INDUCTION OF NITRIC OXIDE SYNTHASE IN THE J774.7 MACROPHAGE CELL LINE

A thesis submitted for the degree of Doctor of Philosophy in the University of Glasgow

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Summary

When used alone, LPS and IFN-γ were weak stimuli for inducing production of nitrite by J774.7 macrophages, but when used together, a powerful synergistic rise in nitrite accumulation was seen. Nitrite was derived from the L-arginine/NO pathway since its production was reduced by the NO synthase inhibitors, L-NMMA and L-NAME. L-NMMA was much more effective than L-NAME but the reason for this was obscure. The peptide polymyxin B also reduced nitrite accumulation by inhibiting the action of LPS on the cells. It is likely that NO was synthesised by the inducible form of NO synthase since nitrite production was low in the absence of stimuli and was reduced by dexamethasone in the presence of stimuli. Dexamethasone reduced nitrite accumulation, though whether via a direct action on the transcription process or through the formation of lipocortin 1 is unknown.

Elevation of cyclic AMP levels reduced the production of nitrite by the cells, but only by a maximum of around 30 %. Most agents had little effect on nitrite accumulation when added after LPS and IFN-γ, and produced most of their effects when added before the induction of NO synthase by LPS and IFN-γ. Thus, cyclic AMP can only regulate the induction process, probably at the level of transcription, to a slight degree.

Elevation of cyclic GMP levels reduced accumulation of nitrite but only by around 30 %. The effects of GTN may be mediated both via toxic effects on the NO synthase enzyme by NO and via a cyclic GMP-dependent mechanism. It is unknown if elevated cyclic GMP levels can induce phosphorylation of the NO synthase enzyme and so modulate its activity.

It appears that both PKC and tyrosine kinase play a role in the induction of NO synthase by LPS and IFN-γ, although evidence for the latter is more straightforward.

The smooth muscle relaxant released from J774.7 cells following stimulation by LPS and IFN-γ was sensitive to attack from superoxide anions and blocked by haemoglobin. Furthermore, production of the relaxant was blocked following incubation with the inhibitor of NO synthase, L-NMMA. Thus, in all respects, this relaxant substance behaves like free NO. The mechanism by which macrophages protect themselves from the toxic effects of the high concentrations of NO they produce is unknown.

The release of superoxide anions from J774.7 cells was undetectable, although the cells appear to release spontaneously a powerful oxidant. The nature of this oxidant is unknown, but it appears not to be hydrogen peroxide, peroxynitrite, hydroxyl radical or hypochlorous acid.

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Abbreviations

AMP	adenosine monophosphate
8 br	8-bromo cyclic AMP
BPIP	bactericidal/permeability-increasing protein
CaM	calmodulin
CFU-GM	colony-forming unit, granulocyte macrophage
CAT	catalase
CHEL	chelerythrine chloride
CYC	cycloheximide
DAG	diacylglycerol
dbcAMP	dibutyryl cyclic AMP
DEX	dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DMTU	1,3-dimethyl-2-thiourea
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDRF	endothelium-derived relaxing factor
EGF	epidermal growth factor
EPO	eosinophil peroxidase
fMLP	formyl-methionyl-leucyl-phenylalanine
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
FOR	forskolin
GEN	genistein
GMP	guanosine monophosphate
GM-CSF	granulocyte/macrophage-colony stimulating factor

GTN	glyceryl trinitrate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HERB	herbimycin A
HDL	high-density lipoprotein
HX	hypoxanthine
HYD	hydrocortisone
IFN-α	interferon-alpha
IFN-β	interferon-beta
IFN-γ	interferon-gamma
IBMX	isobutyl methylxanthine
IL-1	interleukin-1
IL-1β	interleukin-1 beta
IL-2	interleukin-2
IL-4	interleukin 4
IL-6	interleukin-6
IL-12	interleukin 12
IP_3	inositol 1,4,5-triphosphate
LBP	lipopolysaccharide binding protein
L-NAME	N^G-L-nitroarginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MAP kinase	mitogen-activated protein kinase
MPO	myeloperoxidase
MLCK	myosin light chain kinase
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
kD	kilodaltons

NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NANC	non-adrenergic non-cholinergic
NGF	nerve growth factor
NMDA	N-methyl D-aspartate
NO	nitric oxide
PAF	platelet activating factor
PC-PLC	phosphatidyl choline-specific phospholipase C
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PE	phenylephrine
PGE_2	prostaglandin E_2
PGI_2	prostaglandin I_2
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
POL B	polymyxin B
Ro	Ro 31-8220
ROL	rolipram
SIN-1	3-morpholinosydnonimine-N-ethylcarbamide
SKF	SKF 94120
SNAP	S-nitroso-acetyl-penicillamine
SOD	superoxide dismutase
STAUR	staurosporine
TGF-β	transforming growth factor-beta
TNF-α	tumour necrosis factor-alpha
TNF-β	tumour necrosis factor-beta

XO	xanthine oxidase
ZAP	zaprinast

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INTRODUCTION

CHAPTER 1

1.1 Macrophages

Macrophages are members of the mononuclear phagocytic family which, after lymphocytes, constitutes the second major cell population of the immune system. Macrophages are widely distributed throughout the body, displaying great structural and functional heterogeneity. They originate in the bone marrow where they mature to become monoblasts, and then circulate in the blood as monocytes or migrate into the tissues of the body as macrophages. Macrophages participate in a wide range of physiological and pathological processes and play a significant role in the host defence mechanism. Although murine macrophage cell lines, such as the J774.7 cell line used in this study, have the potential to express nitric oxide (NO) synthase and to produce cytotoxic quantities of nitric oxide, the ability of cytokines and other immune mediators to promote the induction of the enzyme in human macrophages remains problematic. Furthermore, the pathways concerned in the induction process in cells which express the enzyme remain uncertain. Macrophages are also known to release a variety of reactive oxygen intermediates in a process known as the 'respiratory burst' involving metabolism of large quantities of glucose and an increase in oxygen consumption. The respiratory burst results in the production of superoxide anion, hydrogen peroxide, hydroxyl radicals, hypochlorous acid and perhaps also peroxynitrite which are involved in the microbicidal activity of the cells.

1.1.1 Macrophage origin and function

Macrophages originate in the bone marrow. In humans, the bone marrow contains resident macrophages as well as their precursors i.e. monocytes and promonocytes. The macrophage progenitor cell is called the colony-forming unit, granulocyte-macrophage (CFU-GM) which also has the ability to give rise to neutrophils (Metcalf, 1971). The CFU-GM gives rise to a monoblast cell which then develops to a promonocyte and further to a monocyte in the bone marrow (van Furth & Diesselhoff-den Dulk, 1970). Newly formed monocytes remain in the bone marrow for less than 24 hours before entering the circulation (Meuret & Hoffman, 1973). Monocytes circulate in the blood until they reach their target organ, where they differentiate into macrophages (van Furth & Cohn, 1968). Mononuclear phagocytes reside in a wide variety of tissues including the liver, where they are known as Kupffer cells, in bone as osteoclasts, in the brain as microglia and in the lung as alveolar macrophages.

Macrophages are relatively large cells whose diameter ranges from 25-50 μm. The surface of the cells is covered with microvilli. The cytoplasm contains vacuoles, lysosomes and phagosomes. Mononuclear phagocytes provide a major defence against invading microbes. Although mononuclear phagocytes are effective at phagocytosis, neutrophils are generally more efficient. Mononuclear phagocytes have the ability to secrete over 100 substances, ranging from those of low molecular weight, e.g. NO and superoxide anion, to fibronectin with a mass of 440 kD. Table 1.1 demonstrates some of the substances that are secreted (Auger & Ross, 1992).

Reactive oxygen intermediates e.g. superoxide anion, hydrogen peroxide, hydroxyl radical
Complement components e.g. C1, C4 and active fragments C3a, C3b , C5a
Cytokine and enzyme inhibitors e.g. IL-1 inhibitors, phospholipase inhibitor, collagenase inhibitor
Lipases e.g. lipoprotein lipase, phospholipase A_2
Neutral Proteases e.g. collagenase, elastase
Lysosomal acid hydrolases e.g. phosphatases, sulphatases, lipases
Cyclo-oxygenase/lipooxygenase products e.g. PGE_2, thromboxane, leukotrienes, platelet-activating factors
Cytokines e.g. TNF-α, transforming growth factor β, IL-1, IL-6
Coagulation factors e.g. tissue factor, plasminogen activators
Miscellaneous e.g. fibronectin, lipocortin, transferrin, NO

Table 1.1 Examples of biologically active substances secreted by macrophages (modified from Auger & Ross, 1992).

1.1.2 Role of the macrophage in the host defence mechanism

Macrophages have the ability both to clear viral pathogens from the circulation and to inactivate the viruses. Macrophages identify virus particles by specific and non-specific viral receptors, and the virus is then taken up into the macrophage in a number of different ways including fusion and phagocytosis (Gendelman & Morahan, 1992). The virus is inactivated via the secretion of various hydrolytic cellular enzymes contained in the macrophage phagolysosomes (Mims, 1964). The ability of macrophages to secrete IFN-α and IFN-β contributes to their anti-viral effects as the interferons play a major role in restricting viral replication in infected macrophages and in neighbouring cells (Bukowski & Welsh, 1986). Interferons exert their anti-viral mechanism by inducing the host cell to produce enzymes capable of inhibiting translation of viral mRNA to viral protein, thus inhibiting reproduction of the virus.

Macrophages play an important role in the elimination of bacterial infections. Alveolar macrophages protect the lungs against inhaled microorganisms (Laskin & Pendino, 1995), whereas macrophages within the liver and spleen provide an effective defence against bacterial infection in the blood (Laskin & Pendino, 1995). Microorganisms may be susceptible to phagocytosis by macrophages, or may come under attack from oxidative or non-oxidative mechanisms. Macrophages release a wide variety of reactive oxygen species (see section 1.2) which exert bactericidal effects on the invading organism. Non-oxidative mechanisms include attack by lysosomal enzymes (Darte & Beaufay, 1983).

Parasites are also sensitive to attack by reactive oxygen and nitrogen intermediates produced by macrophages (Liew *et al.*, 1990), and it is

thought that macrophages represent one of the first lines of defence against neoplastic cell growth. When in contact with tumour cells, macrophages release reactive oxygen and nitrogen intermediates and inhibit DNA synthesis, aconitase and mitochondrial respiration in addition to other mechanisms. These events normally lead to destruction of the tumour cells (Rees & Parry, 1992).

Atherosclerosis, the principal cause of myocardial and cerebral infarction, accounts for the majority of deaths by cardiovascular disease in the western world. Atherosclerosis is a disease of the arteries where there is occlusion of the lumen by a thickening of the artery wall. The thickening consists of lipid deposits, extracellular matrix and cellular components. Cholesterol circulates around the body mainly in the form of low-density lipoprotein (LDL). Macrophages have the ability to take up LDL through high affinity and low affinity receptors. Overloading of the macrophage leads to the development of foam cells, and these are present around the edges of atherosclerotic plaques (Ball *et al.*, 1995). Thus macrophages appear to play an important role in the development of plaques as they constitute a route for transportation of LDL into the intima of arteries (Klurfeld, 1985). Once in the intima, macrophages secrete cytokines which act as chemoattractants for smooth muscle cells and monocytes. In addition, growth factors for smooth muscle cells are secreted, which leads to proliferation of these cells and thus thickening of the occlusion. Enzymes are also secreted, which render the plaque more thrombogenic and likely to calcify (Parums, 1992). Furthermore, reactive oxygen species are released which may modify LDL rendering it antigenic and able to damage membranes, cause necrosis and even damage the macrophage itself (Reid & Mitchinson, 1993). Thus, macrophages appear to be important mediators of cardiovascular disease.

1.2 Free radical release

In addition to production of NO as a host defence mechanism, macrophages produce superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hypohalous acids (HOX), for example hypochlorous acid (HOCl). Production of superoxide and hydrogen peroxide can lead to the additional production of singlet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH^-$) and peroxynitrite ($ONOO^-$). Each of these reactive oxygen species is needed for optimal microbicidal activity towards invading organisms (Rosen *et al.*, 1995).

1.2.1 Superoxide

Superoxide anions are generated by almost all aerobic cells and a major source is leakage of electrons from various components of the mitochondrial and endoplasmic reticulum electron transport chains (Salvemini & Botting, 1993). Superoxide is also generated during the 'respiratory burst' by an electron transport chain that transfers electrons from NADPH in the cytosol to oxygen to form superoxide and subsequently hydrogen peroxide and other oxidants in the phagocytic vacuole. This NADPH oxidase is most abundant in phagocytic cells i.e. neutrophils (Thelen *et al.*, 1993; Carreras *et al.*, 1994), eosinophils (Souness *et al.*, 1991), monocytes (Landmann *et al.*, 1995) and macrophages (Assreuy *et al.*, 1994). The electron transport chain in these cells consists of a flavocytochrome b located in the plasma membrane as well as in the membrane of specific granules (Segal, 1995). Superoxide can diffuse to different sites from its origin to produce toxic effects. It is likely that superoxide plays a role in endothelial dysfunction in endotoxaemic rats (Siegfried *et al.*, 1992). Although superoxide has been shown to inactivate some bacterial enzymes, for example aconitase, most of

its toxic effects are mediated via generation of hydroxyl radicals and hydrogen peroxide. Production of superoxide may inhibit NO synthase (Rengasamy & Johns, 1993b), a process which occurs via competition for oxygen between NO synthase and NADPH oxidase.

A variety of stimuli activate NADPH oxidase and thereby promote superoxide production. Examples of such stimuli include phorbol esters and calcium ionophores, and the combination of these synergistically stimulates superoxide release from neutrophils (Robinson *et al.*, 1984). Moreover, LPS stimulates human monocytes to release superoxide via the CD14 receptor and is dependent on binding with LBP (Landmann *et al.*, 1995). TNF also stimulates activation of human macrophages to release superoxide anion (Bermudez & Young, 1992). Further, arachidonic acid (Sakata *et al.*, 1987) and the chemotaxin, formyl-methionyl-leucyl-phenylalanine (fMLP)(Turner *et al.*, 1993), stimulates superoxide production in peritoneal macrophages. Protein kinase C (PKC) appears to be an important pathway in mediating activation of NADPH oxidase by a variety of stimuli in neutrophils including the phorbol ester, PMA, the chemotactic peptides, fMLP and C5a (Dewald *et al.*, 1989), opsonised zymosan, heat aggregated IgG (Twomey *et al.*, 1990a & b), and fluoride (Twomey *et al.*, 1991). Macrophages from guinea pigs are also stimulated with fMLP, PAF and opsonised zymosan (Turner & Wood, 1994) and those from rat lung are stimulated by PMA (Mayer *et al.*, 1993). Indeed, some isoforms of PKC activate NADPH oxidase at resting calcium concentrations (Sharma *et al.*, 1991). In addition to PKC, superoxide production can be regulated by other second messengers. Superoxide production can be reduced by elevated cyclic AMP levels (Turner *et al.*, 1993; Dent *et al.*, 1994) and tyrosine kinase, whose stimulation is important in the induction of NO synthase, plays a role in modifying superoxide production. However, in contrast to the effects seen

with NO synthase, activation of tyrosine kinase suppresses superoxide production (Conde *et al.*, 1995). Thus, production of superoxide is controlled by a range of different regulatory systems.

1.2.2 Hydrogen Peroxide

At physiological pH, superoxide rapidly dismutates to give hydrogen peroxide (Halliwell & Gutteridge, 1984).

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

Hydrogen peroxide is also formed under the influence of superoxide dismutase, which speeds up the reaction by 10^4 times (Miller & Britigan, 1995a).

$$\bullet O_2^- + 2H_2O \rightarrow 2H_2O_2$$

Hydrogen peroxide induces peroxidation of membranes, oxidises cellular enzymes and mediates DNA damage and mutagenesis.

1.2.3 Peroxynitrite

Peroxynitrite is formed by the combination of NO and superoxide (Freeman, 1994).

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

Peroxynitrite is a potent oxidant which can react by several oxidative mechanisms (Radi *et al.*, 1991a), and it is likely that these mediate at least

some of the cytostatic and cytotoxic action of NO. For example, peroxynitrite has been shown to catalyse membrane lipid peroxidation in an iron-independent manner (Radi *et al.*, 1991b), oxidise DNA bases (Beckman *et al.*, 1990) and react with metals or metalloproteins to form the toxic nitronium ion (Ischiropoulos *et al.*, 1992a). Peroxynitrite also has been shown to inhibit epithelial ion channels (Bauer *et al.*, 1992), inhibit cell motility, induce cellular swelling (Denicola *et al.*, 1993) and play a part in mediating human lung injury (Kooy *et al.*, 1995). Simultaneous production of superoxide and NO leading to peroxynitrite formation by J774 cells is reported to mediate the auto-inhibition of mitochondrial respiration in immunostimulated cells (Szabó & Salzman, 1995). Additionally, NO released from astrocytes, combining with superoxide to form peroxynitrite, may diffuse to neighbouring neuronal cells producing mitochondrial dysfunction and neurodegenerative effects (Bolaños *et al.*, 1995). Furthermore, peroxynitrite has the ability to stimulate cyclic GMP formation, although it is less potent than NO at doing so (Tarpey *et al.*, 1995), and this has been proposed to contribute to the profound hypotension of septic shock and may contribute to its deleterious effects (Szabó *et al.*, 1995), in addition to mediating oxidative damage. Moreover, due to its ability to modify proteins by nitration of tyrosine residues, peroxynitrite has been shown to inactivate some important proteins including manganese-containing superoxide dismutase (SOD) (Ischiropoulos & Al-Medhi, 1995), thus compromising an important component of the intrinsic antioxidant defence mechanism of the cell.

1.2.4 Hydroxyl Radical

Hydroxyl radical is formed by the one-electron reduction of hydrogen peroxide.

$$\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + OH^- + O_2$$

However, the rate of this reaction is slow, and a metal catalyst is needed to speed up the reaction. The presence of iron leads to hydroxyl radical formation via the Haber-Weiss reaction.

$$\bullet O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$
$$H_2O_2 + Fe^{2+} \rightarrow \bullet OH + OH^- + Fe^{3+}$$

Almost all iron *in vivo* is tightly bound to binding proteins. Transferrin is the main iron-binding protein in serum, and lactoferrin binds iron at mucosal surfaces. It is unlikely that either lactoferrin or transferrin serve as the catalyst (Aruoma & Halliwell, 1987). It appears that the cell itself is the major source of iron for catalysis, where the iron is stored in the form of ferritin (Halliwell & Gutteridge, 1984). The mechanism by which the iron is released for catalysis is uncertain, however, superoxide is said to remove Fe^{3+} from ferritin as well as cause its release from protein as Fe^{2+}.

