IL-1α on nitrite accumulation into the serum-free culture medium bathing RASMC

In order to investigate possible interactions between IFN-γ and IL-1α the effects of combinations of both drugs were examined.

Incubation for 24 h with the combination of IFN-γ (30 u ml^{-1}), which itself stimulated nitrite accumulation, and a sub-threshold concentration of IL-1α (1 u ml^{-1}), produced no further increase in nitrite accumulation into the serum-free culture medium bathing RASMC over that produced by IFN-γ alone (fig 7.15a). In contrast, when the effective concentration of IFN-γ (30 u ml^{-1}) was combined with a concentration of IL-1α (10 u ml^{-1}), which alone increased nitrite accumulation, a larger increase in nitrite accumulation was obtained than that induced by either alone (fig 7.15b). Thus, additivity was seen between IFN-γ and IL-1α but only when both were used at concentrations which themselves were effective.

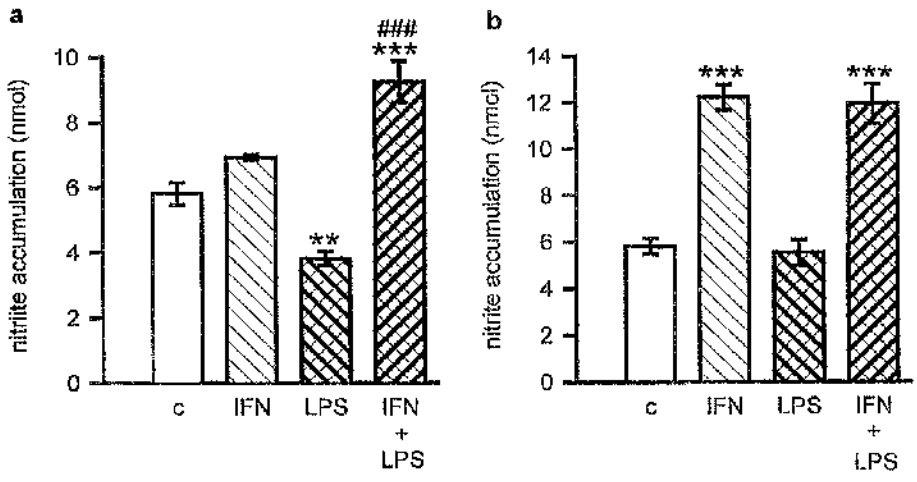


Figure 7.14: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with lipopolysaccharide (LPS; 100 ng ml^{-1}) or interferon-γ (IFN; **a**, 10 u ml^{-1} and **b**, 100 u ml^{-1}) alone and in combination. Each value is the mean of 6 observations with vertical bars representing the s.e. mean. *** $P<0.005$ and ** $P<0.01$, indicate a significant difference from control (c) and ### $P<0.005$, indicates a significant difference from either drug alone.

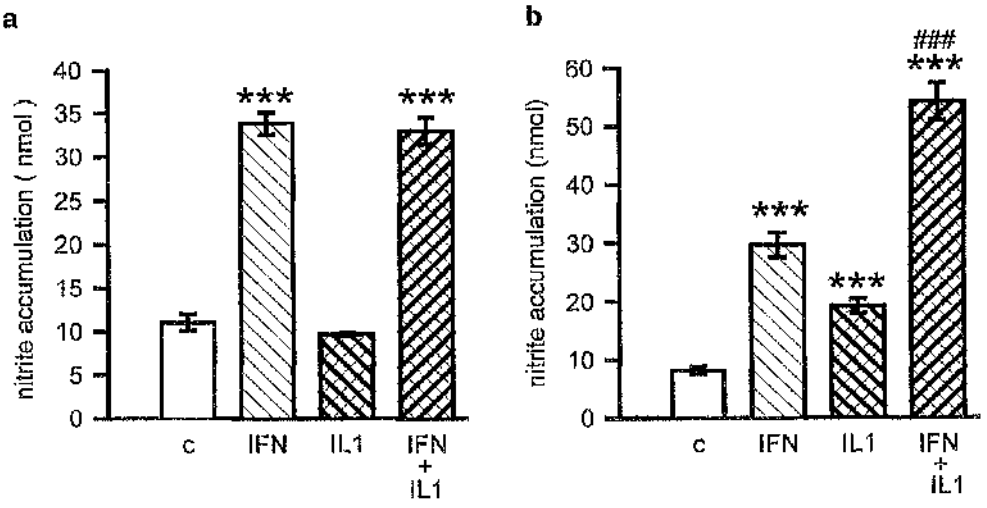


Figure 7.15: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with interferon-γ (IFN; 30 u ml^{-1}) and interleukin-1α (IL1; **a**, 1 u $ml^{-1}$and **b**, 10 u ml^{-1}) alone and in combination. Each value is the mean of 4 - 5 observations with vertical bars representing the s.e. mean. *** $P<0.005$ and ### $P<0.005$, indicate a significant difference from control (c) and from either drug alone, respectively.

Inhibition of the increase in nitrite accumulation into serum-free culture medium bathing RASMC stimulated by cytokines

Having established that the cytokines IFN-γ and IL-1α increased accumulation of nitrite into the serum-free culture medium bathing RASMC, a series of experiments were conducted to determine if this nitrite was derived from NO produced by the L-arginine/NO pathway. These experiments involved the use of the inhibitors of NOS, L-NAME and L-NMMA (Gross *et al.,* 1990; Rees *et al.,* 1990b; McCall *et al.,* 1993), as well as agents which inhibit the expression of iNOS, i.e. the corticosteriod, dexamethasone, and the protein synthesis inhibitor, cycloheximide (Di Rosa *et al.,* 1990; Knowles *et al.,* 1990b; Radomski *et al.,* 1990b; Rees *et al.,* 1990a).

Effects of inhibitors of NOS on nitrite accumulation into the serum-free culture medium bathing RASMC

The first inhibitor of NOS examined in these experiments was L-NAME (Rees *et al.,* 1990b; McCall *et al.,* 1993). L-NAME (100 μM) had no effect on basal accumulation of nitrite into the serum-free culture medium bathing RASMC following a 24 h incubation (fig 7.16). However, L-NAME (100 μM) abolished the increase in nitrite accumulation produced by IFN-γ (30 u ml^{-1}).

Similarly, L-NAME (100 μM), when present during a 24 h incubation also inhibited the increase in nitrite accumulation into the serum-free culture medium bathing RASMC produced by the joint stimulus of LPS (1000 ng ml^{-1}) and IFN-γ (30 u ml^{-1}; fig 7.17).

The second inhibitor of NOS examined was L-NMMA (Rees *et al.,* 1990b; McCall *et al.,* 1993), which produced roughly similar results to L-NAME.

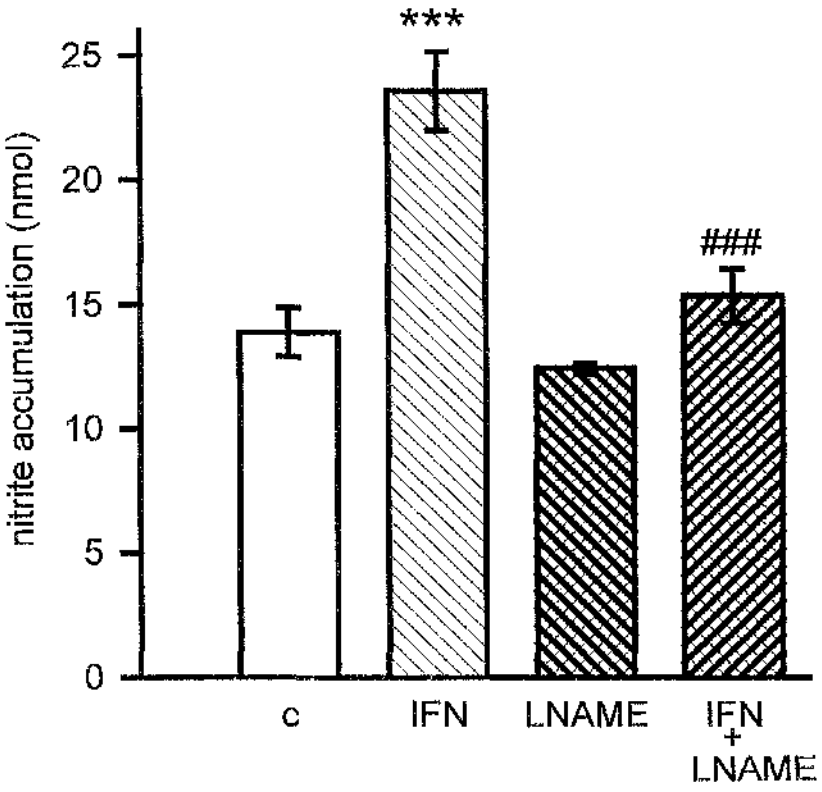


Figure 7.16: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with interferon-γ (IFN; 30 u ml^{-1}) and N^G-nitro-L-arginine methyl ester (LNAME; 100 μM) alone and in combination. Each value is the mean of 4 - 5 observations with vertical bars representing the s.e. mean. *** $P<0.005$ and ### $P<0.005$, indicate a significant difference from control (c) and from IFN alone, respectively.

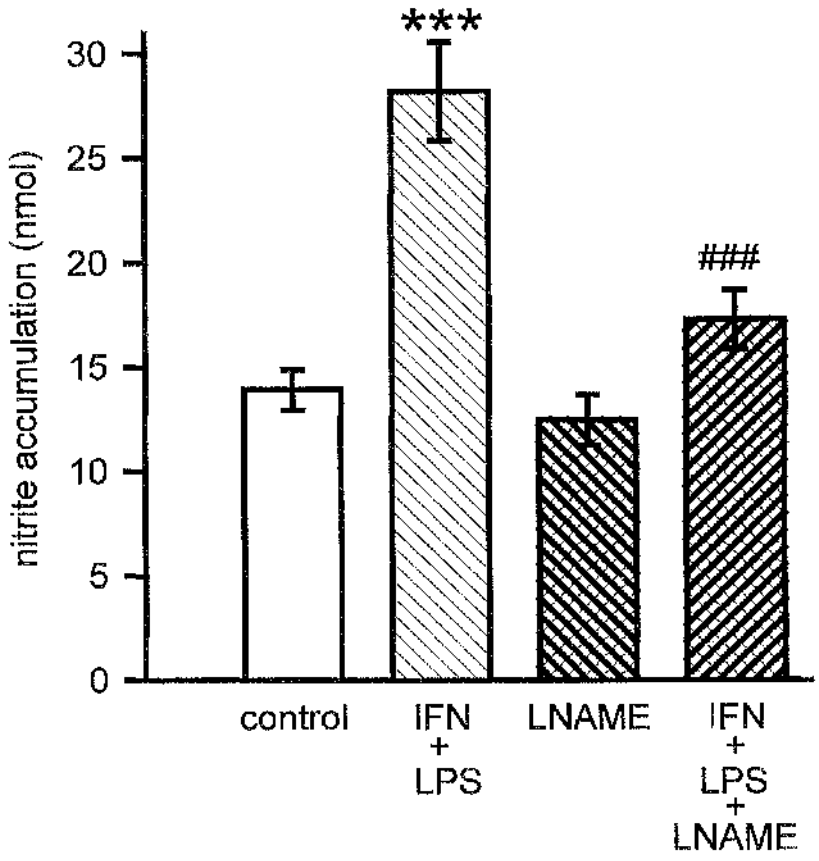


Figure 7.17: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with the joint stimulus of interferon-γ (IFN; 30 u ml^{-1}) and lipopolysaccharide (LPS; 1000 ng ml^{-1}) alone and in combination with N^G-nitro-L-arginine methyl ester (LNAME; 100 μM). Each value is the mean of 6 observations with vertical bars representing the s.e. mean. *** $P<0.005$ and ### $P<0.005$, indicate a significant difference from control and from IFN together with LPS, respectively.

Specifically, L-NMMA (300 μM) produced a small decrease in basal accumulation of nitrite into the serum-free culture medium bathing RASMC following a 24 h incubation (fig 7.18). Furthermore, L-NMMA (300 μM) also abolished the increase in nitrite accumulation produced by IFN-γ (30 u ml^{-1}).

Thus, the effects of L-NAME and L-NMMA suggested that the increased accumulation of nitrite stimulated by cytokines arose from the L-arginine/NO pathway. They also suggest that this pathway was responsible for only a small component of the nitrite generated under basal conditions.

Effects of inhibitors of the expression of iNOS on nitrite accumulation into the serum-free culture medium bathing RASMC

The first inhibitor of expression of iNOS examined in these experiments was dexamethasone (Di Rosa *et al.*, 1990; Knowles *et al.*, 1990b; Rees *et al.*, 1990a). Dexamethasone (0.1 μM and 1 μM) produced concentration-dependent decreases in basal accumulation of nitrite into the serum-free culture medium bathing RASMC following a 24 h incubation (fig 7.19). Furthermore, dexamethasone (0.1 μM and 1 μM) abolished the increase in nitrite accumulation produced by IFN-γ (30 u ml^{-1}).

Roughly similar results were obtained with a second inhibitor of the expression of iNOS, the protein synthesis inhibitor, cycloheximide (Radomski *et al.*, 1990b). Cycloheximide (1 μg ml^{-1}) had no effect on basal accumulation of nitrite into the serum-free culture medium bathing RASMC following a 24 h incubation (fig 7.20a). However, cycloheximide (1 μg ml^{-1}) had a tendency to reduce the increase in nitrite accumulation produced by IFN-γ (30 u ml^{-1}). A higher concentrations of cycloheximide (10 μg ml^{-1}) did decrease basal accumulation of nitrite as well as inhibit the increase produced by IFN-γ (30 u

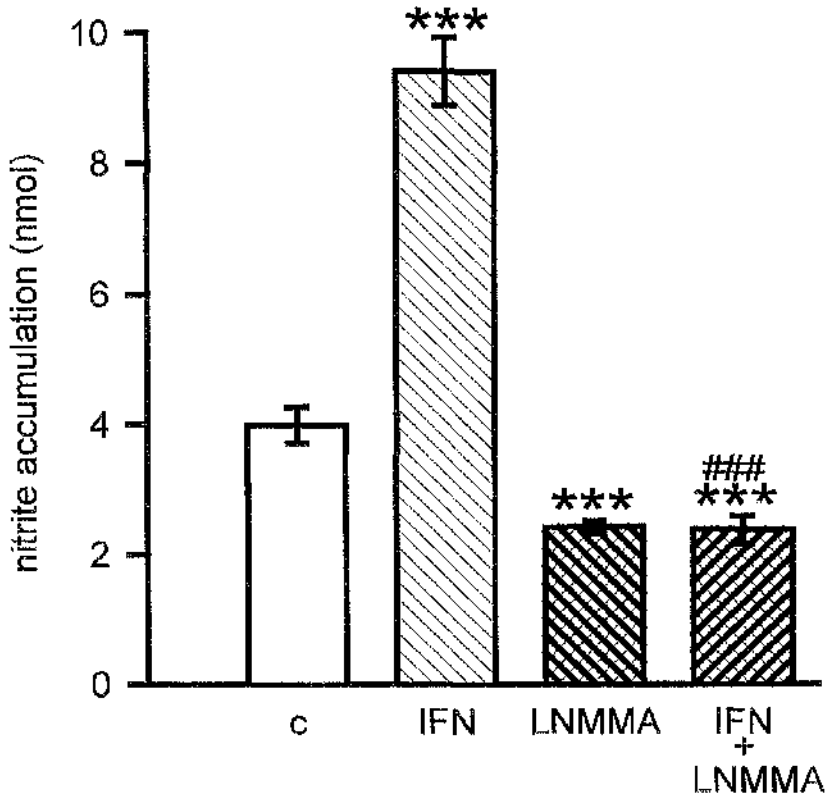


Figure 7.18: Nitrite accumulation (nmol per 10^6 cells) into the serum free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with interferon-γ (IFN; 30 u ml^{-1}) and N^G-monomethyl-L-arginine (LNMMA; 300 μM) alone and in combination. Each value is the mean of 4 observations with vertical bars representing the s.e. mean. *** $P<0.005$ and ### $P<0.005$, indicate a significant difference from control (c) and from IFN, respectively.