Hydroxyl radical can also be generated by an iron-independent pathway, via the decomposition of peroxynitrite (Beckman & Crow, 1993).

$$ONOO^- + H^+ \rightarrow ONOOH$$
$$ONOOH \rightarrow \bullet OH^- + NO_2\bullet$$

Hydroxyl radical is reputed to be the mediator of most of the cell damage mediated by macrophage-derived oxidants (Miller & Britigan, 1995a). Hydroxyl radical is highly reactive, with a rate of reaction of 10^7-10^{10} mol s^{-1}. A major mechanism by which it produces cell injury is via the initiation of lipid peroxidation (Farber, 1994). This peroxidation of unsaturated fatty

acids within a lipid membrane produces peroxyl radical which in turn can react with other lipid molecules to produced further lipid radicals (Halliwell, 1995). The new lipid radicals then react with other unsaturated lipids, and the cascade proceeds until a free radical chain reaction is established leading to membrane damage and cell death. Also, the amino acids tryptophan, tyrosine, phenylalanine, histidine, methionine and cysteine are prone to free radical-mediated modification (Freeman & Crapo, 1982). Moreover, hydroxyl radicals react indiscriminately with all components of the DNA molecule producing base-free sites, deletions, frameshifts, strand breaks, DNA-protein crosslinks and chromosomal rearrangements (Guyton & Kensler, 1993). Hydroxyl radicals also can mediate injury to pulmonary endothelial cells (Varani *et al.*, 1985).

1.2.5 Hypohalous acid

Neutrophils and monocytes (perhaps also macrophages) release the enzyme myeloperoxidase (MPO) from cytoplasmic granules upon activation, whereas eosinophils release eosinophil peroxidase (EPO). Interaction of MPO and EPO with hydrogen peroxide forms hypohalous acid (Rosen *et al.*, 1995).

$$H_2O_2 + HX \rightarrow HOX + H_2O$$

(where X= a halide)

Chlorine is the preferred halide for MPO which forms hypochlorous acid. Hypochlorous acid has been shown to disrupt cell membrane integrity via lipid peroxidation and decarboxylation of membrane proteins (Albrich *et al.*, 1986). It affects components of the bacterial respiratory chain by inhibiting

succinate dehydrogenase (Rakita *et al.*, 1989) and by inducing membrane peroxidation (Winterbourn *et al.*, 1992) and may in addition inhibit DNA synthesis (Rosen *et al.*, 1990).

1.2.6 Priming for free radical release

IFN-γ is known to stimulate the respiratory burst of phagocytes and thus enhance their cytotoxic activity. Macrophages enter a primed state following exposure to a macrophage activating factor (see 1.4.9). Pretreatment with IFN-γ before exposure to a further stimulus, for example PMA, leads to a synergistic enhancement of superoxide production (Wolfson *et al.*, 1993). Priming is thought to involve alterations in calcium levels and activation of protein kinase C (see 1.4.9; Celada & Schreiber, 1986; Somers *et al.*, 1986; Forehand *et al.*, 1989).

1.2.7 Defences against free radicals

As reactive oxygen species are continuously produced in animal cells, and the effects of these species can be damaging, defences against the radicals have evolved, and are called antioxidant defences. There are two main strategies of antioxidant defence; one is to prevent the production of free radicals and the other is to scavenge the radicals once they have been produced (Cotgreave *et al.*, 1988). Transition metals can act as catalysts in the production of free radicals, and thus, the metals are held tightly bound to special binding proteins. Iron, for example, is bound to transferrin, ferritin and lactoferritin, and is not easily accessible to act as a catalyst (Miller & Britigan, 1995b). Scavenging free radicals once they have been formed is a

further mechanism of antioxidant defence. Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide. SOD exists in two forms; a manganese-containing enzyme found in the mitochondrial matrix of eukaryotic cells (Peeters-Joris *et al.*, 1975) and a copper-zinc cytosolic form (Wilkins & Leake, 1994). Another defence mechanism is to remove the peroxides that react with transition metals to produce free radicals. Catalase, which is present in most mammalian cells, decomposes hydrogen peroxide (Salvemini & Botting, 1993).

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

In addition, glutathione peroxidase removes any lipid hydroperoxides that are formed during lipid peroxidation (Singh, 1981).

$$ROOH + 2GSH \xrightarrow{\text{glutathione peroxidase}} ROH + GSSG + H_2O$$

α-tocopherol, a low molecular weight molecule found in cell membranes, is the major member of the vitamin E family. α-tocopherol intercepts lipid peroxyl radicals thus terminating lipid peroxidation chain reactions (Halliwell, 1995). Ascorbic acid (vitamin C), a water soluble molecule found both intracellularly and extracellularly in most biological systems, has been shown to act as an antioxidant in the plasma and also within cells (Cheeseman & Slater, 1993). β-carotene, a metabolic precursor to vitamin A, has been shown to quench singlet oxygen and react directly with free radicals (Cotgreave *et al.*, 1988).

1.3 Nitric oxide

Endothelium-derived relaxing factor (EDRF), was first described in 1980 by Furchgott and Zawadzki, who demonstrated that the vascular relaxation induced by acetylcholine was dependent on the presence of the endothelium. By employing techniques such as cascade bioassay in which the effluent from perfused endothelial cells was used to reperfuse an artery denuded of endothelium (Cocks *et al.*, 1985; Gryglewski *et al.*, 1986a), it was established that EDRF was a very short-lived substance, with a half-life of only a few seconds. Endothelium-dependent relaxation occurs in response to a variety of substances in addition to acetylcholine, such as adenine nucleotides, thrombin, substance P and bradykinin, and these all cause the release of the same EDRF (Cocks *et al.*, 1985). However, other agents such as the nitrovasodilators, atrial natriuretic factor and bovine retractor penis inhibitory factor, cause vascular relaxation by an endothelium-independent mechanism (Griffith *et al.*, 1984; Furchgott, 1984). Nitrovasodilators e.g. sodium nitroprusside and glyceryl trinitrate, exert their effects via NO which is released from the parent compound by reductive enzymes (Ignarro, 1989). The nitrovasodilators mimic the effects of EDRF, and the NO released from the endothelial cells is indistinguishable from EDRF in terms of stability, biological activity and susceptibility to inhibitors and potentiators. Most importantly, however, using a chemiluminescence assay, it was found that NO is released from vascular endothelial cells in amounts sufficient to account for the biological action of EDRF (Palmer *et al.*, 1987; Moncada *et al.*, 1988). It was therefore suggested by these workers that EDRF may in fact be NO. NO has many diverse biological functions in the central and peripheral nervous systems, the immune system and the cardiovascular system.

1.3.1 Mechanism of vascular relaxation by NO

NO causes relaxation in vascular smooth muscle cells by activation of soluble guanylate cyclase (Kukovetz *et al.*, 1979). NO is lipophillic and can easily diffuse from the endothelial cell to smooth muscle cells where it binds to the haem group of soluble guanylate cyclase to yield the nitrosyl-haem-enzyme ternary complex which represents the activated state of guanylate cyclase (Ignarro, 1989). Binding of NO produces an immediate increase of 50-200-fold in catalytic activity. NO activates the enzyme by pulling the iron molecule away from the enzyme and out of plane from the planar porphyrin ring configuration (Ignarro *et al.*, 1984). The substrate, magnesium guanosine 5'-triphosphate is converted to guanosine 3',5'-monophosphate (cyclic GMP) (Ignarro, 1989), and it is widely accepted that the subsequent activation of protein kinase G induces a sequence of protein phosphorylation steps. Elevated cyclic GMP levels inhibit calcium mobilisation within the cells; the release of intracellular calcium is inhibited, calcium entry into cells is inhibited and the rate of calcium extrusion from cells is increased leading to a relaxation of the contractile elements (Wennmalm, 1994).

1.3.2 Synthesis of NO

It is well known that NO is synthesised from one of the terminal guanidino nitrogen atoms of the amino acid L-arginine by the enzyme NO synthase (Fig. 1.1)(Marletta, 1994).

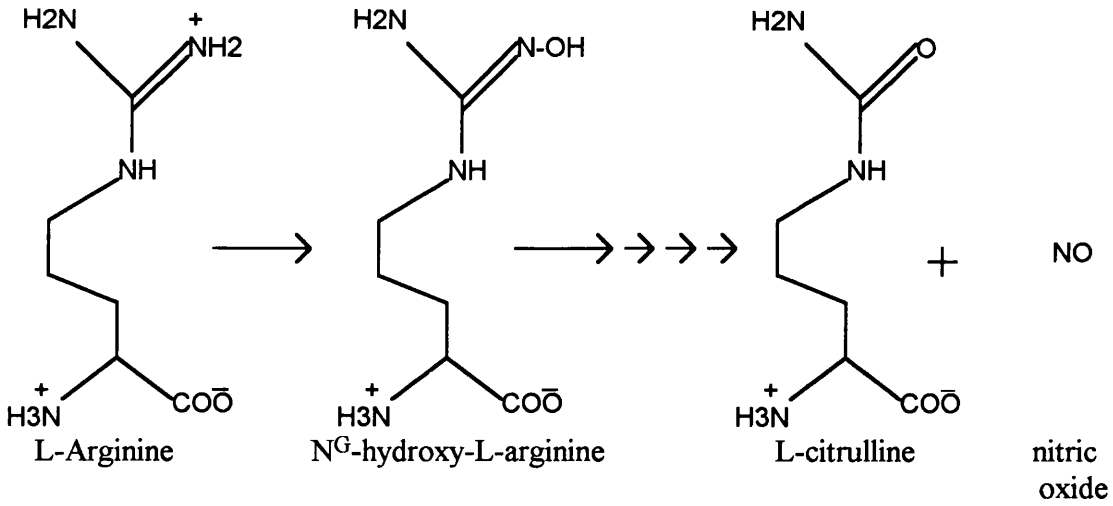


Figure 1.1 Reaction catalysed by the nitric oxide synthases. →→→ indicates intermediate steps still to be elucidated (modified from Marletta, 1994).

The metabolism of L-arginine to L-citrulline and NO involves a five electron oxidation and requires the cofactors nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin, flavin adenine nucleotide (FAD) flavin mononucleotide (FMN) (Nathan & Xie, 1994a). The activity of the constitutive isoforms of NO synthase is strictly regulated by calcium and calmodulin whereas, although the inducible isoform tightly binds calmodulin, its activity is independent of calcium (Marletta, 1994). The reaction to form NO is known to involve an initial hydroxylation of L-arginine to give N^G-hydroxy-L-arginine, although the steps in the conversion of N^G-hydroxy-L-arginine to NO and L-citrulline are not known (Marletta, 1994). It is thought that a haem group in the enzyme is directly involved in the oxidation of N^G-hydroxy-L-arginine (Marletta, 1993).

1.3.3 Isoforms of nitric oxide synthase

Three isozymes of nitric oxide synthase (NO synthase) have been identified and the corresponding genes have been cloned, sequenced and expressed (see Fig 1.2). Neuronal constitutive, endothelial constitutive and inducible NO synthase are the three main isoforms of the enzyme (Förstermann *et al.*, 1991). Flavins and biopterin are found bound to all three isozymes, with biopterin always found as the totally reduced biopterin derivative 6-(R)-5,6,7,8- tetrahydrobiopterin (Stuehr *et al.*, 1991a). All three of the isozymes also contain haem (Förstermann *et al.*, 1994). The activity of NO synthase found in endothelial cells and neuronal cells is regulated by calcium and calmodulin, and NO is released over several minutes (Maliński & Taha, 1992). On the other hand, the inducible isoform found in a variety of different cell types is not regulated by calcium, although it has calmodulin tightly bound, and requires activation of the cells by a variety of stimuli resulting in the *de novo* biosynthesis of the enzyme (Morris & Billiar, 1994). The inducible form generates large amounts of NO over periods as long as 5 days as it is independent of intracellular calcium levels (Vodovotz *et al.*, 1994). Calmodulin appears to play a role in electron transport, as binding of calmodulin allows NADPH-derived electrons to pass onto the haem group of NO synthase (Abu-Soud & Stuehr, 1993). Across species, amino acid sequences are well conserved, with over 90 % homology for the neuronal and endothelial forms and over 80 % for the inducible isozyme (Förstermann *et al.*, 1994).

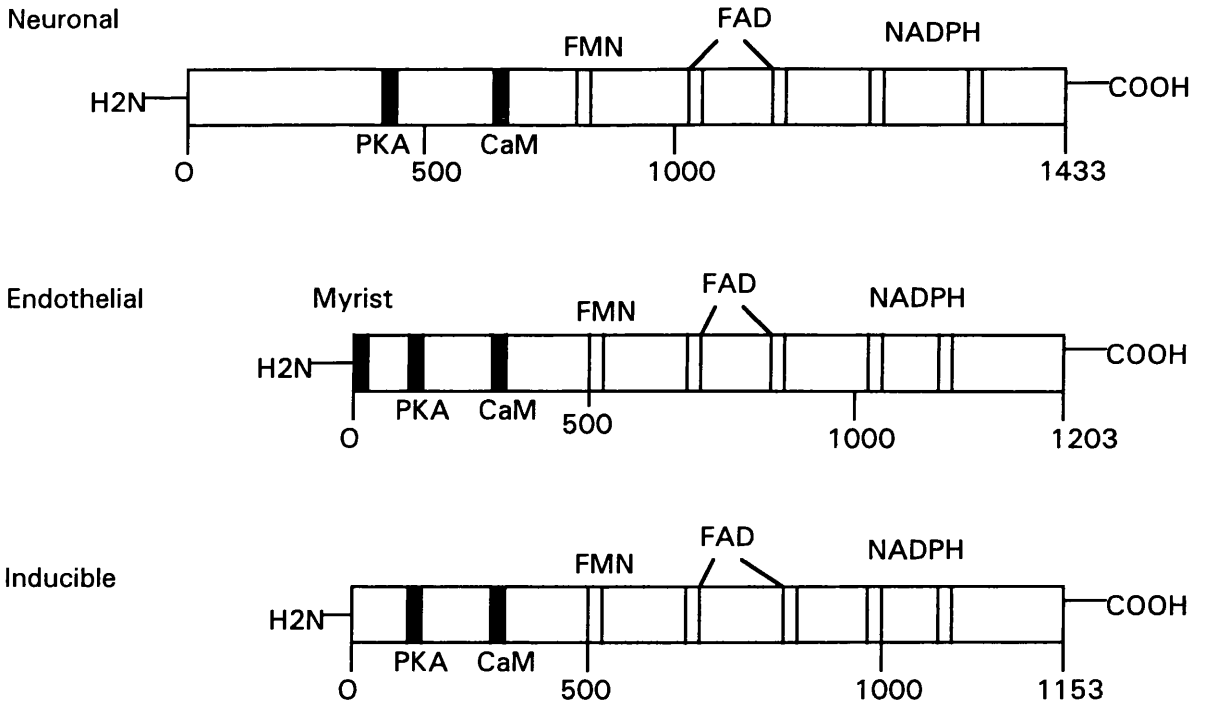


Figure 1.2 Localisation of functional sites on the three isoenzymes of NO synthase. PKA, CaM and Myrist indicate the sites of consensus sequences for phosphorylation by protein kinase A, calmodulin binding and myristoylation, respectively. Amino acids are numbered from the N-terminus. (Modified from Zhang & Snyder, 1995).

1.3.4 Neuronal NO Synthase

The neuronal form of NO synthase is constitutively expressed in the central and peripheral nervous systems and also in some epithelial cells. It was first purified from rat cerebellum (Bredt & Snyder, 1990). This isoform is mostly a soluble enzyme that migrates with a molecular mass of 150-160 kD on SDS polyacrylamide gel electrophoresis (Bredt & Snyder, 1990; Schmidt *et al.*, 1991) and the amino acid sequence appears to be highly conserved between species. It is a calcium and calmodulin-dependent enzyme that is inactive at 10^{-7} M calcium and fully active at $5x10^{-7}$ M (Förstermann *et al.*, 1990), which represent typical changes in intracellular calcium

concentrations following action potential generation or receptor stimulation of excitatory cells such as neurones. This isoform is also present in peripheral non-adrenergic non-cholinergic (NANC) inhibitory neurones found for example in the rat anococcygeus (Gillespie *et al.*, 1989) and the bovine retractor penis muscle (Martin *et al.*, 1988). Unlike central neuronal tissue, the nerves in the bovine retractor penis muscle and the rat anococcygeus contain both soluble and particulate NO synthase (Förstermann *et al.*, 1991). Garthwaite *et al.*, (1988) discovered that activation of a subtype of the glutamate receptor, the N-methyl-D-aspartate (NMDA) receptor, led to release of NO from cerebellar neurones. Furthermore, glutamate-induced cerebral vasodilatation is mediated by NO (Meng *et al.*, 1995). In the CNS, the enzyme can be phosphorylated by calcium-calmodulin-dependent protein kinase II, protein kinase C and cyclic AMP-dependent protein kinase (Bredt *et al.*, 1992). Phosphorylation by PKC leads to either an increase or decrease in activity (Nakane *et al.*, 1991; Bredt *et al.*, 1992) whereas phosphorylation by calcium calmodulin-dependent kinase decreases activity (Nakane *et al.*, 1991; Schmidt *et al.*, 1992a). The activity of neuronal NO synthase is unaffected upon phosphorylation by PKA (Brüne & Lapetina, 1991).

1.3.5 Endothelial NO Synthase

The endothelial form of NO synthase has been reported to be in the soluble and particulate fractions, but is 90 % particulate. This isoform has a molecular mass of 135 kD (Pollock *et al.*, 1991), is calcium/calmodulin dependent and the amino acid sequence of bovine and human isoforms show 94 % homology (Lamas *et al.*, 1992, Nathan & Xie, 1994b). There is a consensus motif for N-terminal myristoylation, a feature which renders the

enzyme particulate (Busconi & Michel, 1993). Substituting glycine with alanine in the sequence converts the isoform to a 92 % cytosolic enzyme (Sessa *et al.*, 1993). The activity of endothelial NO synthase has been shown to be reduced by protein kinase C (Davda *et al.*, 1994; Hirata *et al.*, 1995; Ohara *et al.*, 1995).

1.3.6 Inducible NO Synthase

The inducible form of NO synthase is not normally present in cells. It is induced upon exposure to a number of different immunological stimuli and it is not dependent on calcium (Busse & Mülsch, 1990), although the enzyme has calmodulin tightly bound (Cho *et al.*, 1992). Like the neuronal isoform, the inducible form of the enzyme is soluble, and the protein has a mass of 130 kD when run on SDS polyacrylamide gels (Hevel *et al.*, 1991). The inducible enzyme is expressed in cell types such as macrophages (Marletta *et al.*, 1988), endothelial cells (Radomski *et al.*, 1990a), cardiac myocytes (Balligand *et al.*, 1994), vascular smooth muscle cells (Busse & Mülsch, 1990), neutrophils (van Dervort *et al.*, 1994), Kupffer cells (Billiar *et al.*, 1989) and hepatocytes (Curran *et al.*, 1989).

Although the synthesis of nitric oxide from L-arginine has been demonstrated in a number of cell types, the pathway was first identified in the macrophage as a reaction involving the production of nitrite and nitrate (Iyengar *et al.*, 1987; Stuehr & Marletta, 1987a; 1987b). Further work demonstrated that NO was the source of production of nitrate and nitrite (Hibbs *et al.*, 1988; Marletta *et al.*, 1988).

Macrophage NO synthase is a dimeric enzyme comprising two identical 130 kD subunits, and contains 1 mole each of iron protoporphyrin IX (haem),

FAD and FMN per subunit, variable amounts (0.1-1 mole/subunit) of tetrahydrobiopterin and an unspecified amount of tightly bound calmodulin (Ghosh & Stuehr, 1995). The proposal of a 'head-to head' arrangement in the structure of macrophage NO synthase from the RAW 264.7 cell line suggests the possibility that the N-terminal domain of each of the subunits of the polypeptide chain, representing the catalytic domains, lie side to side and contain binding sites for L-arginine, haem and tetrahydrobiopterin (Fig 1.3, Ghosh & Stuehr, 1995).

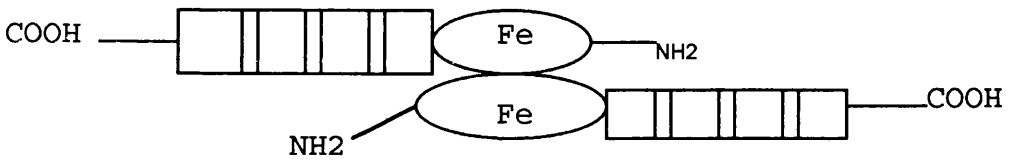


Figure 1.3 Proposed 'head-to-head' structure of macrophage NO synthase (modified from Ghosh & Stuehr, 1995).

Tetrahydrobiopterin appears to be an essential cofactor for the formation of NO (Kwon *et al.*, 1989), as its depletion by the drug, 2,4-diamino-6-hydroxy-pyrimidine, which blocks the synthesis of tetrahydrobiopterin, reduces the production of NO (Bogdan *et al.*, 1995; Schoedon *et al.*, 1993; Sakai & Milstien, 1993). In addition to their participation in catalysis, L-arginine, tetrahydrobiopterin and haem also help to form and maintain the dimeric structure of macrophage NO synthase (Baek *et al.*, 1993). The precise role that tetrahydrobiopterin plays in catalysis, however, remains unclear.

1.3.7 Pharmacological Manipulation of NO synthases and NO actions

Agents able to mimic the effects of NO include the organic nitrates. Since the discovery of nitroglycerin for the treatment of angina pectoris, organic nitrates have been used widely in the management of cardiovascular diseases (Fung, 1993). Some of the nitrovasodilators include nitroglycerin, sodium nitroprusside, isosorbide dinitrate, isosorbide mononitrate, 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1) and amyl nitrite (Harrison & Bates, 1993). The organic nitrates act by being converted to NO by metabolic enzymes and evidence suggests that some of the organic nitrates, for example nitroglycerine and isosorbide dinitrate, are converted to NO by the same enzyme (Chung & Fung, 1992). Two of the favoured enzymes are glutathione S-transferase (Armstrong *et al.*, 1980) and cytochrome P-450 (Schröder, 1992), but Feelisch (1991) has proposed that an enzyme may not be necessary and that reduction may take place by chemical interaction with tissue thiols i.e. L-cysteine and glutathione. However, the enzyme is not responsible for the conversion of non-nitrate nitrovasodilators e.g. sodium nitroprusside and the S-nitrosothiols (Kowaluk & Fung, 1991). The nitrovasodilators azide and hydroxylamine are converted to NO by catalase (Arnold *et al.*, 1977). NO activates guanylate cyclase in vascular smooth muscle cells producing vasodilatation (see 1.3.1).

A variety of drugs can inhibit the enzyme NO synthase. Many are analogues of L-arginine and act as competitive inhibitors of the enzyme if added simultaneously with the substrate, L-arginine, but some, because their rate of dissociation is so slow, prove to be essentially irreversible if added in advance. One of the most widely used inhibitors is N^G-monomethyl-L-arginine (L-NMMA)(Hibbs *et al.*, 1987a). L-NMMA inhibits both

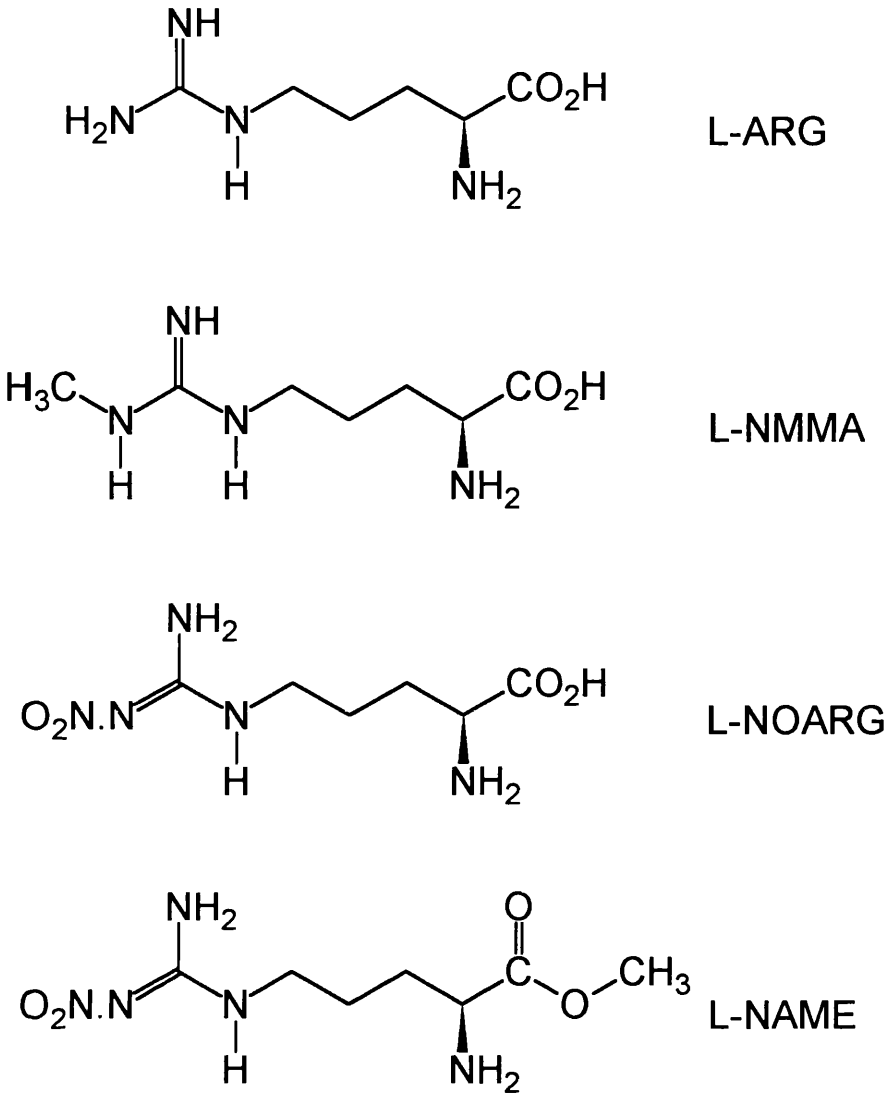


Figure 1.4 Examples of substrate analogue inhibitors of NO synthase: N^G-monomethyl L-arginine (L-NMMA); N^G-nitro L-arginine (L-NOARG); N^G-nitro-L-arginine methyl ester (L-NAME), together with L-arginine (L-ARG).

constitutive and inducible isoforms of NO synthase; the former competitively, but the latter irreversibly (Olken *et al.*, 1991). It is thought that L-NMMA undergoes oxidation catalysed by NO synthase to generate N^G-hydroxy-N-methyl-L-arginine, which upon further conversion, generates an irreversible binding species (Feldman *et al.*, 1993). Another analogue, N^G-nitro-L-arginine acts as an irreversible inhibitor of the constitutive isoform both in endothelial cells and neurones, but its effects have been shown to be fully reversible on the inducible isoform (Furfine *et al.*, 1993). Arginine analogues exhibit some degree of isoform selectivity. N^G-amino-L-arginine and N^G-nitro-L-arginine are about 100 times more potent that L-NMMA at inhibiting constitutive NO synthase in endothelial cells (Gross *et al.*, 1990; 1991). However, the N^G-amino and N^G-methyl analogues are equally effective at inhibiting inducible NO synthase in macrophages, whereas the N^G-nitro analogue is less potent (Gross *et al.*, 1990; 1991). Another study demonstrated that the potency for inhibiting NO synthase in macrophages was N^G-amino≥ N^G-methyl >> N^G-nitro, but for the constitutive enzyme, the order of potency was N^G-nitro>N^G-amino≥N^G-methyl (Lambert *et al.*, 1991). N-cyclopropyl-L-arginine is 300-500 times more selective for the neuronal constitutive isoform, whereas N-amino-L-homoarginine exhibits similar potency for the neuronal constitutive and inducible forms in smooth muscle cells and macrophages (Lambert *et al.*, 1992). McCall *et al.* (1991) found that in J774 macrophages, N-iminoethyl-L-ornithine was a more potent inhibitor of NO synthase than L-NMMA, and that N^G-nitro-L-arginine and L-NAME were much more effective, producing inhibition of around 40 %. Thus, different substrate analogues exhibit varying selectivity for the different NO synthase isoforms.

Due to the need for a variety of cofactors, there are a number of mechanisms of inhibition to which both constitutive and inducible enzymes are

susceptible. One such group are the cofactors, FAD and FMN. Therefore, inhibition of these cofactors would be expected to inhibit NO synthase activity. The drug diphenyleneiodium and several of its analogues, which inhibit flavoproteins, have been shown to inhibit both inducible and constitutive NO synthase activity (Stuehr *et al.*, 1991b). Both forms of the enzyme also are haem containing proteins, where the haem group is needed for catalytic activity. Therefore, it would be expected that inhibition of the haem group would decrease activity. Carbon monoxide has indeed been shown to decrease NO synthase activity, as has cyanide (Klatt *et al.*, 1992) and even NO itself may cause inhibition (Rogers & Ignarro, 1992). Tetrahydrobiopterin is a further cofactor required for activity of both the constitutive and inducible forms of NO synthase. Inhibition of tetrahydrobiopterin biosynthesis would therefore be expected to reduce NO synthase activity. In fact, it has been demonstrated that depletion of tetrahydrobiopterin by the drug, 2,4-diamino-6-hydroxy-pyrimidine, which blocks the synthesis of tetrahydrobiopterin, reduces the production of NO by both the inducible (Bogdan *et al.*, 1995; Schoedon *et al.*, 1993; Sakai & Milstien, 1993) and constitutive isoforms (Gross *et al.*, 1991; Schmidt *et al.*, 1992b).

A variety of nitrogen containing compounds also have the ability to inhibit both the inducible and constitutive isoforms, although the mechanism by which they do so is not known. Aminotriazole is one such inhibitor (Fukuto & Chaudhuri, 1995). Another is aminoguanidine, which contains the guanido group of L-arginine linked to hydrazine; this has been shown to be 10-100 times more potent at inhibiting the inducible isoform of NO synthase compared to the constitutive form (Griffiths *et al.*, 1993; Misko *et al.*, 1993). Further, Nakane *et al.* (1995) demonstrated that two potent inhibitors, S-ethylisothiourea and 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-

thiazine were 10-40 times more selective for macrophage inducible NO synthase compared to neuronal and endothelial constitutive isoforms. The constitutive forms of NO synthase are dependent upon calcium and calmodulin for activity. It has been found that calmodulin inhibitors such as W-7 and fendiline, inhibited the activity of endothelial constitutive NO synthase but they have no effect on the inducible form of the enzyme in vascular smooth muscle cells (Schini & Vanhoutte, 1992).

Interference of the L-arginine/NO pathway can be achieved via the targeting of NO. Haemoglobin can bind and inactivate NO (Martin *et al.*, 1985) as can superoxide anion (Gryglewski *et al.*, 1986b; Rubanyi & Vanhoutte, 1986). As the vasorelaxant effects of NO are mediated through activation of soluble guanylate cyclase and subsequent elevation of cyclic GMP, this can be targeted as a means of inhibiting the effects of NO. Methylene blue is an agent which blocks soluble guanylate cyclase, and thus inhibits the actions of NO (Martin *et al.*, 1985).

Expression of inducible NO synthase can be prevented by treatment with glucocorticoids such as dexamethasone (Di Rosa *et al.*, 1990; Knowles *et al.*, 1990; Assreuy & Moncada, 1992). Dexamethasone inhibits induction of NO synthase probably at the level of transcription of mRNA. Two receptors exist for glucocorticoids; the type I mineralocorticoid receptor where dexamethasone binds at low concentrations, and the type II receptor, which is subdivided into type IIα and IIβ, where dexamethasone will bind after saturation of the type I receptor (Wilckens, 1995). Type I and type II can lead to blockade of transcription enhanced by certain transcription factors (Wilckens, 1995).

1.3.8 Physiological effects of NO

The endothelial form of NO synthase plays a major role in the physiological regulation of blood flow and pressure (Rees *et al.*, 1989). The shear stress of the flowing blood not only increased acute NO release from the endothelial cells (Lamontagne *et al.*, 1992), but also has been shown to up regulate the expression of the NO synthase enzyme (Nishida *et al.*, 1992). In fact, with agonist activation, the enzyme translocates from the particulate to the cytosolic fraction of the cells (Michel *et al.*, 1993). NO dilates all types of blood vessels by elevation of cyclic GMP levels in smooth muscle cells (Förstermann *et al.*, 1986), and is a major endogenous vasodilator system counteracting the vasoconstriction produced by the sympathetic nervous system and the renin-angiotensin system. NO is also a potent inhibitor of platelet aggregation and adhesion to the vascular wall (Radomski *et al.*, 1990b). It has also been shown to inhibit leukocyte adherence to the vascular endothelium (Kubes *et al.*, 1991). As leukocyte adherence is one of the early events in the development of atherosclerosis, NO may protect against the development of this disease.

The neuronal isotype of NO synthase is known to play a role in synaptic plasticity and learning (Böhme *et al.*, 1991). It is thought that NO acts as a retrograde transmitter and is involved in long term potentiation (Schmidt & Walter, 1994), as excitatory amino acid-mediated transmission is accomplished when NO signals the adjacent neurones through a cyclic GMP-dependent mechanism (Garthwaite *et al.*, 1988; Bredt & Snyder, 1989). In addition, centrally released NO appears to play a role in regulating blood pressure by reducing sympathetic tone to blood vessels (Cabrera & Bohr, 1995). In peripheral neurones, NO can mediate relaxation in structures whose nerves release neither adrenergic nor cholinergic transmitters. The

bovine retractor penis muscle (Martin *et al.*, 1988) and the anococcygeus muscle (Gillespie *et al.*, 1989) are two well-documented examples where NO plays an important role in the nerve-mediated relaxation of smooth muscle.

The inducible form of NO synthase is an important component of the immune system, where large amounts of NO produce cytostatic or cytotoxic effects. The ability of NO to produce cytostasis arises from its affinity for protein non haem-bound iron, by which NO attacks and inhibits a number of key metabolic enzymes (Hibbs *et al.*, 1988) (see Pathophysiological effects, 1.3.9). One of the targets for macrophage killing is tumour cells (Stuehr & Nathan, 1989). Although the main mechanism by which monocytes mediate cell killing is through reactive oxygen intermediates, macrophages also kill using reactive nitrogen intermediates (Martin & Edwards, 1993).

1.3.9 Pathophysiological effects of NO

1.3.9.1 Neuronal NO synthase

Activation of the NMDA receptor in the central nervous system leads to release of NO (Garthwaite *et al.*, 1988). Pathophysiological effects of NO in the CNS have been linked to this activation of the NMDA receptor. It is thought that NO is involved in NMDA receptor-mediated cell death, occurring for example during cerebral ischaemia and cerebrovascular stroke. Evidence for involvement of NO comes from the ability of haemoglobin and NO synthase inhibitors to prevent neuronal death (Dawson *et al.*, 1991).

1.3.9.2 Endothelial NO synthase

NO plays an important role in maintaining the endothelium in an anti-thrombotic state. Any reduction in the activity of constitutive NO synthase can lead to increased platelet aggregation as well as increased constriction due to the secretory products of aggregating platelets. Hypercholesterolaemia and atherosclerosis are associated with a reduction in vasodilatation mediated by the endothelium (Förstermann *et al.*, 1988). It is possible that the lipoproteins present may limit L-arginine availability, or increase the breakdown of NO by free radicals formed during lipid peroxidation (Wennmalm, 1994). Reduced endothelium-dependent vasodilatation is also found in hypertensive animals (Calver *et al.*, 1993). Decreased production or activity of NO in the cardiovascular system has thus been associated with the major cardiovascular disorders, i.e. vasospasm, thrombosis and atherosclerosis (Quinn *et al.*, 1995).

1.3.9.3 Inducible NO synthase

Initial events suggesting the involvement of NO in the cytotoxicity of macrophages towards tumour cells came from Hibbs *et al.* (1987b, 1988). These investigators showed that activated macrophages produced inhibition of mitochondrial respiration in target cells and this was associated with large-scale production of nitrite and nitrate. Similarly, it appeared that the large amounts of nitrite and nitrate produced by macrophages, activated by BCG, were associated with fungistatic effects (Granger *et al.*, 1990). However, later it was shown that it was the large amounts of NO released from macrophages that induced cytotoxic and cytostatic effects in target cells (Cui *et al.*, 1994; deRojas-Walker *et al.*, 1995).

The cytostatic and cytotoxic effects of NO include inhibition of a number of enzymes. These include the oxidoreductases of complex I and II of the mitochondrial electron transport chain, aconitase of the Krebs cycle and ribonucleotide reductase, the rate-limiting enzyme for DNA synthesis (Klostergaard, 1993). The high concentrations of NO produced by activated cells also produce damage to the DNA of invading cells, with strand breaks and fragmentation resulting (Green *et al.*, 1994).

Inhibition of certain mitochondrial enzymes occurs due to NO altering the configuration of the catalytic site by co-ordinating with iron at their iron-sulphur clusters (Pellat *et al.*, 1990). Ribonucleotide reductase catalyses the reduction of ribonucleotides to their corresponding deoxyribonucleotides, thus supplying precursors for DNA synthesis. The mechanism for inhibition is thought to be due to nitrosation of a critical cysteine and a tyrosyl radical (Lepoivre *et al.*, 1992; Roy *et al.*, 1995). In addition to its effects alone, NO can react with molecular oxygen to form N_2O_3 or with superoxide anion (see 1.2.3) to form peroxynitrite. Both of these species attack DNA and cause a variety of toxic effects leading to purine and pyrimidine deamination, DNA strand breaks and cross linking (deRojas-Walker *et al.*, 1995). Macrophages also induce apoptosis, or programmed cell death, in tumour cells, and although NO plays a major role, it is likely that apoptosis occurs through more than one mechanism (Cui *et al.*, 1994)(see 1.10).