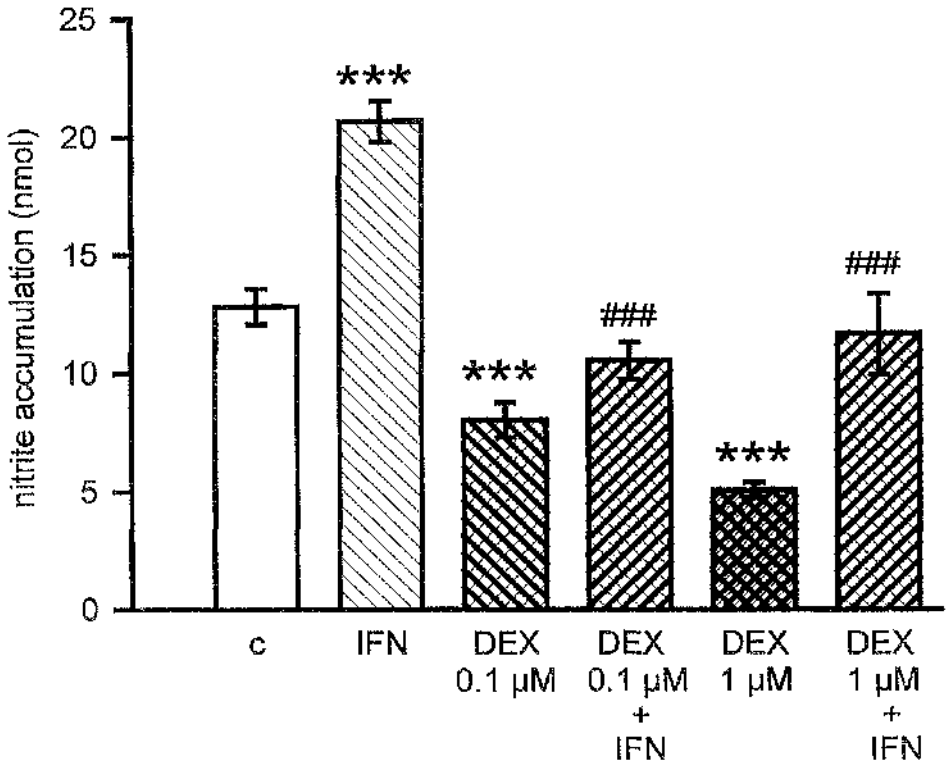


Figure 7.19: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with interferon-γ (IFN; 30 u ml^{-1}) and dexamethasone (DEX ; 0.1 µM or 1 µM) alone and in combination. Each value is the mean of 4 observations with vertical bars representing the s.e. mean. *** $P<0.005$ and ### $P<0.005$, indicate a significant difference from control (c) and from IFN, respectively.

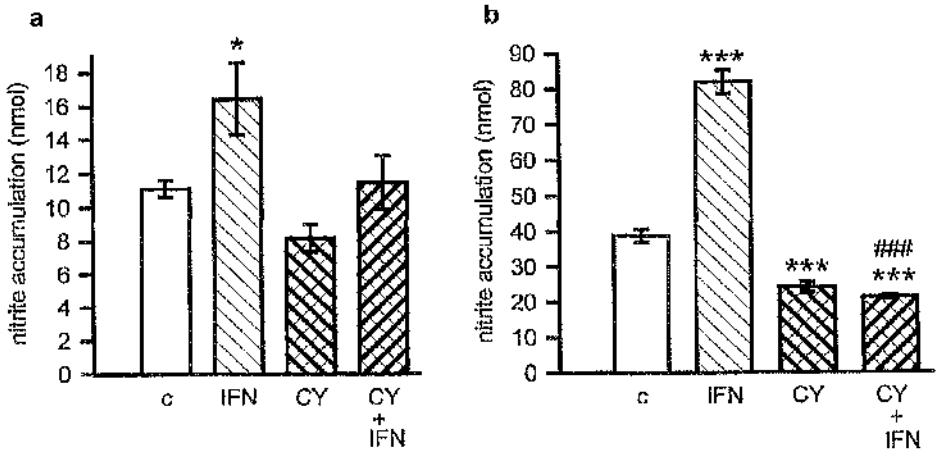


Figure 7.20: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with interferon-γ (IFN; 30 u ml^{-1}) and cycloheximide (CY; **a**, 1 μg ml^{-1} or **b**, 10 μg ml^{-1}) alone and in combination. Each value is the mean of 4 observations with vertical bars representing the s.e. mean. *** $P<0.005$ and ### $P<0.005$, indicate a significant difference from control (c) and from IFN, respectively.

ml^{-1}), but this concentration was associated with a toxic action on the cells, namely detachment of cells from the growth surface (fig 7.20b).

Thus, the effects of dexamethasone and cycloheximide are consistent with the increased accumulation of nitrite stimulated by IFN-γ arising from the expression of iNOS. The data also suggest that the extent to which the L-arginine/NO pathway contributes to basal nitrite accumulation is small if any but varies from experiment to experiment.

Effects of polymixin B, a scavenger of LPS, on nitrite accumulation into the serum-free culture medium bathing RASMC

Although LPS alone failed to stimulate increased accumulation of nitrite into the serum-free culture medium bathing RASMC, it was able to potentiate the actions of sub-threshold concentrations of IFN-γ (fig 7.14a). Experiments were therefore conducted to determine if the effect of IFN-γ alone was influenced to any extent by endotoxin which might be contaminating the culture medium. These experiments were conducted using the antibiotic, polymixin B, which binds and inactivates bacterial LPS (Lasfargues *et al.*, 1989).

Incubation for 24 h with polymixin B (1, 10 or 30 u ml^{-1}) had no effect on the basal accumulation of nitrite into the serum-free culture medium bathing RASMC (fig 7.21a, b and c). Similarly, polymixin B (1, 10 or 30 u ml^{-1}) had no effect on the increase in nitrite accumulation into the serum-free culture medium produced by IFN-γ (30 u ml^{-1}). It was therefore unlikely that contamination of the culture medium by LPS played any part in the increase in nitrite accumulation produced by IFN-γ.

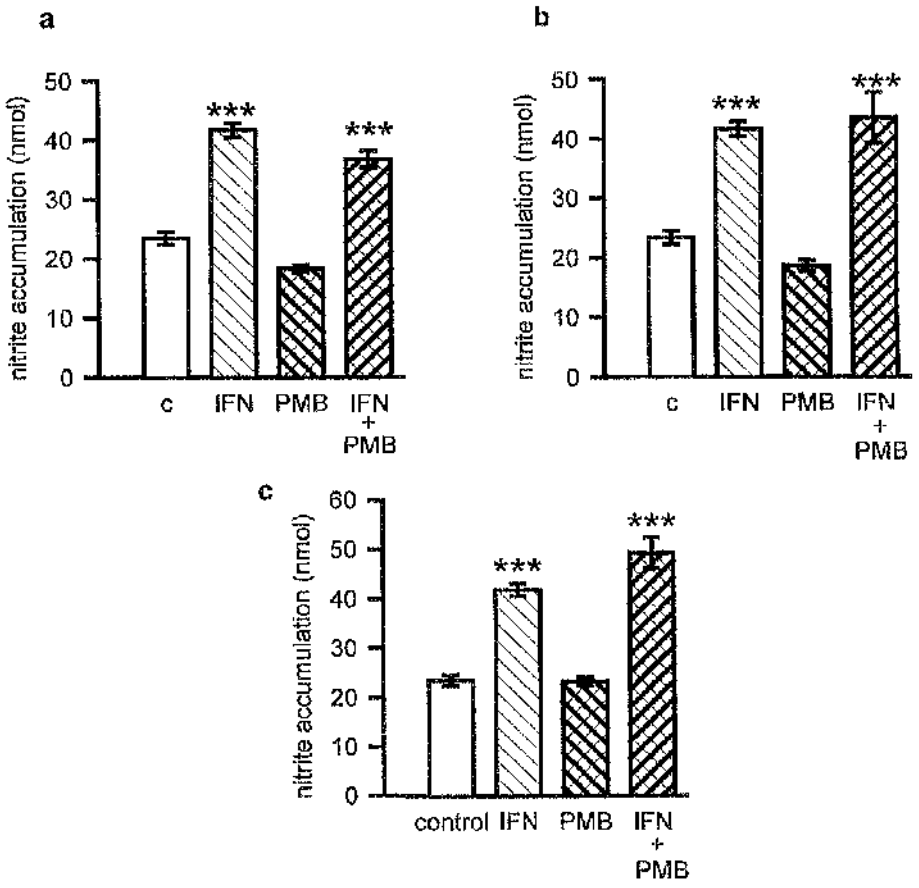


Figure 7.21: Nitrite accumulation (nmol per 10^6 cells) into the serum-free culture medium bathing rat aortic smooth muscle cells following a 24 h incubation with interferon-γ (IFN; 30 u ml^{-1}) and polymixin B (PMB; a, 1 μg ml^{-1}; **b**, 10 μg ml^{-1} or **c**, 30 μg ml^{-1}) alone and in combination. Each value is the mean of 6 observations with vertical bars representing the s.e. mean. *** $P<0.005$, indicates a significant difference from control (c).

Chapter 8

Assessment of inducible nitric oxide synthase expression by measuring cyclic GMP content of rat aortic smooth muscle cells in culture

In the previous chapter it was established that expression of inducible nitric oxide synthase (iNOS) in rat aortic smooth muscle cells (RASMC) stimulated by cytokines could be assessed by measuring the accumulation of nitrite, a major stable breakdown product of nitric oxide (NO) (Wennmalm *et al.*, 1992; Ignarro *et al.*, 1993), into the bathing medium. In this section verification of expression of iNOS was sought by measuring the cellular content of cyclic GMP, the second messenger by which NO produces its actions in vascular smooth muscle (Rapoport & Murad, 1983).

As in the previous chapter, in order to avoid the inhibitory actions of serum on expression of iNOS, all experiments were conducted on cells grown for 24 h in serum-free culture medium. Thereafter, they were washed and fresh serum-free culture medium was added before the experiment was begun.

Effects of LPS on cyclic GMP content of RASMC

In the previous chapter, it was shown that lipopolysaccharide (LPS) from *Salmonella typhosa* had no effect on accumulation of nitrite into the medium bathing RASMC except at extremely high concentrations. A similar action of LPS was seen when measuring the cyclic GMP content of RASMC. Specifically, incubation for 24 h with LPS from *S. typhosa*, in the concentration range 1 - 100 μg ml^{-1} produced no significant change in the cyclic GMP content of RASMC in culture (fig 8.1). However, extremely high concentrations of LPS (300 μg ml^{-1} and 1000 μg ml^{-1}) did increase the cyclic GMP content of RASMC, but this was associated with toxicity, resulting in detachment of the cells from the growth surface.

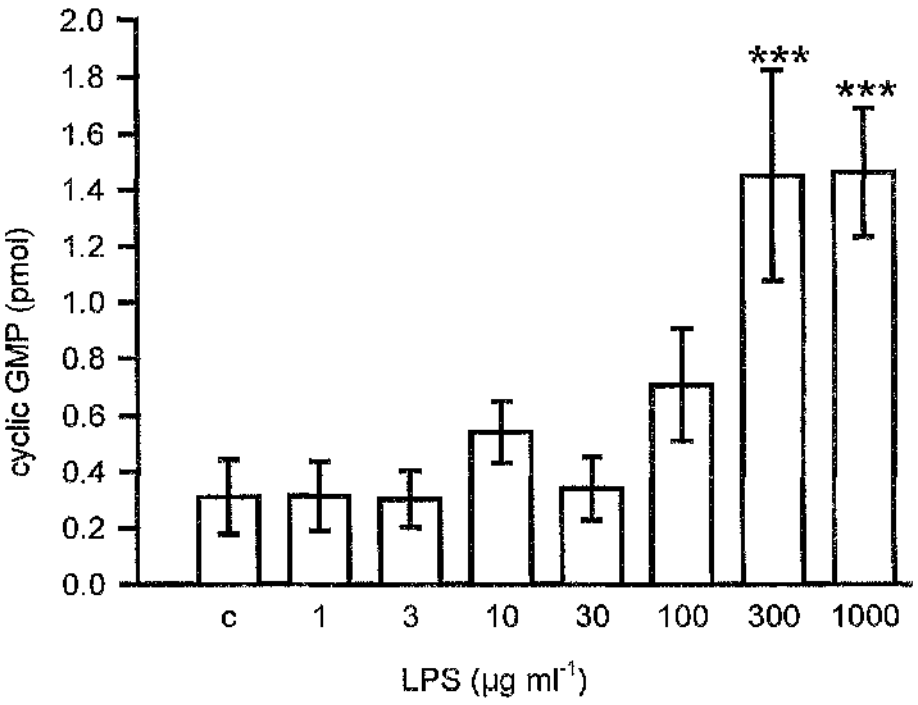


Figure 8.1: Cyclic GMP content (pmol per 10^6 cells) of rat aortic smooth muscle cells in culture following a 24 h incubation with lipopolysaccharide (LPS; 1 - 1000 µg ml^{-1}). Each value is the mean of 4 observations with vertical bars representing the s.e. mean. *** $P<0.005$, indicates a significant difference from control (c), however these concentrations of LPS resulted in detachment of cells from the growth surface indicating a toxic action.

Effects of IL-1α on cyclic GMP content of RASMC

Incubation for 24 h with interleukin-1α (IL-1α) in the concentration range 1 - 10 u ml^{-1} produced a concentration-dependent increase in cyclic GMP content of RASMC in culture (fig 8.2).