Although the large quantities of NO produced by macrophages are aimed at invading organisms, the cells expressing inducible NO synthase have on a few rare occasions been found to be susceptible to the NO that they produce. For example, stimulation of macrophages with IFN-γ and LPS produced inhibition of aconitase, NADH: ubiquinone oxidoreductase and succinate: ubiquinone oxidoreductase in the macrophage itself (Drapier &

Hibbs, 1988). Also, inhibition of cytochrome C oxidase and succinate cytochrome C reductase, after prolonged incubation with LPS and IFN-γ, resulted from the NO produced in rat astrocytes (Bolaños *et al.*, 1994).

Over-production of NO synthase upon systemic exposure to Gram-negative, and also Gram-positive bacteria, leads to the syndrome of septic shock, which can prove rapidly fatal (see Section 1.4.6).

1.4 Factors promoting the induction of NO synthase

A large number of agents are able to promote the induction of NO synthase in a wide variety of cell types (see Table 1.2). Both Gram-positive and Gram-negative bacteria are able to induce increases in NO production. Lipopolysaccharide, i.e. endotoxin, the major component of Gram-negative bacterial cells walls, is a powerful activator either when used alone or in combination with cytokines including IFN-γ, IL-1, IL-2, TNF-α or β.

1.4.1 Gram-positive bacteria

The cell wall of Gram-positive bacteria contains the peptidoglycan, muramyl dipeptide and teichoic acid, which can induce a number of immunostimulatory effects (Noso *et al.*, 1988). In most cases, teichoic acid is a weaker stimulus than peptidoglycan (Mattsson *et al.*, 1993). Gram-positive bacteria can induce the production of IL-1, IL-6 and TNF-α from human monocytes (Riesenfeld-Orn *et al.*, 1989; Heumann *et al.*, 1994), which could promote NO production from the vascular wall and thus contribute to the septic shock induced directly by Gram-positive bacteria.

CELL TYPE	INDUCER	REFERENCE
Macrophage	LPS (J774)	Marotta *et al.*, 1992
		Di Rosa *et al.*, 1990
	IFN-γ+LPS (J774)	Severn *et al.*, 1992
	IFN-γ+TNF-α	Lambert *et al.*, 1991
	(peritoneal)	
Kupffer cell	LPS	Gaillard *et al.*, 1992
Smooth muscle cell	IFN-γ+TNF-α	Geng *et al.*, 1994
	IL-1β	Hirokawa *et al.*, 1994
	LPS+IL-1β	Marczin *et al.*, 1993
Endothelial cell	TNF-α	Estrada *et al.*, 1992
	LPS	Akarasereenont *et al.*, 1994
	LPS+IFN-γ	Kilbourn *et al.*, 1990
Neutrophil	LPS+IFN-γ	van Dervort *et al.*, 1994
Astrocyte	LPS	Feinstein *et al.*, 1993
Hepatocyte	LPS	Hortelano *et al.*, 1992
Cardiac myocyte	IL-1+IFN-γ	Balligand *et al.*, 1994
	IL-1+IFN+TGF-β	
Microglia	LPS+IFN-γ	Jun *et al.*, 1994c
Mesangial cells	IL-1β	Mühl & Pfeilschifter, 1994

Table 1.2 Examples of different cell types which express nitric oxide synthase in response to varying stimuli.

1.4.2 Lipopolysaccharide

LPS is a well documented stimulus for inducing NO synthase in a wide variety of cells types (Marotta *et al.*, 1992; Gaillard *et al.*, 1992; Marczin *et al.*, 1993; Akarasereenont *et al.*, 1994; Feinstein *et al.*, 1993; Hortelano *et al.*, 1992), but little is known regarding the signal transduction pathways involved. Changes in a number of intracellular signal transduction systems have been observed after exposure of cells to LPS. A pertussis toxin-sensitive guanine nucleotide binding protein (G-protein) has been identified which can couple the binding of LPS at the cell surface to the intracellular signal transduction pathway (Jakway & Defranco, 1986; Daniel-Issakani *et al.*, 1989; Wang *et al.*, 1988). However, some actions of LPS in cells are unaffected by pertussis toxin treatment (Dzarski, 1989), suggesting that some of the responses elicited do not utilise a G-protein-dependent mechanism. LPS produces an increase in the turnover of phosphatidyl inositol and hydrolysis of phosphatidyl inositol-4-5-bisphosphate, resulting in increases in inositol-(1,4,5)-triphosphate formation (Ögmundsdottir & Weir, 1979; Prpic *et al.*, 1987). Stimulation of phospholipase D is further proposed as a mechanism by which LPS mediates its action in monocytes and macrophages (Natarajan & Iwamoto, 1994). Stimulation with LPS increases intracellular calcium levels in macrophages (Letari *et al.*, 1991) but in other studies (Drysdale *et al.*, 1987) calcium was found to play no role in activating cytotoxic activity in cells. Furthermore, protein kinase C seems to be part of the signal transduction pathway involved in the LPS-induced expression of NO synthase (see 1.7.1), as does activation of tyrosine kinase (see 1.6.1).

1.4.3 Structure of lipopolysaccharide

Lipopolysaccharide mediates many of its effects through ligand-receptor interaction with a number of immune cells. The Gram-negative bacterial cell surface consists of an inner cytoplasmic membrane and a trilayer outer cell wall structure consisting of a mucopolysaccharide-peptidoglycan layer, a phospholipid protein layer, and an outermost lipopolysaccharide layer (Raetz *et al.*, 1991). The term 'endotoxin' refers to the impure extract of LPS found in combination with different proteins. The active chemical moiety of LPS is the lipid A component, which is unique to each molecule of LPS (Watson *et al.*, 1994), although the critical chemical component of lipid A that makes it immunostimulatory has not been identified. The primary structure of lipid A has been elucidated in great detail for a number of different types of LPS and, in fact, lipid A has been synthetically manufactured, based on the structure of *Escherichia coli* lipid A (Rietschel *et al.*, 1993).

1.4.4 Receptors for LPS

There are three classes of receptor for LPS

(1) CD18 antigens, also known as β2 integrins or leukocyte integrins, bind particulate LPS when it is presented on the surface of bacteria or LPS-coated erythrocytes, and the receptors participate in phagocytosis of the particles. There are various forms of CD18, which comprise ;

CD11a/CD18

CD11b/CD18

CD11c/CD18

It is unlikely that CD18 plays a role in signal transduction of LPS-mediated effects, as its main function is in adhesion events (Wright & Jong, 1986).

(2) A scavenger receptor for LPS exists which plays no role in the signal transduction of LPS mediated effects (Freeman *et al.*, 1990). This receptor which also scavenges acetylated low-density lipoprotein (LDL) may function to remove endotoxin from the circulation and deliver it to lysosomes where it can be metabolised to less active substances (Hampton *et al.*, 1991).

(3) A CD14 receptor. Binding to CD14 is required for macrophage cytokine production (see 1.4.5). This is the only well defined LPS receptor on macrophages that mediates cell activation.

1.4.5 CD14 and LBP

Lipopolysaccharide can stimulate monocytes and macrophages to release a wide array of proteins, free radicals and lipids. The interaction between macrophages and LPS seems to occur via the CD14 receptor (Lee *et al.*, 1992). CD14 is a 53 kD glycoprotein found in two forms; membrane bound and soluble. The two forms have masses of 53 and 48 kD respectively, with the membrane-bound form being anchored via a glycosylphosphatidylinositol link to the cell surface (Hailman *et al.*, 1994). The soluble form of the receptor interacts directly with LPS and may activate cells which are deficient in the membrane-bound form of CD14 (Kielian & Blecha, 1995). The membrane-bound CD14 represents the receptor for LPS and mediates its effects in monocytes, macrophages and polymorphonuclear phagocytes (Mathison *et al.*, 1993). The receptor appears mobile in the plane of the membrane of these cells (Wright *et al.*, 1990). Although LPS can bind to CD14 independently, a protein exists that

renders LPS more accessible for recognition by CD14. This protein, lipopolysaccharide binding protein (LBP), has been isolated from the serum of various animals (Dentener *et al.*, 1993). LBP (isolated from rabbit and human serum) is a 60 kD glycoprotein that has a specific binding site and high affinity for the lipid A moiety of LPS. LBP is present in normal serum at <0.5 μg ml^{-1} and is synthesised in hepatocytes (Schumann *et al.*, 1990), with 69 % homology in the amino acid sequence of rabbit and human versions (Tobias & Ulevitch, 1993). Although it appears that LBP is required for the interaction between LPS and CD14, it has been shown that LPS can still mediate some effects in the absence of LBP, but only at high concentrations. This demonstrates that LBP may serve to bring the LPS-LBP complex close to the cell surface or allow the complex to interact with other cell surface proteins (Watson *et al.*, 1994). Thus, as LBP serves to potentiate the effects of low levels of LPS, a principal function of LBP may be to enhance the ability of the host to detect LPS early in infection. Release of cellular products would then enhance the natural defence mechanisms that combat infection. However, LBP has been shown to play a role in mediating the effects of endotoxaemia (Gallay *et al.*, 1993a), so a method to block the effects of LBP may have some therapeutic use in the treatment of endotoxin shock.

In addition to aiding the transfer of LPS to CD14 to activate cells, LBP catalyses the transfer of LPS to reconstituted high-density lipoprotein (HDL) particles (Wurfel *et al.*, 1994). Lipoprotein particles avidly bind LPS because of partitioning of the ampiphilic LPS molecule into the phospholipid surface of the lipoprotein. Binding of LPS to HDL neutralises its biological activity. LBP can also facilitate a third lipid transfer reaction involving LPS. This catalytic effect of LBP moves LPS from the LPS-CD14 complex to reconstituted HDL particles, neutralising its actions (Wurfel *et al.*, 1995).

Therefore, in addition to causing amplification of the effects of low doses of LPS, LBP also plays a role as an intermediate in the neutralisation of LPS under physiological conditions.

LBP amplifies the effects of LPS, but another protein, bactericidal/permeability increasing protein (BPIP), decreases the stimulatory effects of LPS, including TNF-α synthesis (Heumann *et al.*, 1993). BPIP also has bactericidal activity and permeabilises Gram-negative bacteria (Corradin *et al.*, 1994).

1.4.6 Septic Shock and its treatment

Although NO serves as a key signalling molecule in physiological processes such as control of vascular tone and neuronal communication, excessive and unregulated production is thought to be the cause of several pathophysiological conditions. One such condition is septic shock, where there is overwhelming evidence that LPS-induced increased NO production contributes to the vasodilatation and hypotension seen both in human cases and in animal models of endotoxin shock (Petros *et al.*, 1991; Stoclet *et al.*, 1993; Goode *et al.*, 1995). The majority of cases of septic shock result from infection with Gram-negative bacteria, where the prime initiator of the shock is endotoxin, the LPS component of the bacterial cell wall (Glauser *et al.*, 1994). However, Gram-positive bacteria can contribute from 20-40 % of cases of septic shock (see 1.4.1)(Nogare, 1991). Most of the effects and treatment of septic shock will be dealt with in relation to infection with Gram-negative bacteria since those are better understood than for Gram-positive.

1.4.6.1 Inhibitors of NO synthase

Due to over-production of NO in septic shock, inhibition of inducible NO synthase might be of benefit in this condition. In animal models, NO synthase is induced in smooth muscle cells, endothelial cells, hepatocytes and other cells types, whereas in humans, induction has been demonstrated in blood vessel walls and hepatocytes (Quinn *et al.*, 1995). However, it is likely that the majority of NO contributing to the development of hypotension during septic shock is derived from vascular smooth muscle cells in the vessel wall (Förstermann *et al.*, 1994).

When used at low concentrations, inhibitors of NO synthase produce a reversal of hypotension and increase the survival rate in animal models of septic shock (Kilbourn *et al.*, 1990; Thiemermann & Vane 1990; Nava *et al.*, 1991). This was also the case in a human study where low doses of L-NMMA increased blood pressure in patients (Petros *et al.*, 1994). Also, when administered to healthy patients, L-NMMA increased blood pressure and was well tolerated (Haynes *et al.*, 1993). In mice and rabbits, high doses of L-NMMA led to increased mortality in experimental models probably because the drugs inhibited both the inducible and constitutive forms of the enzyme (Thiemermann, 1993; Wright *et al.*, 1992). In other animal studies, conducted in mice (Minnard *et al.*, 1994) and pigs (Robertson *et al.*, 1994), administration of L-NAME increased mortality. However, Wang *et al.*, (1994b) suggested that there are changes in the amount of endothelium-derived NO released over time after the onset of septic shock. It appears that endothelium-derived NO is elevated 2 hours after onset of septic shock, and decreases 10-20 hours later. Thus, the precise manipulation of NO levels required to treat septic shock is yet to be determined.

As already noted, inhibitors of NO synthase have a role in the treatment of septic shock, although inhibition of both the inducible and constitutive enzymes leads to severe disruption of cardiovascular control, resulting in only a minor overall improvement in mortality (Calver *et al.*, 1993). One potential means of improving survival in septic shock that has been proposed is to co-administer NO-donating agents with an NO synthase inhibitor so as to replace the constitutively-produced endothelial NO (Brady & Poole-Wilson, 1993). Alternatively, an inhibitor with a high degree of selectivity for the inducible form of NO synthase could be used thus minimising disruption of endothelial regulation of vascular tone (Griffiths *et al.*, 1993; Misko *et al.*, 1993). Although certain of the inhibitors discussed previously (1.3.7) have some degree of selectivity for the inducible isoform, compounds with a much higher degree of selectivity may be required to ensure that normal endothelial function is maintained.

1.4.6.2 Effects of glucocorticoids

Glucocorticoids, such as dexamethasone, are commonly used for the treatment of septic shock (Nogare, 1991). Their mechanism of action appears at least in part to involve blockade of NO production by inhibiting the expression of the inducible form of NO synthase in smooth muscle cells in the vessel wall (Rees *et al.*, 1990). In rabbit and rat models of endotoxin shock, pretreatment with dexamethasone inhibited the drop in blood pressure and regional blood flow induced by lipopolysaccharide (Wright *et al.*, 1992; Szabó *et al.*, 1993). However, to be effective in the treatment of septic shock, glucocorticoids must be administered as rapidly as possible after the onset of sepsis (Nogare, 1991; Szabó *et al.*, 1993). In human cases of septic shock, however, even early treatment with glucocorticoids is not always effective and may lead to complications such as infections due to the

concomitant immunosuppression produced by the drugs (Nogare, 1991). Inhibition of expression of inducible NO synthase by glucocorticoids is not restricted to vascular walls, but may be a general phenomenon. For example, inhibition of expression has been found in J774 cells (Di Rosa *et al.*, 1990), various tissues in the rat including lung and liver (Knowles *et al.*, 1990) and peritoneal macrophages (Schoedon *et al.*, 1993).

1.4.6.3 Inhibition of LPS-mediated effects

Agents that inhibit the actions of LPS, for example antibodies directed at the core region of LPS which is conserved in most forms (Baumgartner & Glauser, 1993; Battafarano *et al.*, 1994; Glauser *et al.*, 1994), have also been proposed to have therapeutic potential. However, when used to treat patients with Gram-negative sepsis, the anti core LPS antibodies did not show any definite benefit to patients (Baumgartner & Glauser, 1993). Polymyxin B, a peptide with high affinity for LPS, has often been used to inhibit the actions of endotoxins. However, polymyxin B has other actions including inhibition of PKC (Nel *et al.*, 1985) and disrupts biochemical processes by membrane interactions (Lasfargues *et al.*, 1989) which make it unsuitable for use in humans. Alternatively, structural analogues of LPS could potentially be used that exert competitive inhibition at the CD14 receptor (Watson *et al.*, 1994). An LPS antagonist, B464, has already been shown to reduce LPS-induced production of NO, TNF and IL-6 by RAW 264 cells (Wang *et al.*, 1994a). Another antagonist of LPS, precursor Ia, otherwise known as compound 406, has been shown to inhibit LPS-induced production of IL-1β, TNF and IL-6 by human mononuclear cells (Flad *et al.*, 1993) and may also prove of therapeutic value. CD14 is the receptor through which LPS is proposed to mediate its effects. Antibodies to CD14 may prove to be very effective at blocking activation of macrophages by LPS

blocking cytokine production and expression of inducible NO synthase. Similarly, antibodies to LBP reduced LPS-mediated shock in mice, indicating they may have some therapeutic use (Gallay *et al.*, 1993a). Furthermore, administration of bactericidal/permeability increasing protein, which competes with LBP for binding of LPS, can block-LBP mediated binding of LPS to monocytes, leading to suppression of TNF-α release (Heumann *et al.*, 1993). Which of these strategies will prove to be effective in human septic shock remains to be determined.

1.4.6.4 Inhibition of cytokine-mediated effects

In addition to directly inducing NO synthase, LPS leads to the production of a broad array of cytokines from host macrophages. These include TNF-α, IL-1 and IL-6, and excessive secretion of these cytokines by macrophages contributes to the organ failure and death in Gram-negative sepsis. Of these, IL-1 and TNF-α are potent inducers of NO synthase in the vascular wall (Busse & Mülsch, 1990; Battafarano *et al.*, 1994; Schultz & Triggle, 1994). TNF-α is one of the most potent secreted products of LPS-mediated macrophage activation (Beutler *et al.*, 1985). It is implicated in the pathogenesis of many disease states including septic shock and inflammation, and has a role in the regulation of acute-phase protein gene expression, cellular proliferation and apoptosis (Laskin & Pendino, 1995). TNF-α alone mediates several biological effects which are important in Gram-negative sepsis including stimulation of neutrophil degranulation and increased endothelial permeability to serum proteins (Nogare, 1991). TNF-α stimulates the release of other mediators from macrophages including IL-1, IL-6, PAF, prostaglandins and NO (Beutler & Cerami, 1989), and therefore contributes to the development of septic shock by exerting positive feedback effects on cytokine production. Furthermore, TNF also exerts cytotoxic

effects on endothelial cells and may increase the degradation of endothelial constitutive NO synthase mRNA, thus reducing the NO-mediated cardiovascular tone (Estrada *et al.*, 1992; Yoshizumi *et al.*, 1993). Antibodies to TNF-α protect against death from endotoxaemia in mice, rats and rabbits, and therefore may have a useful therapeutic role in humans (Beutler *et al.*, 1985; Mathison *et al.*, 1988; Thiemermann, 1994). LPS-induced secretion of IL-1 by macrophages can induce tachycardia and hypotension and acts synergistically with TNF to induce tissue damage. Consequently, antagonists of IL-1 could be used to reduce the lethality of LPS-induced septic shock in humans. Use of an IL-1 receptor antagonist, IL-1ra, has already been shown to reduce the mortality of endotoxin-induced shock in rabbits and rats (Ohlsson *et al.*, 1990; Thiemermann, 1994).

1.4.7 Tolerance to LPS

The development of a tolerant state to the pathological effects of LPS can be induced by pre-exposure of cells to low doses of endotoxin. This is a well recognised phenomenon which can develop within hours of LPS injection and requires the presence of the biologically active moiety, lipid A. There appear to be two phases of endotoxin tolerance: (1) an early phase tolerance which begins 24-96 hours after the initial exposure to endotoxin whose mechanism is unclear and (2) a late phase tolerance which develops over several weeks after the initial exposure, and is associated with the production of anti-endotoxin antibodies (Szabó *et al.*, 1994). Down-regulation of CD14 binding sites may also play a part in the desensitisation process, as pre-exposure to LPS can lead to a decrease of up to 70 % in the number of binding sites (Fahmi & Chaby, 1993). However, it is likely that only prolonged exposure to LPS can lead to down-regulation of CD14 receptors since the tolerance to LPS precedes down-regulation (Labeta *et*

al., 1993). It is possible that different regulatory pathways control the response to endotoxin in tolerant and in naive macrophages since pre-exposure of naive macrophages to LPS can prime the cells to release elevated levels of certain inflammatory mediators upon subsequent exposure to LPS. The findings of Seatter *et al.* (1994) demonstrate that pre-exposure to LPS inhibits TNF-α secretion by peritoneal macrophages, but actually increases IL-1 secretion. A similar action was also found by West *et al.* (1994), where pretreatment with LPS led to decreased release of TNF but increased release of NO. Zhang & Morrison (1993) demonstrated that varying the dose of LPS used for pretreatment selectively affects either NO or TNF-α production, depending on the concentration administered. The primary dose of LPS inducing down regulation of NO is lower than that needed for inducing down regulation of TNF-α. The findings of Severn *et al.* (1993) demonstrate that LPS pre-exposure can reduce the production of NO on subsequent exposure to LPS and IFN-γ. One proposal for the mechanism of tolerance to LPS *in vivo* is elevation of plasma glucocorticoid levels which could suppress the effects of further exposure to LPS (Szabó *et al.*, 1994). However, this cannot explain the mechanism of tolerance in macrophages grown in cell culture. Pretreatment with the NO synthase inhibitor, L-NMMA, abolished the ability of microdoses of LPS to protect against further exposure, suggesting a role for NO in the process of tolerance (Rojas *et al.*, 1993). Thus, repetitive exposure to LPS appears to elicit a complex process. LPS not only desensitises the cell to future stimulation, but can selectively orientate the macrophage towards a specific response.

1.4.8 Mechanisms of activation by IFN-γ

Interferons are a family of glycoproteins that, after interaction with specific receptors on the outer surface of target cells, induce a wide array of biological actions. There are three major classes of interferons; leukocyte or alpha interferon (IFN-α), fibroblast or beta interferon (IFN-β) and immune or gamma interferon (IFN-γ) (Pestka & Langer, 1987). IFN-γ interacts with a single class of cell surface receptor. In humans and mice, this receptor is a single chain glycoprotein which has extracellular and intracellular domains and both of these are involved in the binding of the IFN-γ molecule (Szente & Johnson, 1994; Szente *et al.*, 1994). Internalisation and receptor recycling form important components of the ability of IFN-γ to induce non-specific tumouricidal activity in macrophages (Celada & Schreiber, 1987). There are a variety of proposals to explain the mechanisms by which IFN-γ mediates its effects. It has been shown to increase protein kinase C activity (Hamilton *et al.*, 1985; Fan *et al.*, 1988), and this is required for the Fcγ receptor and Ia antigen expression (Politis & Vogel, 1990). IFN-γ elevates intracellular calcium levels and, in addition to activating calcium calmodulin kinases, this may contribute to stimulation of PKC (Weiel *et al.*, 1985). Alteration in the turnover of fatty acids in membrane phospholipids is increased by IFN-γ (Darmani *et al.*, 1993; Jackson *et al.*, 1992), although other findings demonstrate that activation of PKC may be independent of phosphatidylinositol metabolism. Instead, it has been proposed that DAG may be generated only via phosphatidylcholine metabolism (Sebaldt *et al.*, 1990). However, in other studies, the involvement of PKC in IFN-γ-induced activation appears doubtful (Radzioch & Varesio, 1988), as IFN-α and -β, but not IFN-γ produced a rise in DAG and IP_3 levels (Yap *et al.*, 1986). In addition to activating macrophages as part of an inflammatory response,

IFN-γ enhances the adhesiveness of monocytes to endothelial cells (Wang *et al.*, 1994c), by mechanisms involving a number of different adhesion molecules.

1.4.9 Combined stimulation by LPS and IFN-γ

Macrophages resident in tissues in a resting state are relatively resistant to acquiring the ability to destroy neoplastic cells. There are, however, some cases when immature mononuclear phagocytes can respond to inductive signals. Upon exposure to a macrophage activating factor, now known to be IFN-γ, macrophages enter a state where, although not yet cytolytic, they can be made to become so upon addition of a further stimulus. This is referred to as the primed state. Upon subsequent exposure to small amounts of LPS, the macrophages become fully activated and can carry out cytotoxic and cytostatic functions (Hamilton & Adams, 1987).

The sequence of stimulation with LPS and IFN-γ can profoundly influence the extent to which inducible NO synthase is expressed. Either simultaneous exposure to both agents or pre-treatment for 2, 4 or 6 hours with IFN-γ followed by stimulation with LPS results in NO production (Lorsbach & Russell, 1992; Walter *et al.*, 1994). Exposure to LPS followed by stimulation by IFN-γ 2 or 6 hours later gives little response (Lorsbach & Russell, 1992; Walter *et al.*, 1994). Furthermore, pretreatment with IFN-γ followed 24 hours later by stimulation with a mycobacterium (*Mycobacterium bovis*) produces high levels of NO, whereas if the order is reversed, NO production is very low (Hanano & Kaufmann, 1995). A possible mechanism to account for the co-operation between LPS and IFN-γ is that LPS may be able to stabilise the increase in inducible NO synthase

mRNA induced by exposure to IFN-γ (Weisz *et al.*, 1994). Furthermore, IFN-γ may positively regulate the transcriptional activation of the gene for NO synthase in macrophages stimulated by LPS (Lorsbach *et al.*, 1993). It is known that LPS and IFN-γ indirectly activate the promoter region of the mouse gene for NO synthase, and simultaneous activation of the two regions synergistically increases expression of the gene (Lowenstein *et al.*, 1993). In priming for tumouricidal activity which may at least partly involve NO, the effects of IFN-γ are mimicked by the combination of a phorbol ester and a calcium ionophore, suggesting that IFN-γ primes cells via alterations in calcium levels and PKC activity (Celada & Schreiber, 1986; Somers *et al.*, 1986).

1.5 NO and human cells

NO represents an important part of the cytotoxic and cytostatic effector system of monocytes and macrophages in rodents, but there is doubt as to whether a similar system exists in human macrophages (McCall & Vallance, 1992). Human monocytes and macrophages can be activated by LPS to produce cytokines such as TNF-α (Gallay *et al.*, 1993b) and IL-6 (Gessani *et al.*, 1993), but appear to lack the ability to produce NO. Stimulation of monocytes and macrophages with IL-1β (Schneeman *et al.*, 1994), IFN-γ (Martin & Edwards, 1994), LPS plus IFN-γ (Murray & Teitelbaum, 1992), LPS, IFN-γ and TNF alone and in combination (Zembala *et al.*, 1994) or LPS, IFN-γ, GM-CSF and TNF-α alone or in combination (Schneeman *et al.*, 1993), were unable to stimulate the human macrophages or monocytes to produce NO. A possible explanation put forward to explain these findings is the inability of human macrophages to produce tetrahydrobiopterin, an essential cofactor for NO synthase. However, supplementation of the cells

with tetrahydrobiopterin in combination with cytokines (IFN-γ, TNF-α) still did not lead to production of NO (Sakai & Milstien, 1993). The lack of ability to release NO is also found in human polymorphonuclear leukocytes (Yan *et al.*, 1994). Other studies have, however, demonstrated the ability of human monocytes or macrophages to express inducible NO synthase mRNA following stimulation by LPS and IFN-γ (Reiling *et al.*, 1994), to release nitrite following stimulation by IL-4 (Paul-Eugène *et al.*, 1994), or TNF-α plus IFN-γ (Muñoz-Fernández, 1992) and to exhibit anti-parasitic effects upon stimulation with IFN-γ (Gyan *et al.*, 1994). Thus, there is conflicting evidence as to the ability of human monocytes and macrophages to produce NO upon stimulation by LPS and a variety of cytokines.

1.6 Tyrosine kinase

Protein tyrosine kinases play a central role in signal transduction pathways that regulate cell proliferation and differentiation. The kinases can be divided into two main groups based on their structures. One group which possesses extracellular domains is the receptor protein tyrosine kinases such as the receptors for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), nerve growth factor (NGF) and insulin (Ullrich & (Schlessinger, 1990). The second major group is the non-receptor tyrosine kinases which lack extracellular sequences. This group of tyrosine kinases migrate to receptors which lack intrinsic tyrosine kinase activity (Bolen *et al.*, 1992).

1.6.1 LPS and tyrosine kinase

Activation of tyrosine kinase by cytokines is a key event in the signal transduction pathways that mediate some events induced by cytokines (Ullrich & Schlessinger, 1990). Lipopolysaccharide induces tyrosine

phosphorylation in macrophages, and this process mediates many of the inflammatory effects produced by these cells (Dong *et al.*, 1993a; Geng *et al.*, 1993). Initial studies found that LPS rapidly increased tyrosine phosphorylation within 4-5 min of exposure (Weinstein *et al.*, 1991). Further evidence for the involvement of tyrosine kinase arises from the findings that inhibitors of this kinase, including tyrphostin, herbimycin A and genistein, block LPS-induced production of TNF-α, IL-6 and IL-1β by human monocytes and murine macrophages (Beaty *et al.*, 1994; Geng *et al.*, 1993; Novogrodsky *et al.*, 1994), as well as LPS-mediated tumouricidal activity of murine peritoneal macrophages (Dong *et al.*, 1993a). Two of the proteins rapidly phosphorylated following exposure of macrophages to LPS have masses of 41 and 44 kD, and are isoforms of a mitogen-activated protein (MAP) kinase (Ding *et al.*, 1993; Weinstein *et al.*, 1992). In fact, tyrosine phosphorylation of MAP kinase is one of the earliest known responses of macrophages to LPS and, since MAP kinases appear to modulate cellular processes in response to extracellular signals, these kinases may be important targets for LPS action in macrophages (Dong *et al.*, 1993b). There is evidence that the CD14 receptor in human monocytes is involved in the LPS-mediated activation of MAP kinases (Liu *et al.*, 1994a), as well as in the activation of tyrosine kinase resulting in TNF-α and IL-1α release (Stefanová *et al.*, 1993). Furthermore, antibodies to the CD14 receptor inhibit induction of protein tyrosine phosphorylation by LPS (Han *et al.*, 1993; Weinstein *et al.*, 1993), suggesting that LPS-LBP binding to CD14 triggers intracellular protein phosphorylation, which may lead to the expression of anti-bacterial responses.

1.6.2 IFN-γ and tyrosine kinase

IFN-γ signal transduction, which occurs following binding of IFN-γ to cell surface receptors, involves activation of tyrosine kinases (Ralph *et al.*, 1995), which then phosphorylate transcription factors (Pellegrini & Schindler, 1993). Further evidence exists linking tyrosine kinase activity to activation of cells by IFN-γ. For example, IFN-γ has been shown to stimulate IL-12 production via stimulation of tyrosine kinase (Yoshida *et al.*, 1994), a process blocked by the tyrosine kinase inhibitors, herbimycin A and genistein. Further, stimulation of macrophages with IFN-γ has been shown to induce an increase in tyrosine phosphorylation of MAP kinases (Ding *et al.*, 1994). Such tyrosine kinases involved in the IFN-γ-mediated activation include those belonging to the *JAK* family of non receptor protein tyrosine kinases. Although classed as non receptor kinases, many members of this group can associate with some kinds of cell surface ligand binding protein (Bolen *et al.*, 1992). This family comprises the *JAK*1, *JAK*2 and *Tyk*2 subtypes (Argetsinger *et al.*, 1993; Silvennoinen *et al.*, 1993), and it appears that the *JAK*1 and *JAK*2 subtypes play an essential role in IFN-γ response pathways (Müller *et al.*, 1993; Watling *et al.*, 1993; Igrashi *et al.*, 1994).

1.6.3 Tyrosine kinase and NO synthase

Evidence for the involvement of tyrosine kinase leading to the production of NO has been observed by many workers. Stimulation of J774.2 cells with LPS led to generation of NO, a process blocked by two inhibitors of tyrosine kinase, genistein and erbstatin (Akarasereenont *et al.*, 1994). Moreover, the combination of LPS and IFN-γ produced a tyrosine kinase-dependent increase in NO production in RAW 264.7 cells (Paul *et al.*, 1995),

astrocytes (Feinstein *et al.*, 1994) and C3H/HeJ cells (Dong *et al.*, 1993c). In these studies, activity of NO synthase was reduced by various inhibitors of tyrosine kinase, including genistein, herbimycin A and tyrphostin. Furthermore, in rat aortic smooth muscle cells, induction of NO synthase by LPS or IL-1β was blocked by the tyrosine kinase inhibitors genistein and geldanamycin (Marczin *et al.*, 1993). Thus, protein tyrosine kinase activity appears to be involved in the signal transduction pathway for LPS and other immunomodulatory cytokines in the induction of NO synthase in a wide variety of cells.

1.7 Protein kinase C

Protein kinase C (PKC) plays an important role in a wide variety of cellular functions including cell proliferation, gene expression and signal transduction processes (Nishizuka, 1988). The gene structure of several isozymes have been established so far, namely α, βI, βII, γ, δ, ε, η, θ, μ, ι/λ and ζ isoforms (Fujihara *et al.*, 1994). Four of the isozymes, α, βI, βII and γ, require calcium and DAG for activation and are classified as conventional protein kinase C's. The novel isotypes, δ, ε and θ are calcium-independent and DAG-activated and the atypical isoforms ι, λ and ζ are not DAG activated (Parker *et al.*, 1995).

1.7.1 LPS and protein kinase C

Wightman & Raetz (1984) demonstrated the ability of lipid A to activate PKC by measuring changes in the activity of the enzyme. As noted previously, LPS produces an increase in the turnover of phosphatidyl

inositol and hydrolysis of phosphatidyl inositol-4-5-bisphosphate with corresponding increases in IP_3 (Ögmundsdottir & Weir, 1979; Prpic *et al.*, 1987). Macrophages treated with LPS or lipid A exhibit a characteristic pattern of protein phosphorylation (Weiel *et al.*, 1986); five distinct proteins are phosphorylated as shown by gel electrophoresis and radio labelling. Initiation of polyphosphoinositide hydrolysis triggers protein phosphorylation via PKC in many different cell types, as DAG, one of the products of polyphosphoinositide hydrolysis, is a potent activator of PKC (Nishizuka, 1984), and PKC is known to play a role in some of the inflammatory processes in macrophages mediated by LPS (Hamilton & Adams, 1987). Evidence for the involvement of PKC in LPS-mediated effects is fairly widespread. For example, blockade of PKC by the inhibitors H-7 and staurosporine, inhibited production of TNF-α and IL-1β by peritoneal macrophages (Nakano *et al.*, 1993). Moreover, the PKC inhibitors staurosporine and sphingosine reduced LPS-induced TNF production by alveolar macrophages (Tschaikowsky, 1994). Further, H-7 inhibited LPS-induced cytotoxicity in macrophage cell lines (Novotney *et al.*, 1991) and H-7 and staurosporine inhibited LPS-induced increases in arachidonic acid metabolism leading to thromboxane B_2 release by peritoneal macrophages (Geisel *et al.*, 1991). However LPS-stimulated release of TNF-α by rat Kupffer cells was unaffected by H-7 or staurosporine (Weinhold *et al.*, 1991), demonstrating that not all LPS-mediated inflammatory effects are mediated via PKC activation.

1.7.2 IFN-γ and protein kinase C

As noted previously (1.4.8), IFN-γ activates protein kinase C (Sebaldt *et al.*, 1990; Hamilton *et al.*, 1985; Politis & Vogel 1990; Fan *et al.*, 1988) as

demonstrated by a direct increase in enzyme activity, and also by the ability of inhibitors, e.g. staurosporine and H-7, to reduce IFN-γ-mediated effects. Thus, both LPS and IFN-γ can initiate PKC activation.

1.7.3 Protein kinase C and NO synthase

In addition to tyrosine kinase, another pathway implicated in the induction of NO synthase is activation of PKC. Evidence for the involvement of PKC in the induction of NO synthase is widespread. Inhibition of PKC using the inhibitor Ro 31-8220 inhibited LPS- and LPS plus IFN-γ-stimulated increases in NO synthase activity in the RAW 264 cell line (Paul *et al.*, 1995). In the J774 macrophage cell line, NO synthase activity stimulated by the combination of LPS and IFN-γ was also reduced by the PKC inhibitor, Ro 31-8220 (Severn *et al.*, 1992; Sands *et al.*, 1994a). Similarly, treatment of peritoneal macrophages with the PKC inhibitor, staurosporine, abolished the synergistic co-operation between IFN-γ and PMA to induce NO synthase (Jun *et al.*, 1994a).

Activation of phosphatidylcholine-specific phospholipase C (PC-PLC) produces DAG and subsequently activation of PKC (Sands *et al.*, 1994b). Inhibition of PC-PLC by the specific inhibitor D609, inhibited NO production in response to LPS, IFN-γ or a combination of both (Tschaikowsky *et al.*, 1994). Therefore, expression of NO synthase may involve the activation of a PC-PLC rather than a phosphatidyl inositol-specific PLC. Stimulation of J774 cells with LPS and IFN-γ resulted in the translocation from the cytosol to the membrane of predominantly the epsilon form of PKC, suggesting that it is this isoform that is involved in the induction process (Sands *et al.*, 1994a). However, Fujihara *et al.* (1994)

suggested that the βII isoform is involved in the production of NO synthase by J774 cells, due to the down-regulation of the βII isoform and the reduction in NO production by prolonged exposure to PMA (see 1.7.5). Further, blockade of the δ isoform of PKC by elevated NO levels (see 1.9) demonstrated a critical role of this isoform in the induction of NO synthase by LPS and IFN-γ (Jun *et al.*, 1994b).

1.7.4 Inhibitory effects of PKC activation on induction of NO synthase

From the above, it appears that PKC plays a role in the induction of NO synthase. In most cases, expression of NO synthase took place following activation of PKC by LPS alone or by LPS in combination with IFN-γ. However, it has been reported that activation of PKC can produce inhibition of induction of NO synthase in certain circumstances. For example, in rat mesangial cells, either inhibition using the selective PKC inhibitor calphostin C, or down-regulation of PKC by prolonged exposure to phorbol ester (see 1.7.5), potentiated the IL-1β-induced expression of inducible NO synthase (Mühl & Pfeilschifter, 1994). Furthermore, in vascular smooth muscle cells, activation of PKC by a phorbol ester reduced cytokine-induced nitrite accumulation, an effect blocked by the PKC inhibitor, calphostin C (Geng *et al.*, 1994). Moreover, in rat peritoneal macrophages (Hortelano *et al.*, 1993) and rat hepatocytes (Hortelano *et al.*, 1992), it has been shown that a clear antagonism exists between LPS and phorbol esters in inducing the production of NO. The LPS-stimulated increase in NO was reduced in the presence of a phorbol ester, although whether this was due to activation or down-regulation of PKC was not established.