Combined effects of LPS and IFN-γ on cyclic GMP content of RASMC

Incubation for 24 h with interferon-γ (IFN-γ; 30 u ml^{-1}) produced a significant increase in the cyclic GMP content of RASMC (fig 8.3). When LPS (5 μg ml^{-1}), which alone had no effect, was incubated together with IFN-γ (30 u ml^{-1}), the increase in cyclic GMP content was no greater than the increase seen with IFN-γ (30 u ml^{-1}) alone (fig 8.3). Thus, there was no apparent interaction between IFN-γ and LPS on the cyclic GMP content of RASMC.

Effects of inhibitors of NOS on cyclic GMP content of RASMC

Having found that the cytokines, IFN-γ and IL-1α increased the cyclic GMP content of RASMC in culture, experiments were conducted using N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS (Rees *et al.*, 1990b; McCall *et al.*, 1993), to see if this increase was due to actions of NO produced by the L-arginine/NO pathway.

L-NAME (100 μM and 3 mM) had no effect on the cyclic GMP content of unstimulated RASMC incubated for 24 h (fig 8.4a and b). Also, L-NAME (100 μM) had no effect on the increase in cyclic GMP content of RASMC stimulated by IFN-γ (30 u ml^{-1}; fig 8.4a). However, the higher concentration of 3 mM did inhibit the increase in cyclic GMP content of RASMC stimulated by IFN-γ (30 u ml^{-1}; fig 8.4b).

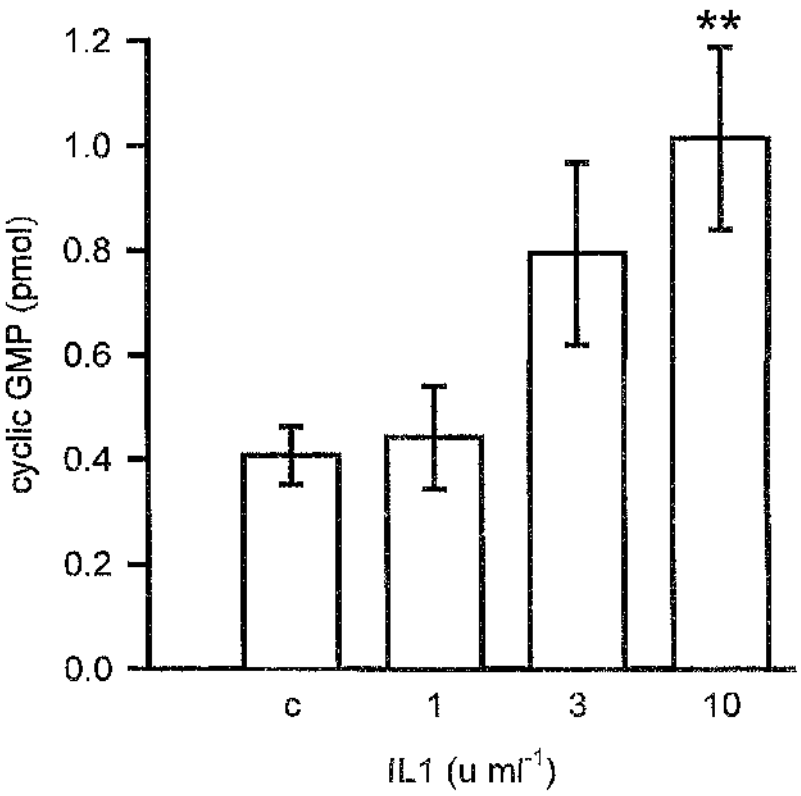


Figure 8.2: Cyclic GMP content (pmol per 10^6 cells) of rat aortic smooth muscle cells in culture following a 24 h incubation with interleukin-1α (IL1; 1 - 10 u ml^{-1}). Each value is the mean of 4 observations with vertical bars representing the s.e. mean. ** $P<0.01$, indicates a significant difference from control (c).

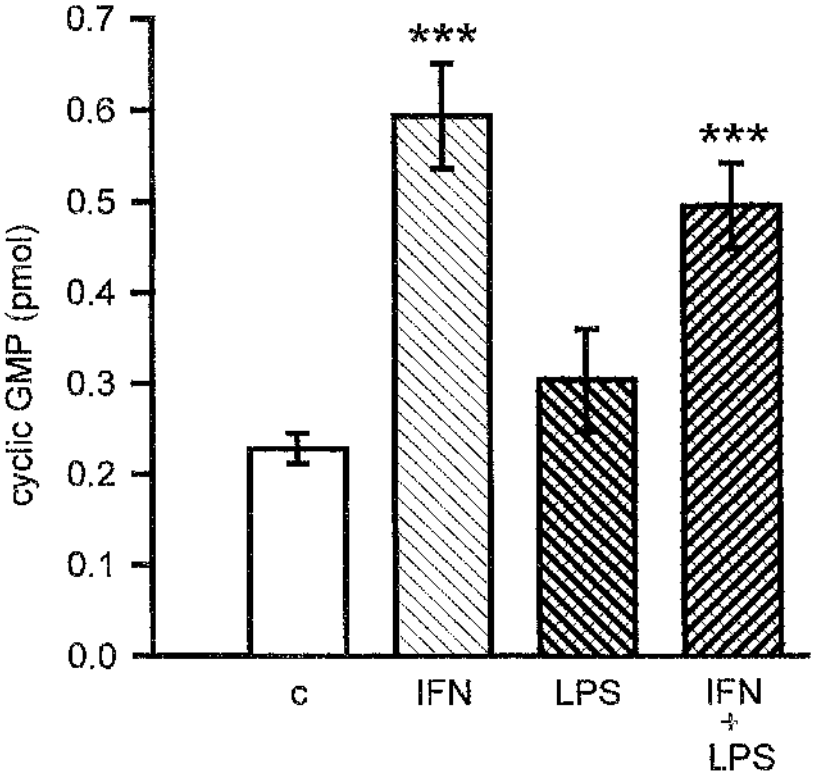


Figure 8.3: Cyclic GMP content (pmol per 10^6 cells) of rat aortic smooth muscle cells in culture following a 24 h incubation with interferon-γ (IFN; 30 u ml^{-1}) and lipopolysaccharide from *Salmonella typhosa* (LPS; 5 µg ml^{-1}) alone and in combination. Each value is the mean of 4 - 6 observations with vertical bars representing the s.e. mean. *** $P<0.005$, indicates a significant difference from control (c).

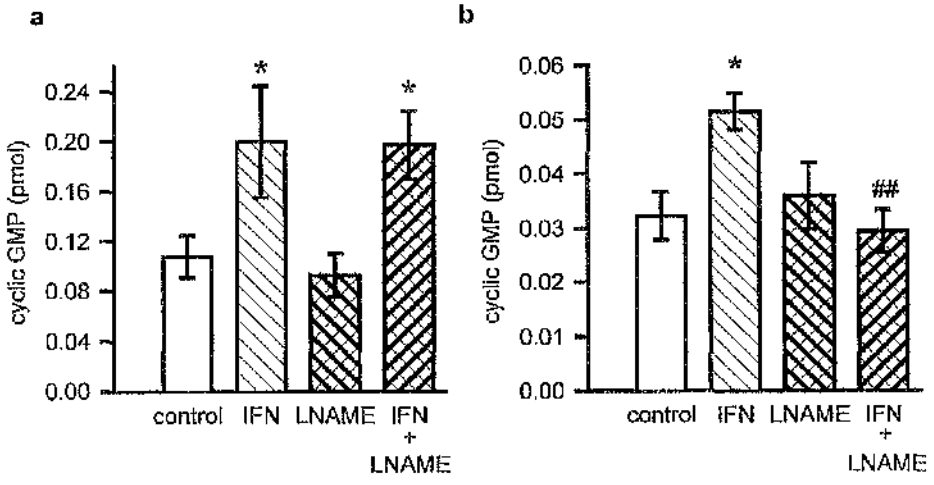


Figure 8.4: Cyclic GMP content (pmol per 10^6 cells) of rat aortic smooth muscle cells in culture following a 24 h incubation with interferon-γ (IFN; 30 u ml^{-1}) and N^G-nitro-L-arginine methyl ester (LNAME; **a**, 100 μM and **b**, 3 mM) alone and in combination. Each value is the mean of 4 - 6 observations with vertical bars representing the s.e. mean. * $P<0.05$ and ## $P<0.01$, indicate a significant difference from control and from IFN (30 u ml^{-1}) alone, respectively.

Similarly, the lower concentration of L-NAME (100 μM) did not inhibit the increase in cyclic GMP content of RASMC following a 24 h incubation with the combined stimulus of LPS (1000 ng ml^{-1}) and IFN-γ (30 u ml^{-1}; fig 8.5).

Effects of dexamethasone on cyclic GMP content of RASMC

In order to further investigate the possibility that the increase in cyclic GMP content of RASMC in culture stimulated by IFN-γ resulted from the actions of NO produced by iNOS the effects of dexamethasone were investigated. Dexamethasone has previously been shown to inhibit the expression of iNOS (Di Rosa *et al.*, 1990; Knowles *et al.*, 1990b; Rees *et al.*, 1990a).

The presence of dexamethasone (1 μM) had no effect on the cyclic GMP content of unstimulated RASMC in culture following a 24 h incubation (fig 8.6). In contrast, a two hour pre-treatment with dexamethasone did prevent the increase in cyclic GMP content of RASMC stimulated by IFN-γ (30 u ml^{-1}).

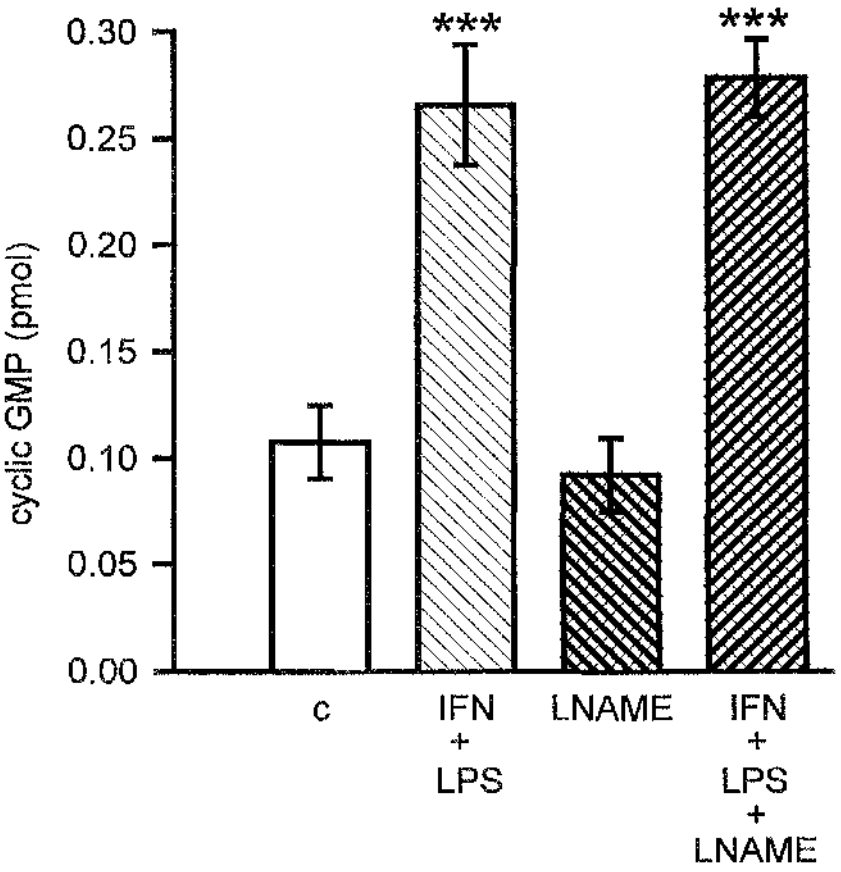


Figure 8.5: Cyclic GMP content (pmol per 10^6 cells) of rat aortic smooth muscle cells in culture following a 24 h incubation with the combined stimulus of interferon-γ (IFN; 30 u ml^{-1}) and lipopolysaccharide (LPS; 1000 ng ml^{-1}) alone and in combination with N^G-nitro-L-arginine methyl ester (LNAME; 100 μM). Each value is the mean of 4 - 6 observations with vertical bars representing the s.e. mean. *** $P<0.005$, indicates a significant difference from control (c).

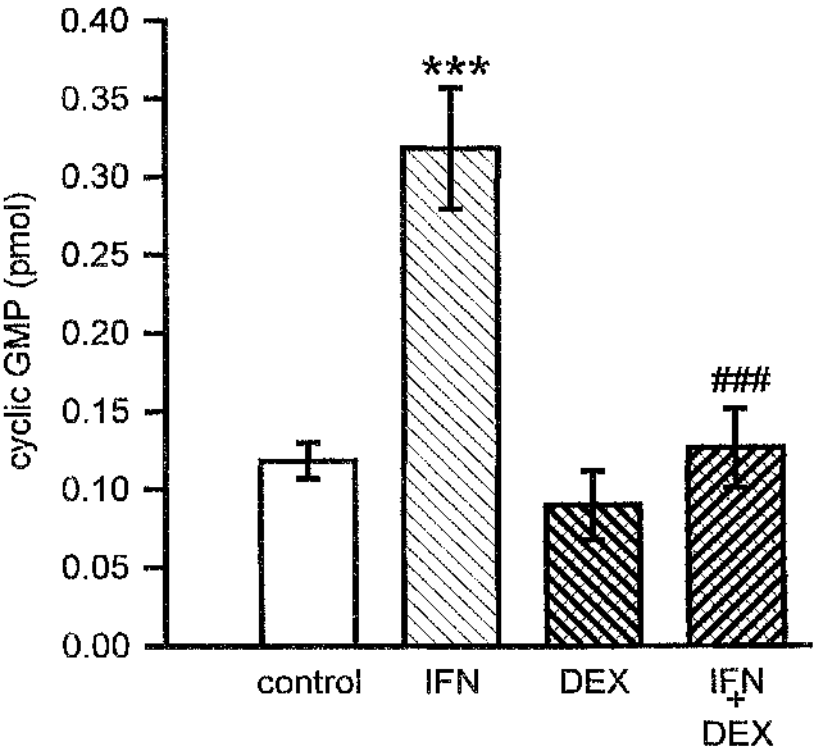


Figure 8.6: Cyclic GMP content (pmol per 10^6 cells) of rat aortic smooth muscle cells in culture following a 24 h incubation with interferon-γ (IFN; 30 u ml^{-1}) and dexamethasone (DEX; 1 μM) alone and in combination. Each value is the mean of 4 observations with vertical bars representing the s.e. mean. *** $P<0.005$ and ### $P<0.005$, indicate a significant difference from control (c) and from IFN (30 u ml^{-1}), respectively.

Chapter 9

Effects of modulating cytokine activity on the depression of phenylephrine-induced contraction of rat aortic rings

The results of the previous chapters have shown that lipopolysaccharide (LPS) alone appeared able to stimulate the expression of inducible nitric oxide synthase (iNOS) in the wall of rat isolated endothelium-denuded aortic rings. In contrast, LPS was unable to stimulate expression in rat aortic smooth muscle cells (RASMC) in culture without the additional presence of a cytokine. One possible explanation for this apparent difference is that non-smooth muscle cells in the aortic wall could respond to LPS by releasing a cytokine which then diffuses to the smooth muscle cells resulting in expression of iNOS. This hypothesis was tested by examining the actions of inhibitors of cytokine activity. The inhibitors used were pentoxifylline, which inhibits transcription of mRNA for tumour necrosis factor-α (TNF-α)(Strieter *et al.*, 1988; Han *et al.*, 1990; Doherty *et al.*, 1991), and a panel of antibodies to the cytokines TNF-α, interleukin- 1β (IL-1β) and interferon-γ (IFN-γ).

Effects of pentoxifylline on the depression of PE-induced contraction

As indicated previously (chapter 6), overnight (18 h) incubation of rat aortic rings in Krebs solution at 37°C led to depression of the ability of phenylephrine (PE, 0.1 nM - 10 μM) to induce contraction and this was partly reversed following addition of the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 100 or 300 μM)(Rees *et al.*, 1990b; McCall *et al.*, 1993), to the tissue baths (fig 9.1). When the inhibitor of TNF-α, pentoxifylline (0.1 - 10 μM) was present during the overnight (18 h) incubation, the depression of PE-induced contraction was completely unaffected (fig 9.1a, b & c). Furthermore, pentoxifylline (0.1 - 10 μM) did not significantly alter the ability of L-NAME (100 μM or 300 μM) to produce a partial reversal of the depression of PE-induced contraction.

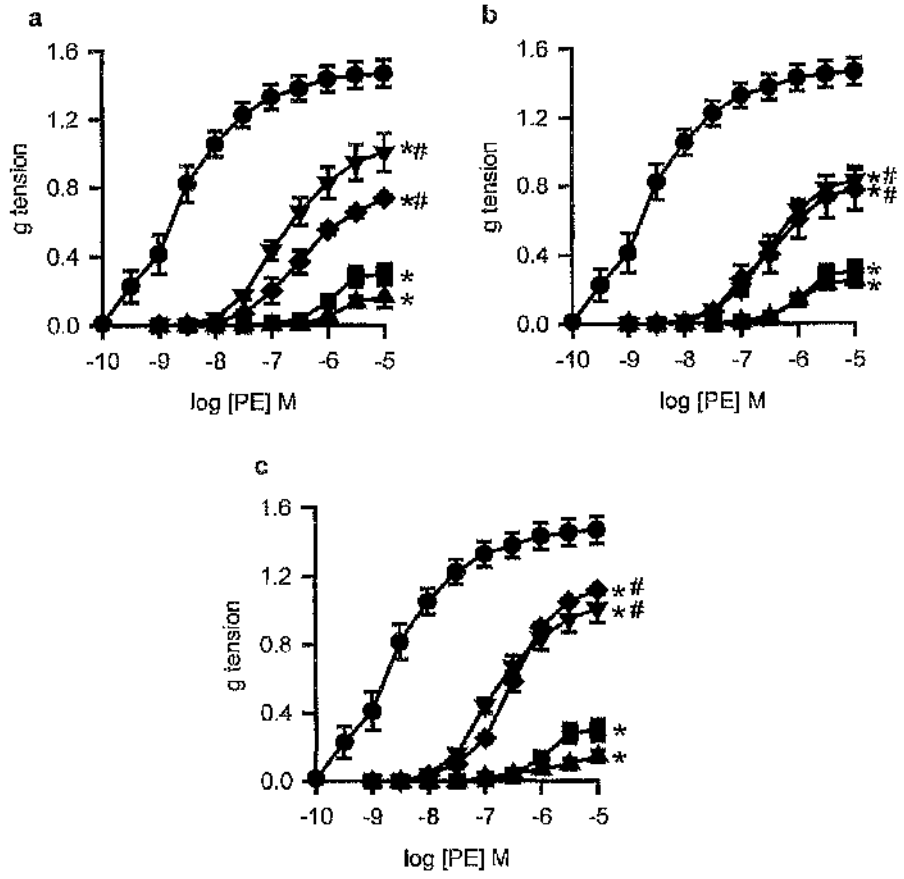


Figure 9.1: Concentration-response curves showing the contractile effects of phenylephrine (PE; 0.1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); rings incubated at 37°C for 18 h in Krebs solution (■); rings incubated overnight in Krebs solution containing pentoxifylline (▲; **a**, 0.1 μM; **b**, 1 μM and **c**,10 μM); rings incubated overnight in Krebs solution followed by treatment with N^G-nitro-L-arginine methyl ester (L-NAME; ▼; **a** and **c**, 300 μM and **b**, 100 μM); and rings incubated with pentoxifylline and then treated with L-NAME (◆; **a** and **c**, 300 μM and **b**, 100 μM). Each point is the mean of 3 - 16 observations with vertical bars representing the s.e. mean. * $P<0.05$, indicates a significant difference from freshly isolated rings and # $P<0.005$ indicates a significant difference from rings incubated at 37°C for 18 h and from rings incubated at 37°C in the presence of pentoxifylline.

Effects of a monoclonal antibody to TNF-α on the depression of PE-induced contraction

When present throughout an overnight (18 h) incubation of rat aortic rings, a monoclonal antibody to mouse TNF-α (anti-m TNF-α, prepared from rat-mouse hybrid cells, clone MP6-XT3) used at 10 and 30 μg ml^{-1} had no effect on the depression of PE (0.1 nM - 10 μM)-induced contraction (fig 9.2). Furthermore, the presence of anti-m TNF-α (10 and 30 μg ml^{-1}) did not significantly alter the ability of L-NAME (100 μM) to reverse the depression of PE (0.1 nM - 10 μM)-induced contraction (fig 9.2).

Effects of a monoclonal antibody to IL-1β on the depression of PE-induced contraction

When present throughout an overnight (18 h) incubation of rat aortic rings a monoclonal antibody to human IL-1β (anti-m IL-1β, prepared from mouse-rat hybrid cells, clone P3x63Ag 8.653) used at 10 and 50 μg ml^{-1} had no effect on the depression of PE (0.1 nM - 10 μM)-induced contraction (fig 9.3). Furthermore, the presence of anti-m IL-1β (10 μg ml^{-1}) did not significantly alter the ability of L-NAME (100 μM) to reverse the depression of PE (0.1 nM - 10 μM)-induced contraction (fig 9.3a). However, anti-m IL-1β at 50 μg ml^{-1} but not 10 μg ml^{-1} appeared to enhance the ability of L-NAME (100 μM) to reverse the depression of PE-induced contraction, but only at sub-maximal levels of contraction, i.e. in the concentration range of PE from 30 - 300 nM (fig 9.3b).

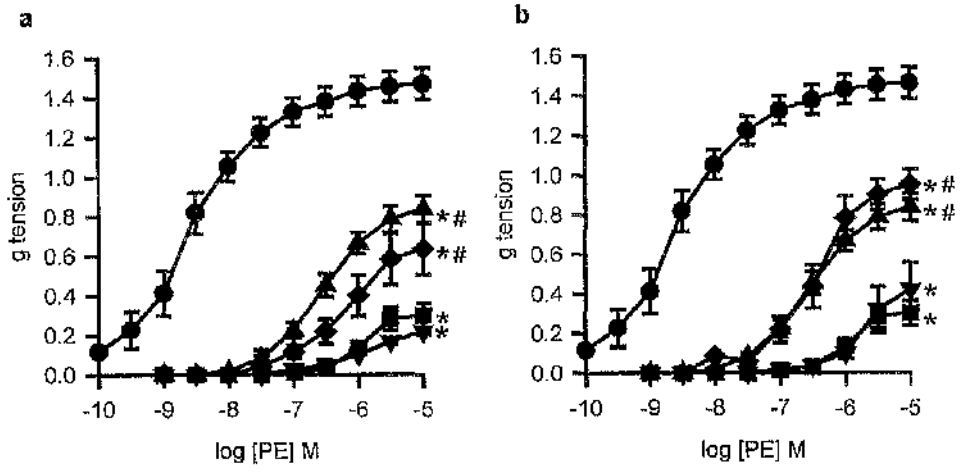


Figure 9.2: Concentration-response curves showing the contractile effects of phenylephrine (PE; 0.1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); rings incubated at 37°C for 18 h in Krebs solution (■); rings incubated overnight in Krebs solution followed by treatment with L-NAME (300 μM; ▲); rings incubated overnight in Krebs solution containing the monoclonal antibody to tumour necrosis factor-α (▼; **a**, 10 μg ml^{-1}, and **b**, 30 μg ml^{-1}); and rings incubated with the monoclonal antibody to tumour necrosis factor-α and then treated with N^G-nitro-L-arginine methyl ester (L-NAME; ♦). Each point is the mean of 3 - 16 observations with vertical bars representing the s.e. mean. * $P<0.005$, indicates a significant difference from freshly isolated rings; # $P<0.005$, indicates a significant difference from rings incubated at 37°C for 18 h and from rings incubated in the presence of pentoxifylline.

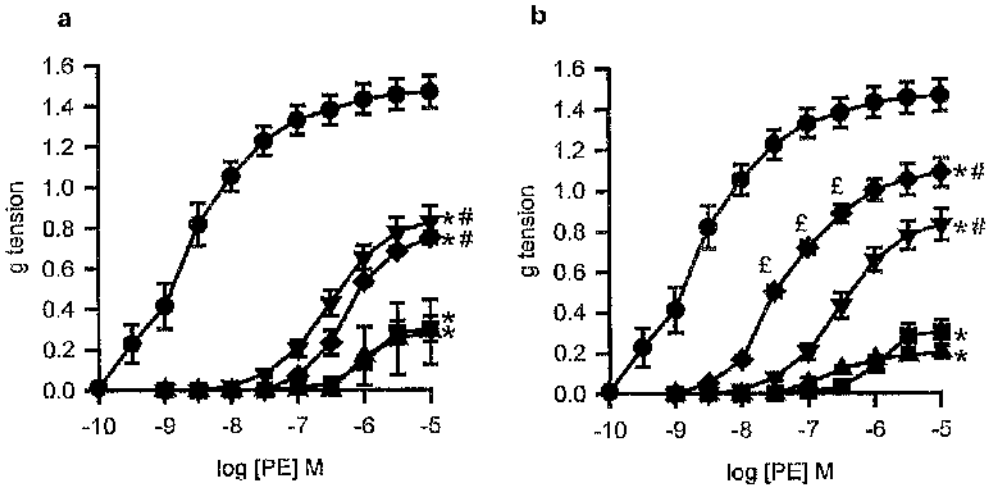


Figure 9.3: Concentration-response curves showing the contractile effects of phenylephrine (PE; 0.1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); rings incubated at 37°C for 18 h in Krebs solution (■); rings incubated overnight in Krebs solution followed by treatment with N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM; ▼); rings incubated overnight in Krebs solution containing the monoclonal antibody to interleukin-1β (▲; a, 10 μg ml^{-1} and b, 50 μg ml^{-1}); and rings incubated with the monoclonal antibody to interleukin-1β and then treated with L-NAME (◆). Each point is the mean of 3 - 16 observations with vertical bars representing the s.e. mean. * $P<0.005$, indicates a significant difference from freshly isolated rings; # $P<0.005$, indicates a significant difference from rings incubated at 37°C for 18 h and from rings incubated in the presence of pentoxifylline; and £ $P<0.005$, indicates a significant difference from rings incubated at 37°C followed by treatment with L-NAME.

Effects of a monoclonal antibody to IFN-γ on the depression of PE-induced contraction

When present throughout an overnight (18 h) incubation a monoclonal antibody to mouse IFN-γ (anti-m IFN-γ, prepared from rat-mouse hybrid, clone XMG1.2) and used at 10 and 30 μg ml^{-1} had no effect on the depression of PE (0.1 nM - 10 μM)-induced contraction (fig 9.4). The presence of anti-m IFN-1γ (10 μg ml^{-1}) did appear, to reduce the ability of L-NAME (100 μM) to reverse the depression of PE-induced contraction, in the concentration range of PE from 1 to 3 μM (fig 9.4a). However, the ability of L-NAME (100 μM) to reverse the depression of PE (0.1 nM - 10 μM)-induced contraction was unaffected by anti-m IFN-1γ at 30 μg ml^{-1} (fig 9.4b).

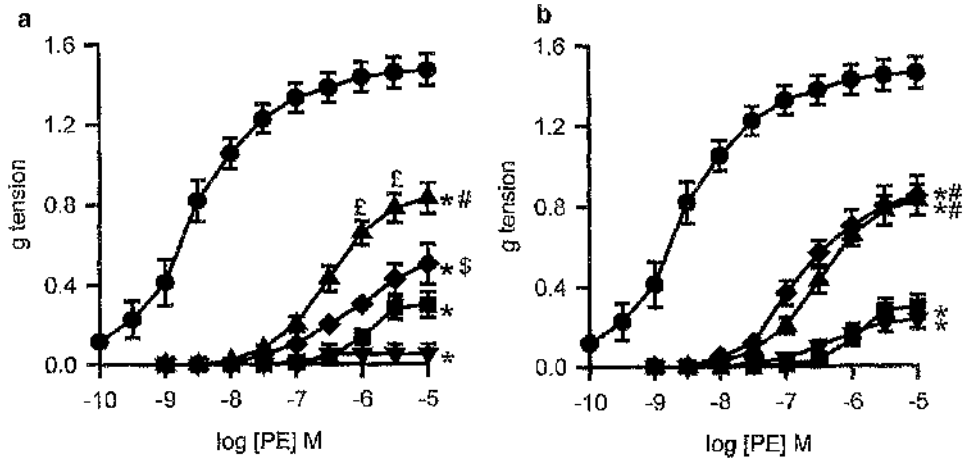


Figure 9.4: Concentration-response curves showing the contractile effects of phenylephrine (PE; 0.1 nM - 10 μM) on endothelium-denuded rings of rat aorta: freshly isolated rings (●); rings incubated at 37°C for 18 h in Krebs solution (■); rings incubated overnight in Krebs solution followed by treatment with N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM; ▲); rings incubated overnight in Krebs solution containing the monoclonal antibody to interferon-γ (▼; **a**, 10 μg ml^{-1} and **b**, 30 μg ml^{-1}); and rings incubated with the monoclonal antibody to interferon-γ and then treated with L-NAME (◆). Each point is the mean of 3 - 16 observations with vertical bars representing the s.e. mean. * $P<0.005$, indicates a significant difference from freshly isolated rings; # $P<0.01$, indicates a significant difference from rings incubated at 37°C for 18 h and from rings incubated in the presence of pentoxifylline; $ $P<0.01$, indicates a significant difference from rings incubated at 37°C for 18 h in presence of pentoxifylline; and £ $P<0.05$, indicates a significant difference from rings incubated with pentoxifylline and treated with L-NAME.