1.7.5 Down-regulation of PKC

Phorbol esters are poorly metabolised tumour promoters, but also have a wide range of biological effects through mimicking the effects of diacyl glycerol in activating PKC (Weiel *et al.*, 1985). However, prolonged treatment with phorbol esters down-regulates PKC (Dieter & Fitzke, 1991). Phorbol ester-induced down regulation is caused by an increased rate of proteolysis (Young *et al.*, 1987), without a change in the rate of synthesis. The proteases involved in the down-regulation process have not been defined, although a leading contender is the calcium-activated neutral protease otherwise known as calpain (Pontremoli *et al.*, 1988). Calpain exists in two forms; type I which is active in the micromolar range of calcium, and type II, which requires a millimolar range of calcium for activity. In rat brain, calpain cleaves the α, β and γ subtypes of PKC. This proteolysis may be the initiating step in the degradation of PKC (Kishimoto *et al.*, 1989). For example, treatment of 3T3 mouse cells with a phorbol ester inhibited enzymatic activity of PKC almost completely after 24 hours (Rodriguez-Pena & Rozengurt, 1984). However, different isoforms of PKC in different cell types can be differentially down-regulated (Parker *et al.*, 1995). For example, in the J774 macrophage cell line, isoform βII was eliminated from the cell via prolonged exposure to phorbol esters (Fujihara *et al.*, 1994).

1.8 Regulation of Induction of NO Synthase by Cyclic Nucleotides

1.8.1 Effects of cyclic AMP on inflammatory function

The ability of macrophages to participate in the inflammatory response is

regulated by cyclic AMP. Cyclic AMP tends to suppress the inflammatory response, and does so by a number of different mechanisms. One such mechanism is to reduce NO production. For example, in J774 cells, prostanoids which stimulate adenylate cyclase, e.g. PGE_2 and PGI_2, inhibit LPS-induced nitrite production by the cells, indicating a reduced expression of the NO synthase enzyme (Marotta *et al.*, 1992). Here, PGE_2 and PGI_2 affected only the induction process, as they had no effect on enzyme activity once the NO synthase had been expressed. Furthermore, elevated cyclic AMP levels in J774 macrophages, achieved by incubating cells with phosphodiesterase inhibitors or 8 bromo cyclic AMP, a membrane permeant analogue of cyclic AMP, inhibited nitrite accumulation stimulated by LPS or by LPS and IFN-γ in combination (Bulut *et al.*, 1993). Again, the agents that elevated cyclic AMP were in contact with the cells when they were stimulated with LPS and IFN-γ, indicating that they were probably affecting the induction process. Moreover, in mouse peritoneal macrophages, elevation of cyclic AMP levels during activation of cells, by the addition of 8 bromo cyclic AMP or the phosphodiesterase inhibitor IBMX, inhibited LPS-mediated nitrite accumulation (Raddassi *et al.*, 1993). In addition, elevation of cyclic AMP by noradrenaline in astroglia cells suppressed LPS-induced nitrite production, thus indicating reduced induction of NO synthase (Feinstein *et al.*, 1993). Again, noradrenaline had to be present at the time of stimulation of the cells by LPS. In addition to induction of NO synthase being suppressed, elevated cyclic AMP levels can reduce the production of cytokines by cells. For example, in human monocytes, elevation of cyclic AMP levels by use of PDE inhibitors, including IBMX and rolipram, inhibited LPS-stimulated production of TNF-α (Endres *et al.*, 1991; Prabhaker *et al.*, 1994) and IL-1β (Verghese *et al.*, 1995). Also, in human astrocytes, LPS-mediated production of IL-1β was inhibited by elevated cyclic AMP levels using 8 bromo cyclic AMP or forskolin, an agent that

stimulates adenylate cyclase (Willis & Nisen, 1995). Further, expression of class II major histocompatibility complex (MHC) gene production induced by IFN-γ was inhibited by elevated cyclic AMP levels using PGE_2 (Figueíredo *et al.*, 1990). Moreover, unstimulated release of TNF-α from macrophages was inhibited by elevating cyclic AMP levels using PGE_2 (Renz *et al.*, 1988) and in mice, cyclic AMP levels elevated by dibutyryl cyclic AMP reduced LPS-mediated TNF-α production (Inoue *et al.*, 1995).

In contrast to inhibiting aspects of the inflammatory response, cyclic AMP can actually potentiate the response in certain cell types including smooth muscle cells. Following elevation of cyclic AMP levels using forskolin and prostaglandins, IFN-γ-induced production of nitrite and expression of NO synthase mRNA levels in vascular smooth muscle cells were enhanced (Koide *et al.*, 1993). Furthermore, dibutyryl cyclic AMP, forskolin and a PDE inhibitor enhanced IL-1β-induced NO production in rat aorta smooth muscle cells (Hirokawa *et al.*, 1994). In rat aortic smooth muscle cells, forskolin and isoprenaline also enhanced IL-1β-induced nitrite production (Schini-Kerth *et al.*, 1994) and using forskolin, IL-Iβ-mediated nitrite production was enhanced (Scott-Burden *et al.*, 1994). Even in the absence of IFN-γ, forskolin produced a small increase in nitrite accumulation when used alone (Koide *et al.*, 1993). Cyclic AMP has also been shown to potentiate NO synthase expression. In peritoneal macrophages, cholera toxin and dibutyryl cyclic AMP each enhanced LPS-stimulated nitrite production, and each also produced nitrite when used in the absence of LPS (Sowa & Przewlocki, 1994). Furthermore, in rat brain microvessel endothelial cells, nitrite production stimulated by IFN-γ and IL-1 was potentiated by 8 bromo cyclic AMP or isoprenaline (Durieu-Trautmann *et al.*, 1993). Finally in rat Kupffer cells, LPS-mediated nitrite production was enhanced in the presence of PGE_2 (Gaillard *et al.*, 1992).

Thus, cyclic AMP is an important intracellular signalling agent involved in regulating expression of various proteins. Elevation of cyclic AMP has a dual role depending on cell type and conditions; it reduces the induction of NO synthase and suppresses the release of inflammatory mediators and cytokines from certain cells involved in the inflammatory reaction, but it can also positively regulate the expression of inducible NO synthase and thus enhance the production of NO in others.

1.8.2 Effects of cyclic GMP on NO production

Cyclic GMP is a mediator in eliciting diverse physiological responses and is especially important in mediating the effects of NO. NO causes relaxation of vascular smooth muscle cells by activation of soluble guanylate cyclase (Kukovetz *et al.*, 1979). NO constitutively produced by endothelial cells diffuses to neighbouring smooth muscle cells where it binds to the haem group of soluble guanylate cyclase and activates it causing the production of cyclic GMP (Ignarro, 1989). There is great potential for cyclic GMP to regulate cell function via a cyclic GMP-dependent protein kinase following the production of NO (Pryzwansky *et al.*, 1995). This is especially the case in vascular smooth muscle where elevated cyclic GMP levels lead to the activation of a protein kinase (PKG) which then lowers intracellular calcium levels by a number of mechanisms. The elevated level of cyclic GMP as a result of constitutively released NO is an important mediator in maintaining the NO dependent vasodilator tone that is essential for the regulation of blood flow and pressure (Vallance *et al*, 1989). Cyclic GMP is also important in maintaining platelets in an anti-aggregatory state via extrusion of intracellular calcium (Johansson & Haynes, 1992). Although elevation of

cyclic AMP levels can profoundly modulate expression of the NO synthase enzyme and can regulate the release of cytokines from cells, cyclic GMP does not appear to have the same modulatory effect. For example, 8 bromo cyclic GMP, a membrane permeant analogue of cyclic GMP has no effect on IFN-γ-induced nitrite production in rat thoracic smooth muscle cells in culture (Koide *et al.*, 1993), and has no effect on nitrite accumulation induced by the combination of TNF and IFN-γ in rat brain microvessel endothelial cells (Durieu-Trautmann *et al.*, 1993). Similarly, dibutyryl cyclic GMP, another membrane permeant analogue of cyclic GMP, has no effect on IL-induced production of nitrite in rat aortic smooth muscle cells (Hirokawa *et al.*, 1994). Thus, cyclic GMP appears not to have the extensive modulatory role that cyclic AMP can command.

1.9 Negative Feedback Role of NO

The process of negative feedback provides an important regulatory system in cells. Such a system appears to exist for NO production by cells, where NO can regulate the extent of its own production. For example, in human mononuclear cells, SIN-1, a generator of NO, suppressed LPS-stimulated synthesis of IL-1β (Endres *et al.*, 1991). Furthermore, the activity of constitutive endothelial NO synthase was reduced by the addition of NO or the NO donor S-nitroso-acetyl-penicillamine (SNAP)(Buga *et al.*, 1993) and the activity of a crude bovine cerebellum NO synthase was inhibited by NO and by the NO donors, SNAP, sodium nitroprusside and GTN (Rengasamy & Johns, 1993a). Moreover, NO synthase induced by LPS plus IFN-γ in alveolar macrophages had its activity reduced by the addition of NO or SNAP (Griscavage *et al.*, 1993) and the activity of neuronal NO synthase from rat cortex was inhibited by the NO donors sodium nitroprusside,

hydroxylamine and GTN (Vickroy & Malphurs, 1995). Further, the activity of muramyldipeptide-induced NO synthase in alveolar macrophages was reduced by the addition of the NO donors SIN-1 and nitrosoglutathione (Morin *et al.*, 1994).

It is not known whether inhibition of NO synthase involves the activity of PKG via elevated cyclic GMP levels, or a direct inhibitory action by NO itself. The available evidence however, suggests that these inhibitory effects are independent of cyclic GMP. Assreuy *et al.* (1993) observed that in J774 cells, NO synthase activity induced by LPS and IFN-γ was inhibited by increasing levels of the NO donor, SNAP. However, this decrease in NO synthase activity was not mimicked by the cyclic GMP analogue, 8 bromo cyclic GMP, ruling out cyclic GMP as the cause of the drop in activity of NO synthase. Mechanisms proposed to explain the ability of NO to inhibit its own production include the possibilities that NO disrupts NO synthase mRNA or blocks transcriptional activity of the NO synthase gene by cytokines (Park *et al.*, 1994a). Alternative explanations have been suggested, however, since NO can bind to the haem iron of nitric oxide synthase to form haem-nitrosyl complexes, leading to inhibition of the enzyme (Hurshman & Marletta, 1995). NO dissociates slowly once it has formed a haem iron-NO complex (Rengasamy & Johns, 1993a), and thus NO-mediated inhibition of NO synthase is relatively long lasting. Also, NO competes with O_2 for nitric oxide synthase, and thus can inhibit the catalytic activity of the enzyme by reducing the availability of oxygen for formation of NO (Hurshman & Marletta, 1995). NO reversibly inhibits PKC (Gopalakrishna *et al.*, 1993) and has been shown to inhibit the δ isoform which is crucially involved in the process of expression of inducible NO synthase (Jun *et al.*, 1994b).

In addition to inhibiting NO synthase, NO has the ability to suppress the production of other biologically active compounds from cells. For example, the addition of the NO donor, SIN-1, inhibited the production of TNF by RAW 264.7 cells (Eigler *et al.*, 1995). Similarly, the production of IL-3 from spleen cells was inhibited by the NO donors SIN-1 and S-nitroso-glutathione (Marcinkiewicz & Chain, 1993). Moreover, the production of extracellularly released oxygen metabolites from activated neutrophils (Forslund & Sundqvist, 1995), the release of histamine from mast cells (Masini *et al.*, 1991) and the chemotactic, degranulation and leukotriene producing functions of human polymorphonuclear leukocytes (Moilanen *et al.*, 1993) were reduced by a variety of different NO donors.

Although most evidence indicates a suppressive feedback of NO on the inflammatory response, a few findings exist which contradict this. NO from the NO donor, sodium nitroprusside, has been shown to increase the release of TNF-α from human neutrophils (van Dervort *et al.*, 1994). In addition, NO derived from the NO donor S-nitroso-glutathione, increased the release of TNF-α and IL-1α from peritoneal macrophages (Marcinkiewicz *et al.*, 1995), thus helping to augment the inflammatory response. Why these reports differ from the majority of findings where NO dampens down cytokine release remains to be established.

1.10 NO and Apoptosis

Apoptosis, a form of programmed cell death, involves condensation of the nucleus of the cell with internucleosomal cleavage of DNA (Gerschenson & Rotello, 1992). NO is thought to be a trigger for apoptosis, and it has been proposed that the NO produced by macrophages induces apoptosis in these

cells (Sarih *et al.*, 1993; Albina *et al.*, 1993). Both endogenously produced NO and exogenously added NO, via the NO donor, NOC-18, produced apoptosis in macrophages, although a far greater concentration of exogenous NO was needed (Shimaoka *et al.*, 1995) . NO-induced apoptosis by four different NO donors was inhibited by activation of PKC with 10-O-tetra-decanoylphorbol-13-acetate, suggesting a role for PKC in the apoptotic process (Messmer *et al.*, 1995). In addition to causing apoptosis in macrophages producing NO, NO has been shown to produce apoptosis in target cells. Tumour cells are one such target, and macrophage-induced apoptosis arises in these cells through both NO-dependent and NO-independent pathways (Cui *et al.*, 1994). In contrast to the majority of findings where NO appears to contribute to the process of apoptosis, NO has been found to protect against apoptosis in human eosinophils derived from peripheral blood (Beauvais *et al.*, 1995).

1.11 Aims of project

A major part of this thesis is concerned with an investigation of the induction of NO synthase in the J774.7 macrophage cell line, and of the stimuli and factors which promote or otherwise regulate its induction. A greater understanding of the mechanisms involved in the induction process may represent a way forward in the treatment of septic shock.

Free radical production represents both a damaging and a protective feature of certain cells of the immune system. Investigation of production of reactive oxygen intermediates by the J774.7 macrophage cell line constitutes a further part of this thesis.

METHODS

CHAPTER 2

Part 1
Macrophages

2.1.1 MACROPHAGE CELL CULTURE

2.1.1.1 RAW 264 cells

The RAW 264 murine macrophage cell line was purchased from The European Collection of Animal Cell Cultures, Porton Down, Salisbury. On delivery, RAW 264 cells, suspended in bicarbonate buffered Dulbecco's Modified Eagle's Medium (DMEM) in a 25 cm^2 flask, were placed in an incubator (NAPCO model no. 5410) at 37°C under an atmosphere of 5 % CO_2 and left in the incubator overnight to recover from transport. Upon reaching confluence, the cells were removed from the flask by means of a cell scraper (Costar) and were transferred to sterile culture tubes (Falcon) where they were spun at 400g for 4 min at 10°C and the medium discarded. The cell pellet was then washed with 30 ml sterile saline (0.9 % sodium chloride, Baxter), spun at 400g for 4 min at 10°C, washed for a second time and then re-suspended in 60 ml of HEPES (20 mM, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) - buffered DMEM supplemented with 10 % foetal calf serum, 2 mM glutamate, 200 u ml^{-1} benzyl penicillin and 200 µg ml^{-1} streptomycin. This is subsequently referred to as full growth medium. The cell suspension was split into three aliquots and each was seeded into a 80 cm^2 flask (Costar/Nunc). The cells were placed in an incubator (Flow model no. 160) at 37°C under an atmosphere of air and grown until confluent.

2.1.1.2 J774.7 cells

J774.7 cells (see Fig 2.1) were kindly donated by the Department of Immunology, University of Glasgow. On delivery, J774.7 cells suspended in bicarbonate-buffered DMEM in a 25 cm^2 flask, were placed in an incubator (NAPCO model no. 5410) at 37°C and under an atmosphere of 5 % CO_2 and left in the incubator overnight to recover from transport. Upon reaching confluence, the cells were removed from the flask by means of a cell scraper (Costar), and were spun and washed with saline as described above. The cells were suspended in 60 ml full growth medium and were split into three aliquots and each was seeded into a 80 cm^2 flask (Costar/Nunc). The cells were placed in an incubator (Flow model no. 160) at 37°C under an atmosphere of air and grown until confluent.

2.1.1.3 Preparation of macrophages for experiments

For each of the two cell lines, the medium was poured off every third day, the cells were then washed twice with 10 ml sterile saline, and 20 ml fresh full growth medium added. On reaching confluence, the cells were removed from the flask, again by means of a cell scraper (Costar), and were transferred to sterile tubes (Falcon) and spun at 400g for 4 min at 10°C and the medium discarded. The cell pellet was then washed with 30 ml sterile saline (Baxter), spun at 400g for 4 min at 10°C, washed for a second time and then re-suspended in 250 ml full growth medium. The cells suspension was transferred to a sterile, siliconised (Repelcote, Hopkin and Williams) Techne stirrer bottle. Here the cells were grown in a 250 ml suspension, stirred at 25 rpm, and incubated at 37°C (Techne biological water bath model no. MWB-10L and Techne Stirrer model no. 104L). The cells were

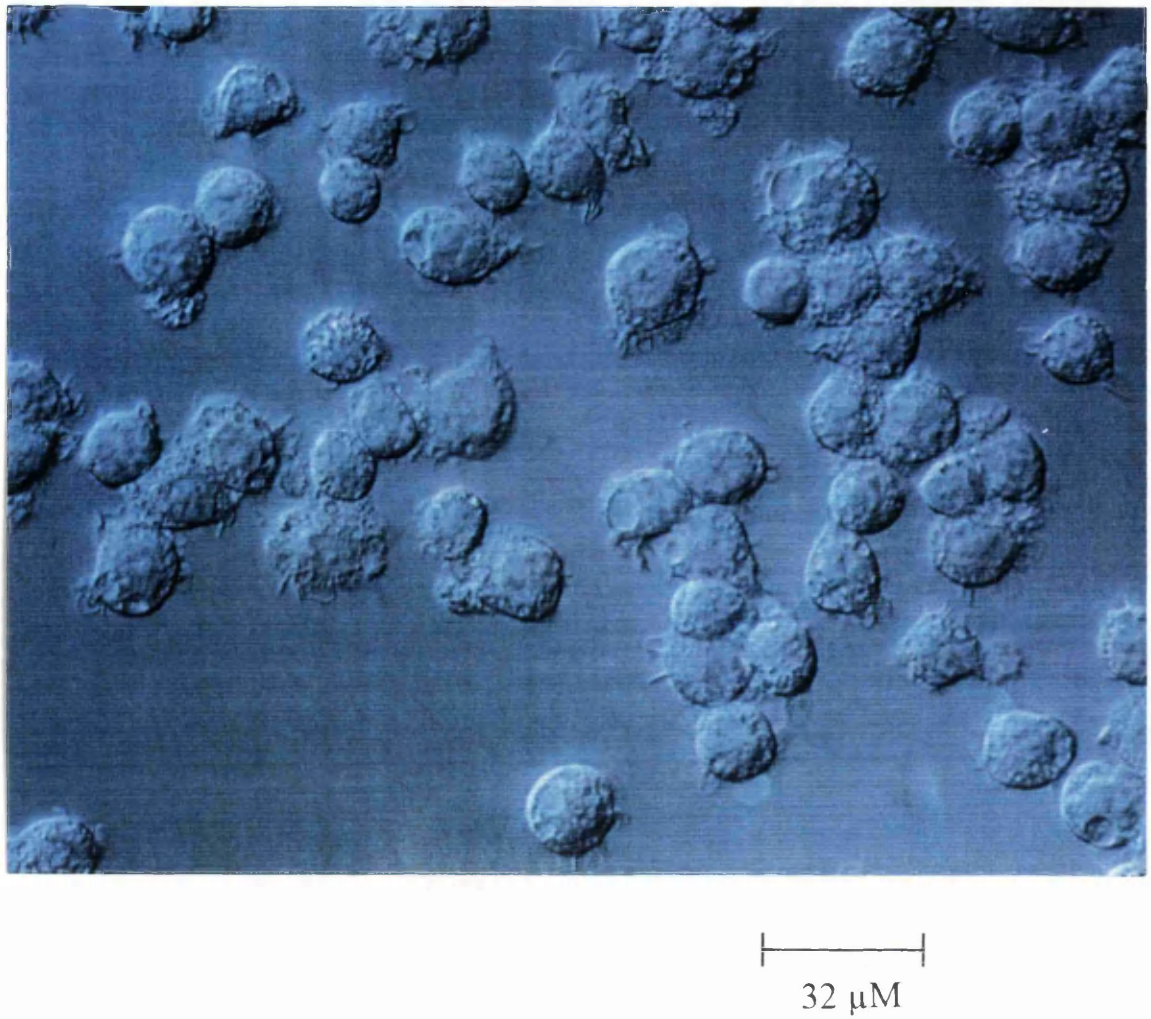


Figure 2.1 Photomicrograph of J774.7 cells grown in full growth medium. Note the calibration bar.

grown in these bottles until they reached numbers that were sufficient to perform an experiment (approximately $3x10^5$ cells ml^{-1}).

For experimentation, the contents of the bottle were vigorously pipetted to break up any clumps of cells. The cells were then counted by means of a haemocytometer. The number of cells in three of the nine large squares of the haemocytometer were counted, and this process was repeated so that cell numbers from at least 6 squares were counted. The average of the cell numbers was then taken. The appropriate volume of cell suspension was removed from the bottle, spun in sterile tubes (Falcon) at 400g for 4 min at 10°C and the medium discarded. The cell pellet was then washed with 30 ml sterile saline, spun at 400g for 4 min at 10°C, washed for a second time and then re-suspended in the appropriate volume of fresh full growth medium to give a density of 10^6 cells ml^{-1}. If any cells remained in the stirrer bottle after removal of that needed for an experiment, they were harvested by spinning at 400g for 4 min at 10°C, washed twice with saline, re-suspended in 250 ml of fresh full growth medium and allowed to grow to provide sufficient numbers of cells for the next experiment.

2.1.2 Freezing and thawing of macrophage suspension

Some batches of macrophages were frozen as a convenient means of storing them for future use. For this purpose, the cells were removed from the 80 cm^2 flasks by means of a cells scraper (Costar), and the 20 ml cell suspension divided equally between two sterile tubes (Falcon). Dimethylsulphoxide (DMSO) was then added dropwise, and the tube agitated after each drop to give a 10% solution. The tubes were then repeatedly inverted for 5 min to ensure thorough mixing of the solution. The tubes were then spun at 400g for 4 min at 10°C. All but 1 ml of the

supernatant was poured off, and the cell pellet was suspended in this remaining volume. The cell suspension was then transferred to a cryovial (Nalgene), which was then labelled and placed in the vapour phase of a container of liquid nitrogen in a bag of cotton wool to provide insulation. After approx. 24 hours the vial was transferred into a holder and placed under the liquid nitrogen.

For thawing, the vials were removed from the liquid nitrogen, and their caps immediately loosened to avoid build up of pressure. The vials were placed in a water bath at 37°C for approx. 2 min, after which the suspension had thawed. Once removed from the water bath, the vials were wiped with a 70 % solution of ethanol. The suspension was then transferred to a culture tube containing 10 ml of full growth medium and spun at 400g for 4 min at 10°C. The cell pellet was then washed with 10 ml saline, spun again and the cell pellet suspended in 20 ml of full growth medium in a culture flask and placed in an incubator under an atmosphere of air until confluent.

2.1.3 Induction of NO synthase

2.1.3.1 Effects of drugs on induction of NO synthase

Induction of NO synthase was assessed by measuring the accumulation of nitrite, the major breakdown product of NO (Stuehr & Marletta, 1987a; Ignarro *et al.*, 1993) in the medium bathing cells. For this purpose, macrophages suspended in full growth medium were seeded in 1 ml volumes into Costar 24-well plates at a density of 10^6 cells ml^{-1}. Cells were pre-treated with drugs at the concentrations and times as detailed in the Results section before the addition of agents known to induce NO synthase (Severn *et al.*, 1992). Specifically, lipopolysaccharide (LPS, 1-100 ng ml^{-1}) from

Salmonella typhosa, mouse recombinant interferon-gamma (IFN-γ, 1-100 u ml^{-1}) or combinations of LPS and IFN-γ were added and incubation continued overnight (approx. 24 hours) in an incubator at 37°C under an atmosphere of air. At the end of this period, the medium was removed from each well and spun at 13,000 rpm for 1 min to remove any cells, and the supernatant was stored in a fresh Eppendorf tube for subsequent assay of nitrite content.

2.1.3.2 Effects of drugs following induction of NO synthase

In order to determine if the drugs used in the above experiments had indeed interfered with the induction as opposed to the activity of NO synthase, a separate series of experiments was conducted in which the drugs were added to cells in which NO synthase had already been induced. Macrophages suspended in full growth medium were maintained in a stirrer bottle at a density of 10^6 cells ml^{-1}. Lipopolysaccharide (LPS, 100 ng ml^{-1}) and interferon-gamma (IFN-γ, 10 u ml^{-1}) were added and left in contact with the cells for 12 hours to promote induction of NO synthase, after which time the cells were washed twice by spinning at 400g for 4 min. and reconstituted in 30 ml sterile saline to remove any trace of LPS and IFN-γ. The cells were then suspended in fresh full growth medium and were seeded into Costar 24-well plates, again at a density of 10^6 cells ml^{-1}. Drugs were then added as detailed in the Results section, and remained in contact for 24 hours in an incubator at 37°C under an atmosphere of air. At the end of this period, the medium was removed from each well and spun at 13,000 rpm for 1 min to remove any cells, and the supernatant was stored in a fresh Eppendorf tube for subsequent assay of nitrite content.

2.1.4 Nitrite Assay

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

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

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

2.1.5 Validation of measurement of nitrite

Although the literature suggested that it was most appropriate to measure nitrite accumulation into the medium bathing the macrophages, experiments were conducted by a colleague in our own laboratory to establish the relative proportions of nitrite and nitrate in samples.

For this purpose, two reducing systems were required to convert nitrite and/ or nitrate to NO before chemiluminescence detection could be applied. The first, a sodium iodide/glacial acetic acid reflux system, consisted of 25 ml of a 6% solution of sodium iodide in 75 ml of glacial acetic acid under reflux at 65°C. This reduces nitrite to NO (Cox, 1980). The second, a vanadium chloride/hydrochloric acid reflux system, consisted of 1.5 g of vanadium

chloride in 100 ml of 2 N HCl under reflux at 95°C. This reduces both nitrite and nitrate to NO (Braman & Hendrix, 1989). Samples were injected into the reaction vessel containing the appropriate reducing solutions. Any NO produced was carried through a condenser by a stream of N_2 and was passed through a liquid trap then through an acid trap into the analyser (Dasibi model no. 2107). Once in the analyser, NO reacted with ozone to form $NO^{\bullet}_2$ which breaks down to emit light. The light emitted was measured by a photomultiplier tube connected to a computer (see McKendrick, 1995). Using the above detection systems, it was found that nitrite content accounted for 82.0 ± 4.3 % (n=6) of the chemiluminescence signal generated by the medium bathing J774.7 cells stimulated for 24 hours with interferon-gamma (100 u ml^{-1}).

Although used to validate the measurement of nitrite as an indirect means of assessing NO production, chemiluminescence was too slow and tedious a technique to use routinely. Consequently, the Griess reaction was adopted since this permitted a more rapid analysis of a large number of samples.

2.1.6 Griess Reaction

The accumulation of nitrite, the major breakdown product of NO, into the growth medium was measured by the formation of a diazo product by a variant of the method of Green *et al.* (1982). Early experiments were conducted using a Shimadzu dual beam spectrophotometer (model no. UV 240) but towards the end of the research, a Dynatech Microplate Reader (model no. DS 2000) was purchased, and this was used for the sake of convenience in place of the spectrophotometer. 96 samples could be read at one time when using the plate reader, whereas only individual samples could be read using the spectrophotometer. Briefly, 400 µl (when using the dual

beam spectrophotometer) or 60 µl (when using the plate reader) of the sample was mixed with equal volumes of the Griess reagents (1% sulphanilic acid in 2M HCl followed by 1% N-(1-napthyl)-ethylenediamine dihydrochloride in H_2O). The absorbance was read at 548 nm using the Shimadzu dual beam recording spectrophotometer or at 550 nm using the Dynatech Microplate Reader. Nitrite concentrations in samples were assessed using a range of standards consisting of sodium nitrite (0, 0.5, 1, 5 and 10 µM) prepared in full growth medium (see Figs 2.2 & 2.3). In each case, the minimum detection limit for nitrite was 0.5 µM and a linear regression line of best fit was used to assess the nitrite content of experimental samples.

2.1.7 Cytochrome C assays for the production of oxidising or reducing species

Macrophages are known to produce a variety of reactive oxygen species as part of the host defence mechanism. Superoxide anions, produced both spontaneously and by activation of the NADPH-dependent oxidase system, are reducing agents (Miller & Britigan, 1995a). However, the other reactive oxygen species that they produce, i.e. hydrogen peroxide, hypochlorous acid, hydroxyl radical and peroxynitrite are all powerful oxidising agents, and are capable of mediating significant cellular damage (Rosen *et al.*, 1995).

Production of reactive oxygen species contributes to the killing of invading pathogens, and thus production of these species gives an indication of the activation state of the cells. Due to the ability of reactive oxygen species to reduce or oxidise compounds in cell targets, one way to assess their production is through their redox properties. This can be accomplished using

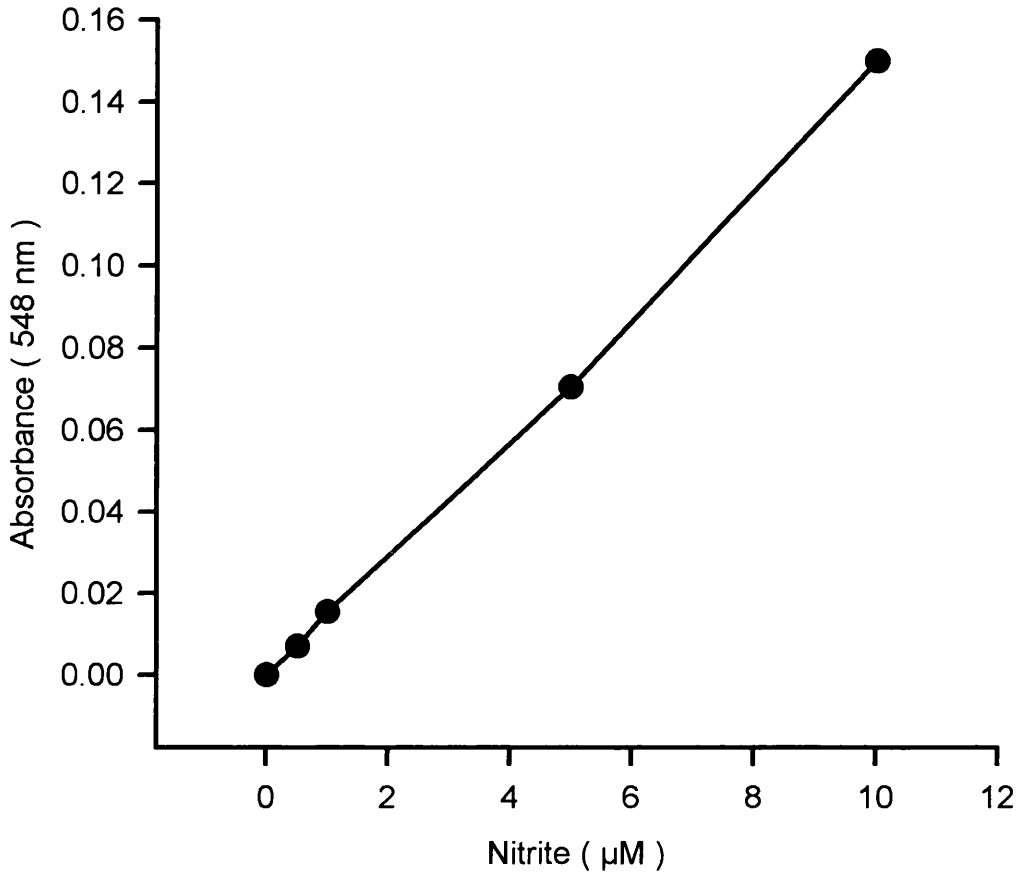


Figure 2.2 Standard curve for measurement of nitrite accumulation in experimental samples using the Shimadzu dual beam spectrophotometer (model no. UV 240). Standards consisted of sodium nitrite in full growth medium. The concentration of nitrite is plotted against absorbance at 548 nm. The instrument was zeroed using blanks consisting of full growth medium.

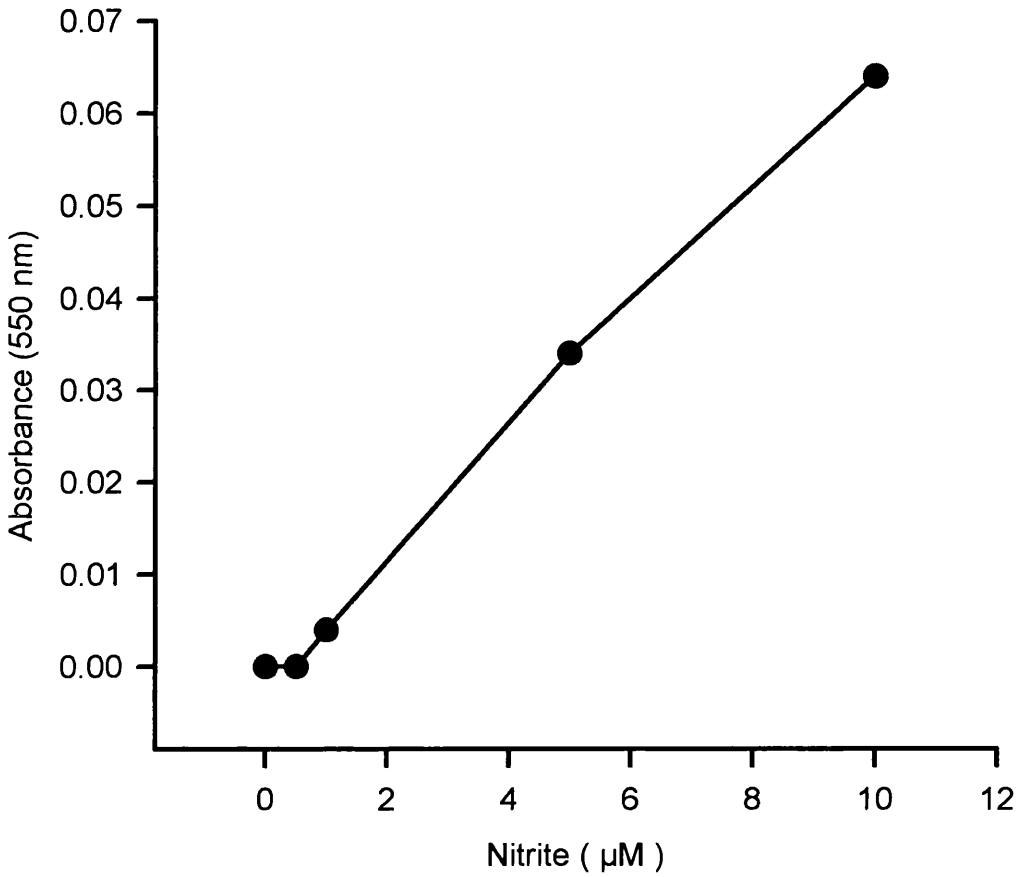


Figure 2.3 Standard curve for measurement of nitrite accumulation in experimental samples using the Dynatech Microplate Reader (model no. DS 2000). Standards consisted of sodium nitrite in full growth medium. The concentration of nitrite (μM) is plotted against absorbance at 550 nm. The instrument was zeroed using blanks consisting of full growth medium.

cytochrome C. Cytochrome C, as commercially supplied, exists predominantly in its oxidised form, ferricytochrome C (Fe^{3+}). Superoxide generation can be determined by the superoxide dismutase-inhibitable reduction of ferricytochrome C (Ischiropoulos *et al.*, 1992b).

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

Cytochrome C in the reduced form has a higher absorbance at 550 nm than its oxidised form (Figure 2.4). Since superoxide anion causes the reduction of cytochrome C, measurement of absorbance at 550 nm will therefore give an indication of the production of this free radical.