Chapter 10
Discussion

In this study an examination and comparison was made of a variety of different techniques for the indirect measurement of nitric oxide (NO) production. None of the detection systems employed measured native NO as this radical has a very short half life of 3 - 50 s (Griffith *et al.*, 1984; McCall & Vallance, 1992). The first systems we examined measured nitrite or combined nitrite/nitrate, the stable metabolites of NO (Wennmalm *et al.*, 1992; Ignarro *et al.*, 1993). The systems employed to measure nitrite were the Griess reaction (Green *et al.*, 1982), a chemiluminescence detection system using two different reducing systems (1,1'-dimethylferrocene/acetonitrile or sodium iodide/glacial acetic acid reflux) (Cox, 1980; Tracey *et al.*, 1990) and a fluorometric detection system (Damiani & Burini, 1986). The Griess reaction and the fluorometric detection system measure nitrite directly but the chemiluminescence system measures NO, thus making a prior reduction step necessary. Combined measurement of the two stable metabolites of NO, nitrite and nitrate, were made by the chemiluminescence detection system but in the presence of a stronger reducing solution (vanadium chloride/hydrochloric acid) (Braman & Hendrix, 1989).

The other detection systems examined, which do not involve measurement of the breakdown products of NO, were cyclic GMP content and relaxation of blood vessels. Cyclic GMP is the second messenger system employed by NO (Rapoport & Murad, 1983). The vasodilator action of NO in blood vessels has also been used as a means of bioassaying the amount of this vasodilator being produced. For example, many investigators have used cascade bioassay systems in which NO produced by endothelium-containing vessels (Griffith *et al.*, 1984) or endothelial cells grown on microcarrier beads (Angus *et al.*, 1986) was detected on endothelium-denuded arteries. Furthermore, the endothelium-dependent depression of contraction seen in many blood vessels has been used as a means of assessing indirectly basal production of NO

(Martin *et al.,* 1986). A variant of this last technique was employed in this study whereby depression of the contraction induced by phenylephrine (PE) was used as a means of assessing production of NO in endothelium-denuded rings of rat aorta following expression of inducible nitric oxide synthase (iNOS).

The sensitivity and utility of each of these systems was evaluated using different cell and tissue preparations, i.e. J774.2 murine macrophages, bovine aortic endothelial cells (BAEC) in culture, rat aortic smooth muscle cells (RASMC) in culture and endothelium-denuded rings of rat aorta. These preparations were chosen because the literature suggests that each could express iNOS and so should produce large quantities of NO.

The second part of this study involved examining in detail the factors controlling expression of iNOS in rat aorta and in cultured RASMC.

The Griess Reaction

The Griess reaction is a well established technique for the measurement of nitrite, the major *in vitro* breakdown product of NO (Wennmalm *et al.,* 1992; Ignarro *et al.,* 1993). It has been widely used to measure nitrite produced by the L-arginine/NO pathway in macrophages (Stuehr & Marletta, 1985; Stuehr & Marletta, 1987a; Di Rosa *et al.,* 1990; Gross *et al.,* 1990), endothelial cells (Schmidt *et al.,* 1988), vascular smooth muscle cells (Schini *et al.,* 1992) and hepatocytes (Geller *et al.,* 1993b). Although this system can only measure nitrite, nitrate can additionally be measured by converting it to nitrite by passage over a cadmium column (Green *et al.,* 1981a; Green *et al.,* 1981b). This technique was used to show that nitrate was synthesised in man (Green *et al.,* 1981a) and in rats (Green *et al.,* 1981b) by measuring the content of the urine.

The Griess reaction involves the formation of a complex between nitroxides and sulfanilic acid which reacts with N-(1-naphthyl) ethylenediamine generating a product easily measured spectrophotometrically (Green *et al.*, 1982). We found that this reaction can easily measure nitrite in biological samples, with a threshold sensitivity of 300 pmol and linearity between 300 - 9000 pmol. Others have stated the threshold sensitivity to be 500 - 1000 pmol (Green *et al.*, 1982; Tracey *et al.*, 1990). In addition to its inability to measure nitrate, as stated previously, the Griess reaction is also not entirely specific for nitrite, as nitrosothiols and nitrosamines also react giving an identical coloured product (Downes *et al.*, 1976; Tracey *et al.*, 1990). The presence of these agents in biological samples would therefore give 'false' readings. The Griess reaction could, however, easily measure large numbers of samples using the same batch of reagents, thus permitting speedy, efficient and convenient conduction of assay. Another advantage was that once the reagents were mixed with the samples, the coloured product was stable for at least 24 h when kept at 4°C, and so measurement did not have to be made immediately; they could have been made many hours later with no fear of loss of sensitivity.

A number of other problems were associated with this measuring technique. These included the fact that the turbidity of the samples could interfere with the spectrophotometric measurements. To reduce turbidity samples were spun in a microcentrifuge before the Griess reagents were added. Also, the colour of the medium resulting from the presence of the pH indicator, phenol red, as well as other constituents may affect the measurements, but to compensate for these the standard curve was prepared by dissolving sodium nitrite in the same culture medium (DMEM) as was used for experimentation.

Chemiluminescence detection systems

Chemiluminescence systems have been widely used and are reported to be more sensitive than the Griess reaction. Another advantage of one of these detection systems is its ability to measure both of the major breakdown products of NO, i.e. nitrite and nitrate, in the presence of strong reducing solutions (Braman & Hendrix, 1989). The reducing solutions tested in this study were 1,1'-dimethylferrocene/acetonitrile (Tracey *et al.*, 1990) and sodium iodide/glacial acetic acid (Cox, 1980), both of which reduce nitrite back to NO, and vanadium chloride/hydrochloric acid (Braman & Hendrix, 1989), which reduces both nitrite and nitrate to NO.

The basis of the mechanism of detection is that NO formed reacts with ozone generated within the chemiluminescence analyser to produce NO^{*}_{2}, which is highly unstable and spontaneously decomposes to NO_2 emitting light. In our study, it was this light that was measured by a photomultiplier tube and displayed on a computer and a Linseis recorder. The amount of light detected was directly proportional to the amount of NO generated from either nitrite and/or nitrate.

1,1'-Dimethylferrocene/acetonitrile system

The first chemiluminescence technique we investigated was the 1,1'-dimethylferrocene/acetonitrile system. This system was described by Termin *et al.* (1992) who used it to measure nitrite production by harvested bovine aortic endothelial cells. The system initially looked attractive as there was no need for reflux involving the potentially dangerous boiling acid during the reduction of nitrite to NO (Termin *et al.*, 1992). Termin's group report that this system was sensitive to nitrite at 50 pmol and above but we found that the signals generated from nitrite were small, difficult to discern from the

background noise and were long in duration. The slow generation of signals suggested a slow and inefficient reduction of nitrite to NO. We also found the sensitivity of this system to be much lower, with a threshold detection limit of nearer 500 pmol, but even then samples were hard to read. Thus, we found this system impractical to use.

Sodium iodide/glacial acetic acid reflux system

The second chemiluminescence technique we investigated was the sodium iodide/glacial acetic acid reflux system. This system was already well established before our study and detects nitrite (Cox, 1980; Palmer *et al.*, 1987). The system had previously been used to measure nitrite produced by porcine aortic endothelial cells (Palmer *et al.*, 1987) and blood platelets (Radomski *et al.*, 1987b). In fact it was this system which Palmer *et* al. (1987) originally used to demonstrate that the amount of NO produced by endothelial cells was sufficient to account for the biological activity of endothelium-derived relaxing factor (EDRF). However, it was also this system that Myers *et al.* (1989) used to claim that the amounts of NO produced by endothelial cells were 7 - 10-fold too low to account for the actions of EDRF. Instead, these authors suggested that EDRF was more likely to be an S-nitrosothiol than free NO (Myers *et al.*, 1989).

One major disadvantage with this system is it involves reflux of boiling acid at high temperature (65°C) to reduce nitrite back to NO (Cox, 1980). This is clearly a dangerous procedure which requires to be performed in a safety cabinet to safeguard the experimenter.

We found that this system produced fast (1 - 2 min to peak) signals which were easily distinguishable from the background noise and could measure nitrite levels as low as 100 pmol, making it 3 times more sensitive than the

Griess reaction. The sensitivity of this system has, however, been reported to vary in the literature. For example, Cox (1980) found a threshold of 300 pmol, but Palmer *et al.* (1987) were able to measure values as low as 70 pmol. The latter group, in fact, reported that electronic modification of their commercially available detector was necessary to obtain such a high sensitivity. Using our system, when the amount of nitrite (100 - 1000 pmol) injected was plotted against the peak signal obtained the resulting relationship though reasonably proportional was not strictly linear. This was probably due to samples with a large volume reducing the temperature of the reflux system thus leading to slow generation of NO. Indeed, large sample volumes were clearly seen to stop the reducing solution from boiling. Theoretically, under these situations it would be expected that measuring the entire area under the curve of the signal would provide a more reliable index and indeed this was the case. When the amount of nitrite injected was plotted against the area under the curve of the signal a good linear relationship was obtained. Consequently, measuring the area under the curve was adopted as the routine means of assessing the signals generated by standards and biological samples.

A major concern using this, as well as other reducing systems, is that they lead to the generation of NO not just from nitrite but also from other nitrogen-containing compounds, e.g. nitrosothiols and nitrosamines (Myers *et al.*, 1990). Consequently, a number of compounds and reagents used in our experiments were examined to determine if they could interfere with the measurement of NO production by cells through the generation of 'false' positive signals. These included nitrate, the major *in vivo* product of NO (Wennmalm *et al.*, 1992); L-arginine, the precursor of NO (Palmer *et al.*, 1988) and a number of L-arginine analogues that inhibit NOS, L-NOARG and L-NMMA (Rees *et al.*, 1990b; Moore *et al.*, 1990). In fact, none of these compounds were found to produce a chemiluminescence signal using this system.

The media used for experimentation were also examined. Bicarbonate- and HEPES-buffered Krebs solutions, similarly produced no signals using this system. However, the culture media and some of the constituents added to culture media did produce chemiluminescence signals. Signals were produced by Dulbecco's Modified Eagles Medium (DMEM), HEPES-buffered DMEM and new born serum (NBS). Serum is known to contain nitrite at 2 - 5 μM and this probably accounts entirely for the signals produced by NBS. In our samples, 100 μl NBS produced a signal of 220 ± 33 ppb which is equivalent to ~250 pmol (i.e. 2.5 μM). However, neither type of DMEM contains nitrite and so another constituent must be responsible for producing the chemiluminescence signals. We were unable to identify the agents responsible but a full list of constituents is shown in table 4.2. These signals due to DMEM, although of unknown origin, and NBS, from its nitrite content, were taken as background and subtracted from those of biological samples to obtain corrected levels of nitrite.

One disadvantage of this system over the Griess reaction was that measuring each sample took approximately 10 min, thus making the measurement of large sample numbers a long process. Thus, only 30 - 40 samples could be assayed conveniently each day compared to 200 using the automated plate reader with the Griess reaction. Also, the reducing solution lasted approximately 2 days which sometimes made it difficult to measure all the samples from a single experiment using the same batch.

Vanadium chloride/hydrochloric acid reflux system

The third chemiluminescence technique we investigated was the vanadium chloride/hydrochloric acid reflux system which is reported to reduce both nitrite and nitrate to NO (Braman & Hendrix, 1989; Bush *et al.*, 1992). Again, like the sodium iodide glacial acetic acid system, reflux at high temperature (95°C)

was required. This system had also been well established before we began our study (Braman & Hendrix, 1989; Bush *et al.*, 1992) and had been used successfully to measure the nitrite/nitrate content of human blood serum and urine (Braman & Hendrix, 1989) as well as to detect NO produced by rat cerebellum (Bush *et al.*, 1992).

We found, in common with the sodium iodide/glacial acetic acid system, that the vanadium chloride/hydrochloric acid reflux system also produced fast (1 - 2 min to peak) signals which were easily distinguishable from the background noise. The system, in fact, could not distinguish between nitrite or nitrate and similar amounts of these produced identical signals. The assay could measure nitrite and/or nitrate levels as low as 200 pmol making it more sensitive than the Griess reaction but not as sensitive as the sodium iodide/glacial acetic acid reflux system. Other papers report a higher sensitivity of around 100 pmol (Braman & Hendrix, 1989; Bush *et al.*, 1992) but again this may be dependent on the nature of the individual detectors used. The ability to measure both metabolites of NO should potentially make this system more efficient than the sodium iodide/glacial acetic acid reflux system. When the amount of nitrite and/or nitrate (200 - 1000 pmol) was plotted against the peak signals obtained the resulting relationship although roughly proportional was not strictly linear. Thus, as with the sodium iodide/glacial acetic acid reflux system, this was likely to be due to large sample volumes lowering the temperature and thereby slowing the rate of reduction to NO. On the other hand, when the amount of nitrite and/or nitrate (200 - 1000 pmol) was plotted against the area under the curve of the signals the resulting line was linear.

Although on a theoretical basis this system should be more appropriate for the measurement of total oxides of nitrogen formed from NO, it suffered from a major drawback. Specifically, a number of compounds and reagents used in experiments, i.e. L-arginine, the endogenous substrate for NOS (Palmer *et al.*,

1988), and L-arginine analogues, which inhibit NOS, L-NOARG and L-NMMA (Moore *et al.*, 1990; Rees *et al.*, 1990b), produced large chemiluminescence signals. These signals may have been due to the vanadium chloride/hydrochloric acid reflux reducing L-arginine and the L-arginine analogues with the consequent release of NO from these compounds. Alternatively, the reducing conditions may have generated other substances from these compounds which reacted with ozone to produce chemiluminescence signals. Whatever their origin, these large signals produced by L-arginine and the L-arginine analogues severely limited the type of experimental sample which could be measured using this system.

The media in which experimentation was conducted were also examined. Bicarbonate-buffered Krebs solution produced no signal in this system. Unlike Krebs, however, culture media and some of the its constituents produced signals using this system. Signals were produced by DMEM, HEPES-buffered DMEM, foetal calf serum (FCS) and NBS. Each type of DMEM contained both nitrate (7400 pmol) and L-arginine (40000 pmol), thus we would expect larger signals if all the nitrate and L-arginine was reduced to NO but this does not seem to be the case. The possibility that other constituents of the culture media also contributed to the signal was not investigated. FCS and NBS both contain nitrite (2 - 5 μM) which should give a signal of 400 - 700 ppb but the signal generated is much larger so some other component of FCS and NBS must be producing a false signal. The signals due to DMEM, FCS and NBS were so large, in fact, that the smaller levels of nitrite and/or nitrate generated by cells were extremely difficult to discern. These difficulties were minimised, however, by ensuring that standards of nitrite and nitrate were made up in exactly the same medium as used for experimentation.

In common with the sodium iodide/glacial acetic acid reflux system, it took approximately 10 min to assay each sample, thus making the measurement of

large sample numbers a long process. Also, the reducing solution lasted approximately 2 days, again often making it difficult to assay all samples from a single large experiment using the same batch.

Fluorometric detection

The fluorometric detection system for nitrite was described by Damiani & Burini (1986). Misko *et al.* (1993) were the first to introduce this assay in the study of NO produced by cells. Specifically, this group used the technique successfully to examine inhibition of NO production by aminoguanidine, a selective iNOS inhibitor, in macrophages.

The fluorometric detection system is based on the reaction of nitrite with 2,3-diaminonaphthalene (DAN) in acidic solution to form 1-[H]-naphthotriazone, a highly fluorescent compound when placed in alkaline conditions (Damiani & Burini, 1986; Misko *et al.*, 1993). This system could, like the Griess reaction and the chemiluminescence system with sodium iodide/glacial acetic acid, measure nitrite but not nitrate. In our experiments, the relationship between the amount of nitrite in standards and the intensity of fluorescence measured at 405 nm when excited at 365 nm was linear. The threshold sensitivity was approximately 120 pmol. Damiani & Burini (1986) reported a similar threshold sensitivity of 150 pmol. This system was thus more sensitive than the Griess reaction and the chemiluminescence detection system using vanadium chloride/hydrochloric acid but of an equal sensitivity to the chemiluminescence system using sodium iodide/glacial acetic acid.

This system too was not without limitations. For example, the 1-[H]-naphthotriazone produced is only stable for approximately 10 min, rendering this system inappropriate for measuring large numbers of samples.

Also, the turbidity of samples interfered with the measurements thus samples required firstly to be microcentrifuged quickly before assay.

Damiani & Burini (1986) have reported that interference in the measurement of nitrite can occur in the presence of cations such as calcium (16 μM), magnesium (0.03 μM) and iron (0.06 μM) (Damiani & Burini, 1986). Certain anions, such as chlorine (1 mM) and sulphate (660 μM), were also reported to interfere but higher concentrations were required except for sulphide (0.3 μM) which causes interference at relatively low concentrations (Damiani & Burini, 1986). Bicarbonate (16 μM) also interferes. In our system certain ions were present in the Krebs solution including chlorine as NaCl (118 mM), KCl (4.8 mM) and $CaCl_2$ (2.4 mM); calcium as $CaCl_2$ (2.4 mM); sulphate as $MgSO_4$ (1.2 mM); magnesium as $MgSO_4$ (1.2 mM); and bicarbonate as $NaHCO_3$ (2.4 mM). These ions would cause high background readings thus rendering this system hard to employ to measure small changes in nitrite concentrations, but to minimise these difficulties standards of nitrite were made up in exactly the same medium as used for experimentation.

Cyclic GMP detection

The cyclic GMP assay unlike the previous assays described does not measure NO or the breakdown products of NO, i.e. nitrite and nitrate, but measures the content of the second messenger system employed by the mediator (Rapoport & Murad, 1983). This assay is well characterised and has been used by many to demonstrate the activity of NO (Beasley *et al.*, 1991; Geller *et al.*, 1993b). It has also been used to assess the activity of iNOS in a number of cell types such as hepatocytes (Geller *et al.*, 1993b) and rat vascular smooth muscle cells (Beasley *et al.*, 1991) following activation by endotoxin and cytokines.

The basis of this assay involves measuring cyclic GMP using a radioimmunoassay (RIA) where radioactive and non-radioactive antigen compete for a fixed number of binding sites. In our hands, the cyclic GMP assay could readily detect 1 fmol cyclic GMP and this agreed with other reports (Harper & Brooker, 1975). This assay is highly specific for cyclic GMP but it is important to bear in mind that it is not only NO that can activate soluble guanylate cyclase; arachidonic acid is reported to activate this enzyme (Ignarro & Wood, 1987) and so stimulate increases in cyclic GMP content. Furthermore, atrial natriuretic factor (Rapoport, 1986) can activate particulate guanylate cyclase leading to elevation of the second messenger. Consequently, this system should not be used on its own as an index of NO activity. When used, however, in conjunction with another measuring system such as the Griess reaction or chemiluminescence it should provide valuable supportive information.

Relaxation of blood vessels

Relaxation of blood vessels can also be used as a detection system for NO. Such assay systems have been used enormously where NO activity or production of nitrite or nitrite/nitrate were too small to be measured. For example, in the first ever report on EDRF Furchgott and Zawadzki (1980) showed that placing an endothelium-containing strip of rabbit aorta close to an endothelium-denuded strip led to transfer of relaxation activity. Thus, EDRF was shown to be a humoral factor. Numerous cascade bioassay systems in which NO released from endothelium-containing rings (Griffith *et al.*, 1984) or endothelial cells grown on microcarrier beads was assayed on endothelium-denuded vessels have also been described. In addition, work on endothelium-containing and -denuded blood vessels from many species has demonstrated the existence of basal production of NO. Thus, endothelium-containing vessels are often less sensitive to vasoconstrictor agents than their endothelium-

denuded counterpart because of the tonic vasodilator action of basal NO (Allan *et al.*, 1993; Egleme *et al.*, 1984). A variant of this last assay has been used successfully to examine the expression of iNOS in blood vessels. Thus, following overnight incubation in Krebs solution contaminated with bacterial endotoxin (lipopolysaccharide, LPS) a depression of PE-induced tone and relaxation to L-arginine in endothelium-denuded rings, indicative of expression of iNOS was seen (Rees *et al.*, 1990a).

In this study we used the depression of PE-induced contractions of endothelium-denuded rings of rat aorta which occurs after overnight incubation (Rees *et al.*, 1990a) as a means of indirectly assessing expression of iNOS. By using, L-NAME, a known inhibitor of NOS we were able to determine with certainty if this depression of PE-induced contraction resulted from NO production following expression of iNOS. This system therefore can be used to determine if NO is being produced but it is not able to be used to quantify production. Its utility is therefore limited to situations where it might be impossible to measure nitrite.

Comparison of measuring systems tested

All of the measuring systems we examined had advantages and limitations. The Griess reaction was perhaps the simplest and quickest system to use since it permitted assay of hundreds of samples per day especially when the automated plate reader was employed. Furthermore, the samples once mixed with the Griess reagents were stable for at least 24 h so the assay could be read at any point during this time. The chemiluminescence systems were more sensitive but each sample took roughly 10 min to read, thus making these systems inefficient when large numbers of samples were to be measured. Also, the reflux solutions were stable for only 1 - 2 days. The theoretical advantage of measuring both products of NO (nitrite and nitrate) using the

vanadium chloride/hydrochloric acid reflux system was not realised. In practice, the large chemiluminescence signals generated from L-arginine and its analogues together with the constituents of the culture media employed, prevented the use of this system in most experimental situations. The fluorometric system was more sensitive than the Griess reaction and less so than the chemiluminescence techniques, but samples were only stable for 10 min or so, again making it awkward to employ when large numbers of samples were to be measured. As will be seen later in the Discussion, relaxation of a segment of blood vessel provided a good physical sign of NO production from iNOS. It was thus, useful for tissue work where levels of nitrite/nitrate were below detection limits for even our most sensitive chemiluminescence assays. As will also be seen, the measurement of cyclic GMP was good as a secondary detection system to show that NO was indeed active, but it provided no direct quantitative information on how much was produced. Fortunately, in many of our experiments the added sensitivity of the chemiluminescence detection systems were not required so it was prudent to routinely adopt the fast and reliable Griess reaction as the assay of choice.

Comparison with other measuring systems

A number of systems other than those described above are available for the measurement of NO or activity of the L-arginine/NO pathway. These include the L-citrulline assay (Rees *et al.*, 1990b; Pollock *et al.*, 1991), methaemoglobin spectrophotometry (Kelm & Schrader, 1988; Ignarro *et al.*, 1987; Kelm *et al.*, 1988), electron paramagnetic resonance (EPR) (Arroyo & Kohno, 1991; Maples *et al.*, 1991), gas chromatography (Pai *et al.*, 1987), mass spectroscopy (Gustafsson *et al.*, 1991) and various NO-sensitive microelectrodes (Shibuki, 1990; Malinski & Taha, 1992; Ichimori *et al.*, 1994).

The L-citrulline assay in fact measures [^{3}H]-L-citrulline formation, i.e. the by-product of NO production from [^{3}H]-L-arginine (Hibbs *et al.*, 1987). This does not give a direct measurement of NO but has been used extensively to examine NOS activity in a large number of different cells and tissues (Rees *et al.*, 1990b; Pollock *et al.*, 1991; Bush *et al.*, 1992; Yu, 1994).

Methaemoglobin detection is based on the rapid oxidation of reduced haemoglobin to methaemoglobin by NO (Kelm *et al.*, 1988; Kelm & Schrader, 1988; Ignarro *et al.*, 1987). This is a highly sensitive technique, having a threshold sensitivity of around 1 nM and is thus as powerful as the most sensitive of the chemiluminescence techniques. Its other major advantage is that it measures NO and not one of its breakdown products. Methaemoglobin spectrophotometry has in fact been used by Kelm & Schrader (1988) to support the view of Palmer *et al.* (1987) that NO is produced in amounts sufficient to account for the actions of EDRF.

NO is a paramagnetic molecule with an unpaired electron. Thus, application of a magnetic field of an appropriate strength will promote this electron to a higher energy level, and relaxation from this state will produce a characteristic spectrum (Maples *et al.*, 1991). In view of the unstable nature of NO, EPR detection of NO *per se* has not been applied to biological experiments. When bound to haemoglobin, however, the complex formed is quite stable and retains paramagnetic properties. Consequently, detection of the haemoglobin-NO complex by EPR has been applied successfully in a number of biological situations. Specifically, EPR has been successfully used to demonstrate the expression of iNOS in macrophages upon activation with IFN-γ, and this was inhibited in the presence of L-NMMA (Henry *et al.*, 1991). Interestingly, Rubanyi *et al.* (1991) reported using EPR that EDRF was probably a S-nitrosothiol rather than native NO. This was based on their finding that

authentic NO gave a paramagnetic signal when combined with haemoglobin but EDRF did not.

Gas chromatography using Varian gas chromatography with a Parapak Q column and electron capture was less sensitive than chemiluminescence (Pai *et al.*, 1987). In this study the production of NO from nitrite was examined using nitrite reductase purified from *Achomobacter cycloclastes* (Pai *et al.*, 1987).

Mass spectroscopy has been used to detect NO in the breath of rabbits (Gustafsson *et al.*, 1991) and was also used by Palmer in conjunction with a cascade bioassay and chemiluminescence to show that NO was derived from the precursor, L-arginine (Palmer *et al.*, 1988). The detection limit for mass spectroscopy is 9 pmol (Gustafsson *et al.*, 1991) and thus more sensitive than chemiluminescence.

Two different microelectrode detection systems for NO have been described. The first was able to detect nanomolar levels of NO by measuring the oxidation of NO at the electrode tip (Shibuki, 1990; Ichimori *et al.*, 1994). This system is highly specific for NO, with nitrite and nitrate producing no interference. The inhibitor of NOS, L-NAME, however, causes a significant non-specific current when used in conjunction with this form of microelectrode (Ichimori *et al.*, 1994) thus reducing its utility. This system has, however, been employed to measure acetylcholine (1 μM)-induced release of NO (30 nM) from rat aortic rings, which was inhibited by L-NMMA (50 μM)(Ichimori *et al.*, 1994), and on release of NO (28 nM) from rat cerebellum following electrical stimulation (Shibuki, 1990). The underlying principle on which the second type of microelectrode is based is the metalloporphyrin-catalysed oxidation of NO. This technique is even more sensitive, measuring NO at concentrations of 10^{-20} M and above (Malinski & Taha, 1992). This is therefore the most

powerful of all the techniques yet described and can be used to detect NO (450 nM) release from individual endothelial cells exposed to bradykinin (2 nmol). A major advantage of both types of microelectrode is that they detect NO *per se* and not one of its breakdown products. Furthermore, their use can give an insight into the kinetics of NO production in real time (Malinski & Taha, 1992).

Having established the sensitivity of a number of different measuring systems we then went on to examine their utility to measure the activity of the L-arginine/NO system in a number of different tissues and cell preparations. Those studied were J744.2 murine macrophages, BAEC, endothelium-denuded rings of rat aorta and RASMC in culture.

Expression of iNOS in J774.2 murine macrophages

In resting macrophages, NOS is not normally present but the inducible form can be expressed following stimulation with LPS and cytokines, such as interferon-γ (IFN-γ) (Stuehr & Marletta, 1987a; Stuehr & Marletta, 1987b). In our experiments we used J774.2 murine macrophages, and when 10^6 unstimulated cells were incubated in 1 ml of tissue culture medium, accumulation of nitrite into the medium reached 5.3 ± 0.3 nmol, which was easily measured by the Griess reaction. Others have also successfully used the Griess reaction to measure nitrite production by macrophages (Hibbs *et al.*, 1987; Stuehr & Marletta, 1987a; Stuehr & Marletta, 1987b). For comparison, we also measured basal nitrite production using chemiluminescence with the sodium iodide/glacial acetic acid reflux system and found a similar resting level of nitrite (4.5 ± 0.5 nmol). Furthermore, when we measured combined nitrite and nitrate production using the vanadium chloride/hydrochloric acid reflux system we found a basal level of 5.3 ± 4.7 nmol. These data, therefore, suggest that the major metabolite of NO formed

by resting cells is nitrite with only very small levels of nitrate. Such findings are in keeping with the literature which suggests that when NO reacts with O_2 in aqueous solution *in vitro*, nitrite is the major product formed (Ignarro *et al.*, 1993; Wennmalm *et al.*, 1992). Nitrate, would only be expected if NO was destroyed by superoxide anion (Beckman *et al.*, 1990) or if it reacted with haemoglobin (Wennmalm *et al.*, 1992). Consequently, nitrate has been found to be the major product of NO present in plasma *in vivo* (Wennmalm *et al.*, 1992).

When the macrophages were stimulated with IFN-γ, nitrite and combined nitrite/nitrate accumulation during the overnight incubation increased significantly in a concentration-dependent manner. Again the Griess reaction, the sodium iodide/glacial acetic acid reflux system and the vanadium chloride/hydrochloric acid reflux system produced similar results for maximal nitrite or nitrite/nitrate production by 100 u ml^{-1} IFN-γ (44.4 ± 0.6 nmol, 54.5 ± 3.5 nmol and 52.5 ± 7.3 nmol, respectively). The data suggested that, as with resting cells, the major metabolite of NO produced by activated cells was nitrite. Thus, as the major metabolite of both unstimulated and stimulated cells was nitrite, all further experiments were performed using the Griess reaction or the sodium iodide/glacial acetic acid reflux system only. A second reason for not using the vanadium chloride/hydrochloric acid system was that it resulted in the production of high background readings when DMEM, FCS, NBS, L-arginine and L-arginine analogues were present, thus making precise measurement of cellular nitrite/nitrate production difficult. The ability of IFN-γ to increase nitrite accumulation in our experiments is thus consistent with the stimulated expression of iNOS in these cells, as has previously been reported for other macrophage cell lines (Stuehr & Marletta, 1987a).

LPS from *Salmonella typhosa*, like IFN-γ, stimulated a concentration-dependent increase in nitrite production by J774.2 murine macrophages

during an overnight incubation. The maximum increase at 1000 ng ml^{-1} was 3-fold. These data are therefore in agreement with a previous report by Di Rosa *et al.* (1990). Moreover, when LPS and IFN-γ were used in combination we found, as did Stuehr and Marletta (1987a), that these stimuli acted in a synergistic manner to increase nitrite production.

Thus, in J774.2 murine macrophages the production of NO could be assessed by measuring its major stable product, nitrite, using either the Griess reaction or chemiluminescence coupled with the sodium iodide/glacial acetic acid reducing system under reflux. No added benefit was derived from the combined measurement of nitrite and nitrate using the vanadium chloride/hydrochloric acid reflux system as the main metabolite of NO produced by J774.2 murine macrophages was nitrite. By far the most convenient way to measure accumulation of nitrite into the medium bathing cells was using the Griess reaction since large numbers of samples could be measured simultaneously. Chemiluminescence, although just as accurate and reliable was extremely slow by comparison. Having established that the Griess reaction was the most appropriate system for measuring NO production by J774.2 murine macrophages which produce large quantities of nitrite we then wished to examine if we could measure NO production after expression of iNOS in BAEC using the more sensitive chemiluminescence detection systems.

Attempted expression of iNOS in BAEC

It is well established that NOS is constitutively present in endothelial cells and is responsible for production of NO, the EDRF (Mülsch & Busse, 1991). It has been reported, as for other cells such as macrophages described above, that iNOS can also be expressed in endothelial cells from porcine aorta following stimulation with LPS and IFN-γ (Radomski *et al.*, 1990b). Also, expression of

iNOS has been shown in murine brain microvascular endothelial cells after stimulation with the cytokines, TNF-α and IFN-γ (Gross *et al.*, 1991). Thus, it appeared that vascular endothelial cells could be made to express both cNOS and iNOS.

As outlined in the next section, there is growing evidence that the hypotension of septic shock is due to massive production in the blood vessels of NO following expression of iNOS (Parker & Adams, 1993). Associated with this hypotension is a profound increase in vascular permeability, but the role of NO and iNOS in this action had not been investigated when our study was underway. Such a role was suggested, however, by reports showing that the cytokines, interleukin-1 and interleukin-2 (IL-1 and IL-2), which promote expression of iNOS, also stimulate massive increases in vascular permeability (Campbell *et al.*, 1992). I therefore undertook a joint project with other members of the laboratory to investigate the role of iNOS in the increased vascular leakage produced by LPS.

We found that overnight incubation with LPS from *Salmonella typhosa* stimulated a massive increase in albumin transfer across monolayers of BAEC grown on polycarbonate membranes (Berman & Martin, 1993a; Berman & Martin, 1993b; Berman *et al.*, 1993). This increase in vascular leakage did not, however, appear to involve the L-arginine/NO pathway, because L-NMMA and L-NOARG, inhibitors of NOS, failed to inhibit the increase in endothelial permeability (Berman *et al.*, 1993)

Although it seemed apparent that the increase in endothelial permeability produced by LPS did not appear to involve NO, it was still important to establish if concomitant iNOS expression had occurred. Using chemiluminescence coupled with sodium iodide/glacial acetic acid reflux, nitrite (27.8 $\pm$ 4 nmol 10^6 cells^{-1}) was found to accumulate into the medium

bathing unstimulated cells during an overnight incubation and when combined nitrite/nitrate was measured using vanadium chloride/hydrochloric acid reflux there was no significant difference in the measurements (31.2 ± 4 nmol.10^6 cells^{-1}). We found that the presence of LPS in the bathing medium failed to increase the accumulation of nitrite or nitrite/nitrate suggesting that iNOS expression had not taken place. There may be a species difference between bovine and porcine aortic endothelial cells.

Basal production of nitrite by BAEC (27.8 ± 4 nmol) was greater than the basal production by J774.2 macrophages (5.3 ± 0.3 nmol) for the same number of cells. Surprisingly, we could further conclude that the basal production of nitrite by BAEC was not derived from the L-arginine/NO pathway as the inhibitors of NOS, L-NAME and L-NMMA (Moore *et al.*, 1990; Rees *et al.*, 1990b), had no effect when present during the overnight incubation. The biochemical pathway by which basal nitrite production took place was therefore unknown. The inhibitor of expression of iNOS, dexamethasone (Di Rosa *et al.*, 1990; Knowles *et al.*, 1990b; Rees *et al.*, 1990a), similarly had no effect on basal accumulation of nitrite.

On the basis of these findings it was likely that expression of iNOS was not responsible for the increased permeability of the endothelium associated with endotoxic shock.

Role of iNOS in septic shock

Septic shock is a devastating systemic inflammatory response which results in 60 - 90% mortality (Hess *et al.*, 1981). A number of different stimuli cause septic shock including Gram-negative bacteraemia, Gram-positive bacteraemia, fungi, viruses and parasites (Bone *et al.*, 1987; Ispahani *et al.*, 1987; Calandra *et al.*, 1988; Glauser *et al.*, 1991). In this study we examined

the effects of LPS, the active component of Gram-negative bacterial cell walls (Glauser *et al.*, 1991; Rietschel *et al.*, 1993).

Exposure to LPS has led to the expression of iNOS in a number of different cells in culture including macrophages (Di Rosa *et al.*, 1990) and rat vascular smooth muscle cells (Marczin *et al.*, 1993; Szabó *et al.*, 1993a). Expression of iNOS has also been demonstrated *in vivo* in rats, mice and dogs treated with LPS (Kilbourn *et al.*, 1990b; Thiemermann & Vane, 1990; Kosaka *et al.*, 1992). The increased production of NO results in hypotension and this can be reversed by inhibitors of NOS, e.g. L-NMMA (Kilbourn *et al.*, 1990b; Thiemermann & Vane, 1990; Meyer *et al.*, 1992). However, L-NMMA has its disadvantages as it also inhibits the actions not only of iNOS but of cNOS, thus preventing endothelial control of blood vessel tone, resulting in intense vasoconstriction and increased vascular resistance which adversely affects survival (Nava *et al.*, 1992; Wright *et al.*, 1992). Some of these detrimental effects of L-NMMA can be prevented by co-administration of the NO donor S-nitroso-N-acetylpenicillamine (Nava *et al.*, 1992; Wright *et al.*, 1992). Selective inhibitors of iNOS might also be advantageous in the treatment of septic shock since they would be unlikely to interfere with the normal endothelial function. Certain selective inhibitors of iNOS have been described such as L-canavanine (Misko *et al.*, 1993) and aminoguanidines (Hasan *et al.*, 1993; Misko *et al.*, 1993) but these have not yet been evaluated for the treatment of septic shock.

The effects of inhibitors of NOS have been tested on man. L-NMMA and L-NAME have been given to patients suffering from septic shock, and both inhibitors produced a dose-dependent increase in blood pressure and peripheral resistance (Petros *et al.*, 1991). In a larger clinical trial L-NMMA produced similar results but was also noted to cause a fall in cardiac output

(Petros *et al.,* 1994). Thus, again selective inhibitors of iNOS require evaluation.

In experimental animals, as already stated, LPS induces septic shock-like symptoms (Kilbourn *et al.,* 1990b; Thiemermann & Vane, 1990; Gray *et al.,* 1991; Kosaka *et al.,* 1992), however, other compounds have also been shown to produce similar effects. These include cytokines, TNF-α, IFN-γ, IL-1 and IL-2(Kilbourn *et al.,* 1990a; Hibbs *et al.,* 1992; Ochoa *et al.,* 1992; Szabó *et al.,* 1993c). As with LPS, certain of the septic shock-like symptoms induced by these cytokines can be reversed by administration of L-NMMA or L-NAME (Kilbourn *et al.,* 1990a).

The hypotension of septic shock is also associated with a reduction in the pressor response to vasoconstrictor agents (Parratt, 1973; Teale & Atkinson, 1992). Depression of contraction to a number of different pressor agents, including noradrenaline (Parratt, 1973), vasopressin (Schaller *et al.,* 1985), angiotensin II (Schaller *et al.,* 1985) and serotonin (Wakabayashi *et al.,* 1987) can still be observed in blood vessels from treated animals *ex vivo.* Furthermore, in order to examine the mechanism underlying hyporesponsiveness, many groups have examined the reactivity of vascular rings taken from animals treated with LPS. This depression of contraction has been shown to be reversed by the inhibitor of NOS, L-NMMA (Julou-Schaeffer *et al.,* 1990). Hyporeactivity to vasoconstrictors can even be produced following overnight incubation of rings of rat aorta in Krebs solution contaminated with LPS (Rees *et al.,* 1990a). This hyporeactivity is blocked by inhibitors of NOS, L-NMMA (Busse & Mülsch, 1990), and by inhibitors of expression of iNOS, e.g. glucocorticoids (Rees *et al.,* 1990a), suggesting that it is due to expression of iNOS. Agents other than LPS, including IFN-γ, TNF-α, IL-1 and IL-2, have also been shown to produce a similar hyporeactivity to vasoconstrictors (Busse & Mülsch, 1990). Again, this

hyporeactivity was reversed by inhibitors of NOS, L-NAME and L-NMMA (Busse & Mülsch, 1990) suggesting that it resulted from the production of NO by iNOS in the vascular wall.

The data described above provide powerful evidence that the hyporeactivity of septic shock is due to the over production of NO in the vascular wall. Although expression of iNOS could have taken place in many different cell types in the vessel wall, most attention has been focused on the smooth muscle cells. This is principally because expression of iNOS is readily achieved in smooth muscle cells cultured from blood vessels such as rat aorta (Busse & Mülsch, 1990; Fleming *et al.*, 1991). For example, treatment of vascular smooth muscle cells with IL-1, TNF-α, IFN-γ and LPS for several hours has led to the production of nitrite, indicative of expression of iNOS (Busse & Mülsch, 1990; Beasley *et al.*, 1991; Kanno *et al.*, 1993; Koide *et al.*, 1994). In cultured smooth muscle cells there is debate as to whether LPS alone can stimulate expression of iNOS. Marczin *et al.* (1993) and Szabó *et al.* (1993a) report that LPS can stimulate expression of iNOS but Sirsjö *et al.* (1994) dispute this and propose that the additional presence of a cytokine is required. We therefore wished to examine the factors which lead to expression of iNOS in the vascular wall of rat aorta as well as in smooth muscle cells cultured from it.

Expression of iNOS in endothelium-denuded rings of rat aorta

In order to investigate the factors regulating expression of iNOS in the intact blood vessel, rat isolated aortic rings were incubated overnight in Krebs solution as previously described by Rees *et al.* (1990a). All rings were denuded of endothelium to avoid any effects of cNOS. Our experiments showed a clear depression of the contractile actions of PE following incubation in Krebs solution at 37°C for 18 h in support of the earlier report (Rees *et al.*, 1990a). Also consistent with earlier reports was our finding that a major

component of this hyporeactivity was abolished following blockade either of the synthesis of NO with L-NAME (Fleming *et al.*, 1991a), or its actions with methylene blue (Fleming *et al.*, 1993). We also found that haemoglobin, which binds and inactivates NO (Gibson & Roughton, 1957; Martin *et al.*, 1985), partially reversed this hyporeactivity. Surprisingly, although nitrite accumulation was detected by chemiluminescence in the Krebs used to incubate the rings, this was completely unaffected by the presence of L-NAME throughout the incubation. The source of this nitrite, like that produced by BAEC, therefore appeared not to be the L-arginine/NO pathway. Thus, production of NO appeared to be the cause of the depression of contraction in incubated rings but no evidence of enhanced production of NO could be obtained by measurement of its major metabolite nitrite.

Actions of polymixin B

Our finding that polymixin B, which binds and inactivates bacterial LPS (Lasfargues *et al.*, 1989), inhibited the development of hyporeactivity to PE supported the previous conclusion that it resulted from the expression of iNOS in the vascular wall stimulated by endotoxin contaminating Krebs solution (Rees *et al.*, 1990a). The levels of LPS found to be contaminating the Krebs solution have been reported to be 9.1 - 18.2 ng ml^{-1} (Rees *et al.*, 1990a). We found that addition of further LPS failed to enhance the degree of depression of PE-induced contraction obtained suggesting that the levels already present in the Krebs solution were maximal for expression of iNOS. Interestingly, although L-NAME and polymixin B each only partially reversed the depression of PE-induced contraction following incubation in Krebs solution, the combined effect of these two treatments was no greater than with either alone. This would suggest that the hyporesponsiveness seen consisted of two components; a major component through expression of iNOS and a lesser

component whose origin is unknown, but may simply represent a decline in tissue integrity following prolonged incubation.

Actions of dexamethasone

In contrast to an earlier report (Rees *et al.*, 1990a), we found that dexamethasone did not inhibit the depression of PE-induced contraction if it was present throughout the overnight incubation. Previous work in our laboratory did, however, find that dexamethasone inhibited the lower degree of depression of PE-induced contraction seen following incubation of aortic rings for 6 - 10 hours (personal communication from S. Eason and K. Mian). The reason for this difference is not clear but we speculate that perhaps our tissues may have been exposed to higher concentrations of endotoxin than those of Rees *et al.* (1990a) and that dexamethasone was unable to oppose this more powerful stimulus.

Actions of superoxide anion

To further determine if NO was being produced and to examine if the expression of iNOS was occurring in the vascular smooth muscle cells or another cell type in the cell wall, we examined the effects of superoxide anion generating systems.

It is well established that superoxide anion destroys the relaxant activity of NO (Gryglewski *et al.*, 1986). Interestingly, we found that the depression of PE-induced contraction following overnight incubation was inhibited in a concentration-dependent manner by LY 83583 (0.1 - 1 μM), which generates superoxide anion both intracellularly and extracellularly (Mülsch *et al.*, 1988; Mülsch *et al.*, 1989), but not by hypoxanthine/xanthine oxidase (McCord & Fridovich, 1968), which produces the free radical only extracellularly. These

actions were thus consistent with NO being produced and then acting within the same smooth muscle cell. Also consistent with this conclusion was the inability of superoxide dismutase, which inactivates superoxide anion, to enhance the depression of PE-induced contraction or to reverse the effects of LY 83583, since in view of its large size, superoxide dismutase can only act extracellularly. Interestingly, a concentration of 3 μM LY 83583 produced a smaller degree of reversal of the depression of PE-induced contraction than 1 μM. The possibility was considered that this paradoxical action may have arisen due to the production of the relaxant, hydrogen peroxide from the superoxide anion (Dowell *et al.,* 1993). However, when catalase, which inactivates hydrogen peroxide, was added it had no effect on the magnitude of the reversal induced by 3 μM LY 83583. Thus, in view of the inability of catalase to enter cells we can conclude that reduced ability of LY 83583 at 3 μM to reverse the depression of PE-induced contraction was not due to the extracellular accumulation of hydrogen peroxide. Whether or not this action results from the intracellular accumulation of hydrogen peroxide or some other unrelated action remains to be determined.

Expression of iNOS in RASMC in culture

In contrast to the agreed actions on isolated vascular rings, there is controversy regarding the ability of LPS alone to stimulate expression of iNOS in vascular smooth muscle cells in culture. Certain groups have found that LPS alone is a sufficient stimulus (Marczin *et al.,* 1993; Szabó *et al.,* 1993a; Beasley & Eldridge, 1994; Donald & Struthers, 1995) for expression of iNOS in RASMC in culture. Others disagree, reporting that LPS alone has no effect on nitrite production or expression of iNOS mRNA and that the additional presence of a cytokine such as IFN-γ is required (Sirsjö *et al.,* 1994). Similar results to Sirsjö *et al.* (1994) were reported for rat pulmonary artery smooth muscle cells in culture (Nakayama *et al.,* 1992). There is general agreement,

however, that certain cytokines including IL-1 and TNF-α reliably stimulate expression of iNOS in RASMC in culture (Junquero *et al.*, 1992; Beasley & Eldridge, 1994; Perrella *et al.*, 1994). On the basis of these conflicting reports on the actions of LPS, we felt that it was important to evaluate the actions of this agent, as well as those of cytokines on RASMC in culture.

Expression of iNOS in RASMC in full culture medium

Our initial experiments were conducted in full culture medium containing serum but we were unable to express iNOS in RASMC, as assessed by increases in nitrite production measured using the Griess reaction, using either LPS, IFN-γ or IL-1α alone over wide concentration ranges. Combinations of IFN-γ and LPS or IL-1α and LPS also failed to increase nitrite accumulation, however, the combination of IL-1α (40 u ml^{-1}) and IFN-γ (100 u ml^{-1}) did increase nitrite accumulation by 2-fold. At about the time this work was being conducted reports came to light which suggested that serum could inhibit the expression of iNOS in RASMC (Junquero *et al.*, 1992; Schini *et al.*, 1992; Hirokawa *et al.*, 1994b). These papers went on to report that the presence of transforming growth factor-β and platelet-derived growth $factor_{AB}$ and $_{BB}$ in the serum were likely to account for the ability of serum to inhibit the expression of iNOS. It was therefore likely that the presence of serum in our initial experiments prevented the expression of iNOS except with the powerful combination of IFN-γ and IL-1α. Consequently, we conducted a series of experiments in serum-free culture medium and found that IFN-γ alone stimulated nitrite accumulation following an overnight incubation, consistent with expression of iNOS. We also found that NBS and FCS inhibited the increase in nitrite accumulation produced by IFN-γ suggesting that serum indeed had the ability to block expression of iNOS. Thus, all further experiments were conducted in serum-free culture medium.

Expression of iNOS in RASMC in serum-free culture medium

We found that bacterial LPS alone from either *Salmonella typhosa* or *Escherichia coli* failed to stimulate expression of iNOS activity in RASMC in culture, as assessed either as accumulation of nitrite in the culture medium or as an increase in smooth muscle cyclic GMP content, except at exceptionally high concentrations (300 and 1000 μg ml^{-1}). These high concentrations of LPS in fact appeared toxic to the cells as they induced detachment from the growth surface. They are also around 100000 times those required to express iNOS in isolated rings of rat aorta (Rees *et al.*, 1990) and 10000 times that required in J774.2 murine macrophages. In contrast, IFN-γ or IL-1α alone were able to produce concentration-dependent increases in the accumulation of nitrite and in cyclic GMP content of the cells, as previously reported (Busse & Mülsch, 1990; Beasley *et al.*, 1991; Kanno *et al.*, 1993; Koide *et al.*, 1994). Although, alone LPS, except at high concentrations produced no effect, low concentrations (100 ng ml^{-1}) could enhance the nitrite accumulation if a low, near threshold concentration of IFN-γ (10 u ml^{-1}) was used. In contrast, a low concentration of LPS (100 ng ml^{-1}) failed to augment the actions of a near-maximal concentration of IFN-γ (30 u ml^{-1}). It seemed therefore that the cells were able to respond to LPS but only in the presence of an additive stimulus. The time course of the expression of iNOS that we found when RASMC were stimulated with IFN-γ was in agreement with other reports (Beasley & Eldridge, 1994; Buttery *et al.*, 1994). We found that nitrite accumulation began to rise after 6 h and continued increasing until at least 72 h. Such a time course is consistent with the need for *de novo* protein synthesis.

Inhibition of the actions and expression of iNOS in RASMC

We concluded that the enhanced production of nitrite by RASMC stimulated by overnight incubation with IFN-γ was clearly due to expression of iNOS since it

was blocked following treatment with L-NMMA or L-NAME, which inhibit NOS (Moore *et al.*, 1990; Rees *et al.*, 1990b). Surprisingly, dexamethasone, which failed to block expression in rat aortic rings, inhibited the expression of iNOS in RASMC. Cycloheximide, which prevents *de novo* synthesis of proteins, also inhibited the production of nitrite by our RASMC, consistent with its reported ability to inhibit expression of iNOS (Di Rosa *et al.*, 1990; Knowles *et al.*, 1990b; Radomski *et al.*, 1990b; Rees *et al.*, 1990a). We also found that polymixin B, which binds and inactivates LPS (Lasfargues *et al.*, 1989), had no effect on the accumulation of nitrite stimulated by IFN-γ alone which suggested that the culture medium, unlike the Krebs solution used to bathe aortic rings, was not contaminated with bacterial LPS.

Other agents are known to inhibit expression of iNOS in other cell types, e.g. macrophages, and these include IL-4 and IL-10 (Oswald *et al.*, 1992; Liew *et al.*, 1991; Chesrown *et al.*, 1994) but the effects of these were not examined on RASMC in our study.

Comparison of RASMC in culture and in situ

Clearly, an explanation is required to account for the findings of this study and of others (Nakayama *et al.*, 1992; Sirsjö *et al.*, 1994) where LPS alone stimulated the expression of iNOS in an isolated blood vessel but not in smooth muscle cells in culture. Sirsjö *et al.* (1994) suggested that the differential effects of LPS could either be due to the presence of heterogeneous cell types in blood vessels which were absent in homogeneous populations of smooth muscle cells in culture or to differences in smooth muscle phenotype in the vessel wall and in culture, and both merit consideration. For example, one of the many cell types which normally reside in the vessel wall could potentially respond to LPS by secreting a cytokine which then acted either alone or in concert with LPS leading to expression of

iNOS in the smooth muscle layer. Of the cell types present, endothelial cells, although able to express iNOS in some species (Knowles *et al.*, 1990b; Radomski *et al.*, 1990b; Gross *et al.*, 1991), can probably be discounted since their complete removal has no effect on the magnitude of the depression of vasoconstriction observed following incubation of blood vessels for prolonged periods in LPS-containing Krebs solution (Rees *et al.*, 1990a). The presence of the endothelium may, however, enhance the sensitivity to LPS as well as the rate of onset of hyporeactivity (Fleming *et al.*, 1993). Monocyte/macrophages or lymphocytes which are normally present in the arterial wall (Joris *et al.*, 1979) appear more likely candidates since they are known to respond to endotoxin by secreting cytokines (Flad *et al.*, 1993). This possibility is further supported by the findings in animal models that shock-like symptoms can be induced by certain cytokines such as IL-1, IL-2 and TNF-α (Kilbourn *et al.*, 1990b; Gray *et al.*, 1991; Hibbs *et al.*, 1992; Ochoa *et al.*, 1992; Szabó *et al.*, 1993c), and that the hypotension induced following administration of LPS is inhibited by the receptor antagonist, interleukin-1_{RA} (Szabó *et al.*, 1993a), as well as by antibodies to IL-1 or TNF-α (Thiemermann *et al.*, 1993). Consequently, we examined the actions of antibodies to TNF-α, IL-1β and IFN-γ as well as the inhibitor of the production of TNF-α, pentoxifylline, on the depression of PE-induced contraction in rat aortic rings obtained following overnight incubation.

Inhibition of TNF-α

TNF-α has been shown to produce shock-like symptoms when injected into animals (Tracey *et al.*, 1987) and has also been shown to be released during shock induced by LPS (Mathison *et al.*, 1988). Inhibition of TNF-α during septic shock has been studied in two main ways. Firstly, an inhibitor of the production of TNF-α, pentoxifylline, has been shown to be an effective pre-treatment and inhibits increases in TNF-α in the plasma of rabbits and rats

(Mathison *et al.*, 1988; Matuschak *et al.*, 1994). Secondly, pre-treatment of rats with a monoclonal antibody to TNF-α has been found to prevent the fall in blood pressure induced by LPS (Thiemermann *et al.*, 1993). This latter pre-treatment also prevented the associated depression in contractile response to noradrenaline *ex vivo* (Thiemermann *et al.*, 1993).

In our experiments we incubated rings overnight in the presence of pentoxifylline. We found, however, that this had no effect on the depression of PE-induced contraction. Thus, although pentoxifylline may reduce plasma TNF-α levels following injection of LPS (Mathison *et al.*, 1988) this cytokine does not appear to be responsible for the depression of contractility seen following overnight incubation of rat aortic rings.

We also examined the effects of including a monoclonal antibody to TNF-α in the Krebs solution throughout the overnight incubation. As with pentoxifylline this had no effect on the depression of PE-induced contraction. Thus, we had no evidence that TNF-α was involved in the depression of contractility. A criticism that could be raised against our experiments, however, is that the antibody used was raised against mouse and not rat TNF-α. Antibodies to rat TNF-α are not, to our knowledge, available. It is therefore possible that the antibody would not cross react with and so would therefore not inhibit rat TNF-α. Others have found that antibodies to TNF-α are effective in protecting animals when used as a 2 h pre-treatment before the addition of LPS (Beutler *et al.*, 1985; Silva *et al.*, 1990; Thiemermann *et al.*, 1993). Silva *et al.* (1990) and Thiemermann *et al.* (1993) used an antibody to murine TNF-α raised in hamsters (TN3.19.12) and this was effective against murine and rat TNF-α. Beutler *et al.* (1985) raised antibodies to murine TNF-α in rabbits. An antibody to TNF-α has also been administered to human patients with sepsis syndrome (Abraham *et al.*, 1995). Here the antibody appeared to reduce mortality but this reduction was not statistically significant. However, these human

experiments, unlike those described above for animal experiments were conducted on patients with existing sepsis and not used as a pre-treatment. The effectiveness of the antibody may be under-estimated.

Inhibition of IFN-γ

IFN-γ is known to produce shock-like symptoms in animals (Heinzel, 1990; Silva & Cohen, 1992). Furthermore, a monoclonal antibody to murine IFN-γ raised in hamsters (H22) has been reported to produce significant protection from LPS-induced septic shock in mice (Evans *et al.*, 1992). These authors showed that it also decreased the expression of iNOS in hepatocytes in those animals (Evans *et al.*, 1992). In our experiments, however, inclusion of a monoclonal antibody to IFN-γ throughout the overnight incubation of rat aortic rings had no effect on the resulting depression of PE-induced contraction. Thus, we had no evidence that the depression of PE-induced contraction involved the release of IFN-γ, although, again, it must be acknowledged that an antibody raised against rat and not the mouse form of the cytokine would have been ideal.

Inhibition of IL-1β

Reports suggest that antagonists of IL-1 can reduce the mortality of endotoxin shock in rabbits, suggesting that this cytokine does play a role (Ohlsson *et al.*, 1990; Ohlsson *et al.*, 1990; Wakabayashi *et al.*, 1991). We therefore examined the effects of a monoclonal antibody to human IL-1β on the depression of PE-induced contraction following an overnight incubation of rat aortic rings. The antibody, despite being present throughout the overnight incubation, had no effect on the depression of PE-induced contraction. It did, however, significantly increase the ability of L-NAME to reverse the depression suggesting that IL-1β may be involved at least to a limited extent.

Overall, however, our combined results using pentoxifylline and a panel of antibodies to cytokines have thus failed to provide convincing evidence that LPS contaminating the Krebs solution leads to depression of contractility via expression of iNOS indirectly via release of a cytokine from a non-smooth muscle cell type in the vascular wall.

Phenotypic state of smooth muscle

The alternative proposal (Sirsjö *et al.*, 1994), i.e. that LPS may only be able to simulate expression of iNOS in smooth muscle in the contractile phenotype (Chamley-Campbell & Campbell, 1989) present in the arterial wall but not in the synthetic phenotype in culture has not been tested and requires further evaluation.

Induction of iNOS in vascular smooth muscle cells in culture by LPS

Another major question that remains to be resolved is why some groups (Marczin *et al.*, 1993; Szabó *et al.*, 1993c) find LPS alone to be sufficient to stimulate expression of iNOS in vascular smooth muscle cells in culture while others do not (Nakayama *et al.*, 1992; Sirsjö *et al.*, 1994 & our study). It is possible that this anomaly too may be explained by the presence of heterogeneous cell types in the smooth muscle culture systems used in different laboratories.

LPS binding protein

Another possible reason for the lack of ability of LPS to stimulate expression of iNOS in smooth muscle in culture in our experiments is probably worthy of consideration. Specifically, in order to bind to its receptor, CD14, LPS must first form a complex with lipopolysaccharide binding protein (LBP) (Wright *et*

al., 1990). This LBP is present in the serum of various animals including rabbits, mice and humans (Schumann *et al.*, 1990). The removal of serum in our experiments to prevent the inhibitory actions of platelet derived growth factor$_{AB}$ and $_{BB}$ and transforming growth factor β would certainly have led to the removal of LBP, thus preventing the binding of LPS to its receptor. We did in fact find that LPS was possibly effective at high concentrations at which LPS can bind to its receptor in the absence of LBP (Hailman *et al.*, 1994). Experiments involving the use of serum, i.e. to provide LBP, but lacking platelet derived growth factor$_{AB}$ and $_{BB}$ and transforming growth factor β, which inhibit iNOS expression would therefore be warranted. Although not attempted in our study, such experiments are possible through the use of plasma-derived serum, i.e. serum formed from the clotting of plasma following the removal of blood platelets (which release platelet derived growth factor and transforming growth factor β).

In conclusion, our findings suggest that bacterial LPS alone is a sufficient stimulus for expression of iNOS in isolated rings of rat aorta but that for cultured RASMC the presence of a cytokine such as IFN-γ is required. Whether this difference results from the heterogeneous nature of the cell types present in the blood vessel or from differences in the phenotypic state of smooth muscle in the arterial wall and in culture or from the absence of LPS binding protein remains to be determined.

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