2.1.8 Validation of the assays involving cytochrome C

2.1.8.1 The hypoxanthine (HX)/xanthine oxidase (XO) superoxide generating system:

$$\begin{array}{lll} & \text{XO} & \\ & \downarrow & \\ \text{HX} & \leftrightarrow \quad \text{X} + & \bullet O_2^- \\ & \downarrow \text{XO} & \\ & \text{uric acid} + & \bullet O_2^- \end{array}$$

Hypoxanthine, in the presence of xanthine oxidase, is converted to xanthine and superoxide anion. Xanthine can be further converted to uric acid, with the production of another superoxide anion. This simple system allows for the convenient production of superoxide anions. Use was made of the HX/XO generating system to validate the use of our assay to detect superoxide production by macrophages. Figure 2.5a demonstrates the effects of HX ($2x10^{-4}$ M) and XO (20 mu ml^{-1}) on the absorbance of oxidised

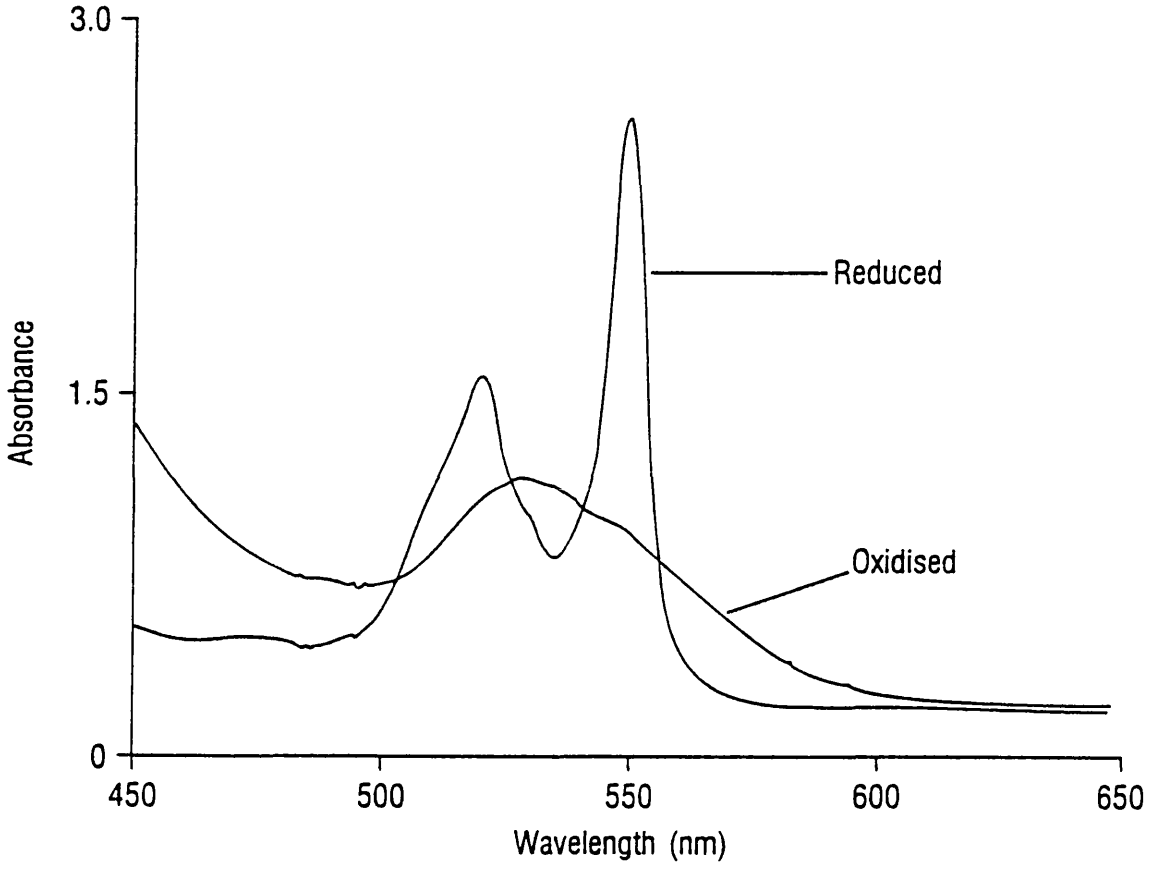


Figure 2.4 Absorption spectra of cytochrome C (10^{-5} M) in both its oxidised and reduced states. Cytochrome C is supplied mainly in the oxidised (Fe^{3+}) state and was reduced using a 10-fold molar excess of sodium dithionite. An increase in absorbance of the oxidised form of cytochrome C was used as a means of assessing production of reducing species and a decrease in absorbance of the reduced form was used to assess production of an oxidant.

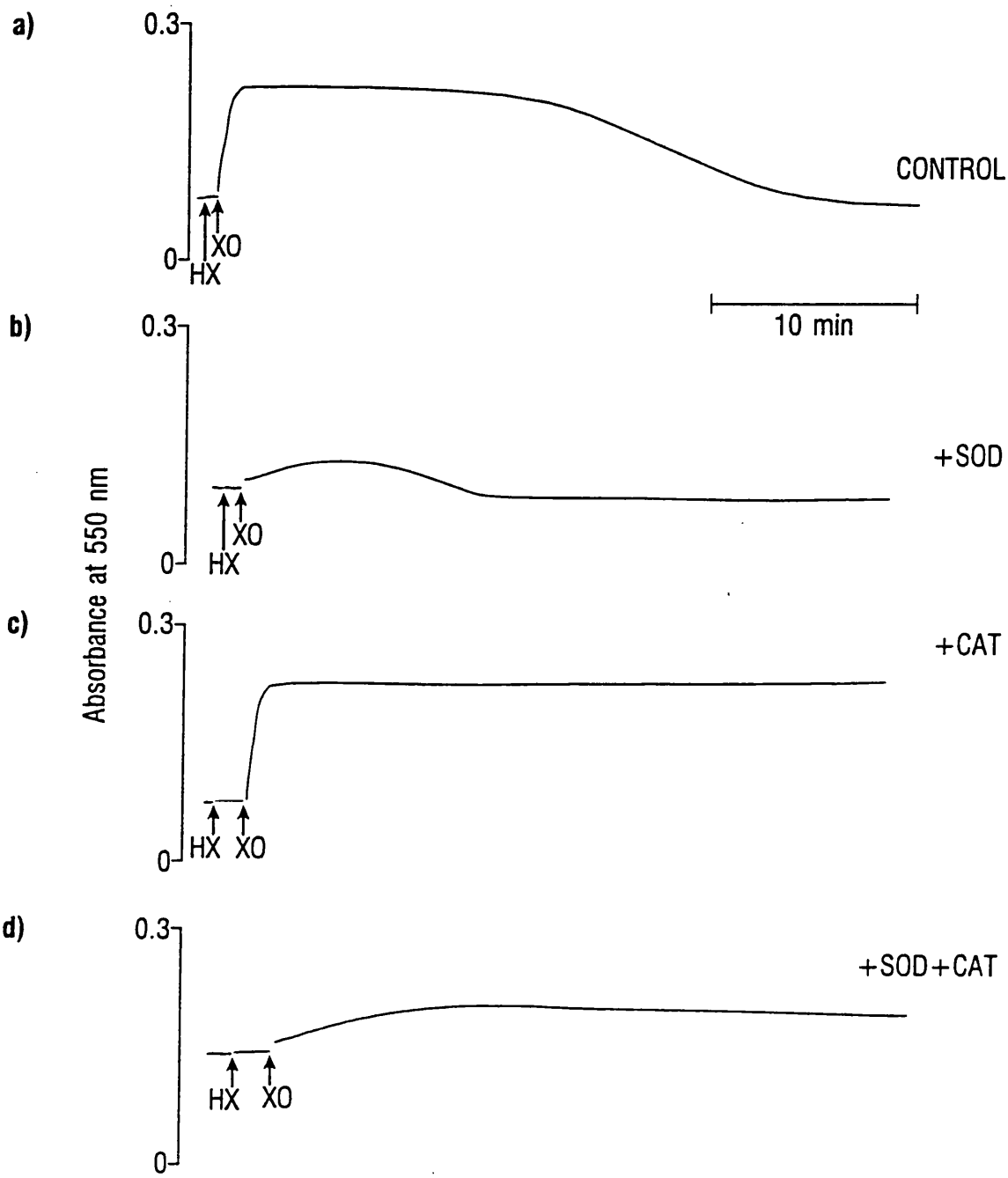


Figure 2.5 Validation of the use of cytochrome C to assess production of oxidising or reducing species. Traces demonstrate the absorption of 10^{-5} M cytochrome C at 550 nm following the addition of $2x10^{-4}$ M hypoxanthine (HX) and 20 mu ml^{-1} xanthine oxidase (XO) (a) alone, or in the presence of (b) 6000 u ml^{-1} superoxide dismutase (SOD), (c) 100 u ml^{-1} catalase (CAT) or (d) both 6000 u ml^{-1} superoxide dismutase (SOD) and 100 u ml^{-1} catalase (CAT). The increase in the absorbance in the presence of HX/XO indicates production of superoxide anions and this is blocked by SOD. The delayed fall in absorbance is due to production of the oxidant, hydrogen peroxide and this is blocked by catalase.

cytochrome C (10^{-5} M). The presence of both XO and HX led to the generation of superoxide anions, producing a rise in absorbance. However, the absorbance fell with time due to the spontaneous conversion of superoxide anions into the oxidant, hydrogen peroxide (see below).

2.1.8.2 Superoxide Dismutase

Superoxide dismutase (SOD) scavenges superoxide anions by converting them to hydrogen peroxide. SOD is found within cells in two forms; a manganese-containing enzyme (Peeters-Joris *et al.*, 1975) and a copper-zinc cytosolic form (Wilkins & Leake, 1994). The presence of SOD within cells is an important antioxidant defence mechanism in preventing the accumulation of superoxide anions which could lead to damage. The addition of SOD will thus remove the reductive influence of superoxide anions from the assay.

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

$$\bullet O_2^- + 2H_20 \rightarrow 2H_2O_2$$

This process can also occur spontaneously, but in the presence of SOD, the reaction takes place approximately 10^4 times faster (Salvemini & Botting, 1993). Figure 2.5b demonstrates the effects of SOD (6000 u ml^{-1}) on the absorbance of cytochrome C in the presence of HX (10^{-4} M) and XO (0.2 mu ml^{-1}). Only a very small transient rise in absorbance is seen, as the reducing ability of superoxide is lost by its conversion into the oxidising agent, hydrogen peroxide.

2.1.8.3 Catalase

Catalase scavenges hydrogen peroxide by catalysing its conversion to oxygen and water. Catalase is present in most mammalian cells and acts as an important defence mechanism against the damaging effects of hydrogen peroxide (Salvemini & Botting, 1993).

$$2H_2O_2 \xrightarrow{\text{catalase}} 2\,H_2O + O_2$$

The presence of catalase (100 u ml^{-1}), as demonstrated in Figure 2.5c, removes the oxidising effects of hydrogen peroxide, thus resulting in a sustained increase in absorbance of cytochrome C in the presence of the superoxide generating system, XO (20 mu ml^{-1}) and HX ($2x10^{-4}$ M).

2.1.9 Experimental protocol for detection of a reducing agent

Macrophages were grown in stirrer bottles in full growth medium as described previously. On the day of use, cells were counted by means of a haemocytometer, transferred to sterile culture tubes (Falcon) and were spun at 400g for 4 min at 10°C and the medium discarded. The cell pellet was then washed with 30 ml sterile saline, spun at 400g for 4 min at 10°C, washed for a second time and then re-suspended in the appropriate volume of HEPES-buffered Krebs to give a density of 10^6 cells ml^{-1}. Cells were suspended in HEPES-buffered Krebs rather than DMEM, due to interference with the absorption of cytochrome C by the pH indicator (phenol red) present in the growth medium. HEPES-buffered Krebs consisted of (mM): HEPES 5, NaCl 118, KCl 4.8, CaCl 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 2.4, glucose

11 and KH_2PO_4 1.2, at pH 7.4. 500µl of the cell suspension was added to each well of Costar 24-well plates, followed by 450 µl of Krebs and finally an appropriate volume of drug solution or Krebs to give a final volume of 1 ml in each well. Two well known activators of superoxide production by cells, the phorbol ester, PMA (Wolfson *et al.*, 1993; Conde *et al.*, 1995) and LPS (Landmann *et al.*, 1995) were used to stimulate the cells. In experiments involving stimulation by PMA (10^{-7} M), the cells were in contact with the drug for 1 hour. In experiments involving stimulation by LPS (100 ng ml^{-1}), the cells were previously exposed to the drug in full growth medium for 24 hours. After these times, the medium was removed and was replaced with fresh Krebs. In each experiment, 10^{-5} M cytochrome C was incubated with the cells for 1 hour, after which the medium was removed from each well, spun at 13,000 rpm, and the absorbance read at 550 nm on a Shimadzu dual beam recording spectrophotometer. The blanks used in this experiment consisted of HEPES-buffered Krebs. Any increase in absorbance detected was assumed to be due to superoxide anion if it was abolished by superoxide dismutase (100 u ml^{-1}).

2.1.10 Experimental protocol for detection of an oxidising agent

Experiments were also conducted to examine the production of oxidising agents by cells. In these, oxidation of reduced cytochrome C to the less absorbant oxidised form at 550 nm was used to assess production of oxidising species. The reduced form of cytochrome C was prepared adding a 10-fold molar excess of sodium dithionite to cytochrome C (10^{-5} M). This was achieved by dissolving sodium dithionite in a small volume of distilled water to achieve minimum dilution, and this was added to the cytochrome C with thorough mixing. The cytochrome C (10^{-5} M) was added to the cells immediately after its reduction to minimise the spontaneous conversion to

the oxidised form that occurs with time. Aliquots without cells were always included to determine the degree of spontaneous oxidation. The cells were suspended in HEPES-buffered Krebs at densities of either 10^6, $3x10^5$ or 10^5 cells ml^{-1}, as indicated in the Results section. 500 µl of the cell suspension was added to each well, followed by 480 µl of Krebs, 10 µl of drug solution and 10 µl of reduced cytochrome C (10^{-5} M). At various time points (5 min, 30 min, 1 hour, 1.5 hours and 2 hours), the medium was removed, spun at 13,000 rpm for 1-2 min, and the supernatants read immediately at 550 nm on a Shimadzu recording spectrophotometer. Cell-mediated oxidation was obtained by subtracting the spontaneous oxidation (in the absence of cells) from the total oxidation obtained in the presence of cells.

PART 2
Assessment of nitric oxide production by macrophages by bioassay on rat aortic rings

In addition to measuring nitrite as an index of NO production by macrophages using the Griess reaction, the production was also assessed from the relaxation produced by macrophages when added to rat aortic rings. In addition, this system permitted an investigation of the properties of the relaxant released from activated macrophages.

2.2.1 Preparation of aortic rings

Male Wistar rats of approximately 200-300 g were used throughout. Rats were killed by stunning and cervical dislocation. The thoracic aorta was dissected out, cleared of any adipose and connective tissue and any remaining blood in the lumen was removed. The vessel was cut into transverse rings 2.3 mm wide using a cutting device of parallel razors. In every experiment, the endothelium was removed, to ensure there was no

contribution of constitutively produced NO. This was achieved by locating the aortic ring between two stainless steel hooks, placing a 2 g weight in the bottom hook and gently rubbing the intimal surface with a moist matchstick. The rings were then suspended on stainless steel hooks in 12 ml organ baths containing bicarbonate-buffered Krebs consisting of (mM): NaCl 117.8, KCl 4.8, $MgSO_4$ 2.5, KH_2PO_4 1.2, $CaCl_2$ 2, $NaHCO_3$ 23.8 and glucose 11.1 mM. The Krebs solution was gassed with 95% O_2 and 5 % CO_2. Resting tension was set at approximately 1g and was readjusted to this before starting each separate experiment. Rings were left to recover for approx. 30 min before the addition of any drugs. A concentration-response curve to phenylephrine was carried out at the start of each day to sensitise the tissue and verify that the rings were responsive to contractile agents. A lack of relaxation following addition of acetylcholine (10^{-6} M) was used to verify the absence of endothelial cells. Tension was measured by Grass force displacement transducers (FT03C) and traces were displayed on a Grass polygraph via a transducer amplifier.

Phenylephrine (PE, 10^{-8}-10^{-7} M) was used to induce tone in the rings. Once the contraction to PE had stabilised, the relaxant stimuli were added. Relaxation is expressed throughout as % relaxation of PE-induced tone.

2.2.2 Preparation of macrophages

The relaxant effects of NO released from macrophages on PE-induced tone was investigated. In each experiment, the relaxant effects of activated and unactivated macrophages were compared.

J774.7 macrophages were grown in stirrer bottles in full growth medium as described previously. On the day of use, the cells were counted by means of

a haemocytometer. The appropriate volume of cell suspension was transferred to sterile culture tubes (normally four), spun at 400g at 10°C for 4 min, washed twice with sterile saline, and the cell pellet in each of the four culture tubes suspended in 20 ml of full growth medium. Each of the 20 ml cell suspensions was then transferred to an 80 cm^2 culture flask, two of which received lipopolysaccharide (LPS, 100 ng ml^{-1}) and interferon-gamma (IFN-γ, 10 u ml^{-1}) to provide activated cells and the others received no drugs to provide unactivated cells. The flasks were then placed in an incubator under an atmosphere of air at 37°C overnight. The following day, the flasks were removed from the incubator, and the cells removed from the flasks by means of a cell scraper (Costar). The cell suspensions were then spun at 400 g at 10°C for 4 min, the medium was poured off and the cell pellet resuspended in the appropriate volume of prewarmed (to approx. 37°C) sterilised Krebs to give the appropriate cell concentration ($8.4x10^7$-$1.2x10^6$ cells ml^{-1}). Krebs was sterilised by passing it through a Flowpore (ICN Biomedicals, 0.22 μm filter). The cell suspensions were then diluted further in sterile Krebs to give a range of cell concentrations that would allow for cumulative addition of cells into the tissue baths containing the aortic rings. The cells were placed in separate wells of a six-well plate (Nunc) at their appropriate densities ($8.4x10^7$-$1.2x10^6$ cells ml^{-1}), and stored in an incubator at 37°C under an atmosphere of 5 % CO_2 and 95 % air until required.

2.2.3 Addition of macrophages to organ baths

Immediately prior to use, the six-well plates were removed from the incubator and the wells were scraped with the rubber end of a syringe plunger to remove the cells from the bottom of the well. The resulting cell suspensions were added to the organ baths in a cumulative fashion and relaxation was plotted as a function of cell concentration.

Part 3
Materials

2.3.1 Tissue culture materials

HEPES-buffered DMEM was obtained from ICN Biomedicals Ltd., Bucks, and bicarbonate-buffered DMEM from Life Technologies Ltd., Paisley. Foetal calf serum, glutamine and penicillin/streptomycin were obtained from Life Technologies Ltd., Paisley. Sterile saline (0.9 % sodium chloride) was purchased from Baxter Health Care, Hillington. All culture tubes (Falcon) were purchased from R&J Wood Laboratory Supplies, Paisley. 24-well and 6-well plates were purchased either from Nunc, Glasgow University Biochemistry stores or Costar, Costar (UK) Ltd., High Wycombe. Cell scrapers and culture flasks (25 and 80 cm^2) were purchased from Costar (UK) Ltd., High Wycombe. Nalgene cryovials were purchased from Sigma, Poole.

2.3.2 Drugs

Lipopolysaccharide (LPS) from *Salmonella typhosa* (phenol extracted), polymyxin B sulphate, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), dexamethasone, cytochrome C (horse heart), hypoxanthine, superoxide dismutase (SOD, bovine erythrocyte), xanthine oxidase (buttermilk), catalase (bovine liver), phorbol 12-myristate 13-acetate (PMA), mannitol, dithiothreitol, interferon-gamma (IFN-γ, mouse recombinant), ionophore A23187, herbimycin A (from *Streptomyces hygroscopicus*), genistein, acetylcholine chloride, phenylephrine hydrochloride, dimethylsulphoxide (DMSO), sulphanilic acid, N-(1-napthyl)-ethylenediamine dihydrochloride, trypan blue, dibutyryl adenosine 3', 5'-cyclic monophosphate (sodium salt), 8 bromo guanosine 3', 5'-cyclic

monophosphate (sodium salt), sodium orthovanadate and hydrogen peroxide were all obtained from Sigma, Poole (UK).

Staurosporine, chelerythrine chloride, genistein, forskolin and LY 83583 were purchased from Calbiochem Novabiochem, Nottingham (UK).

Ro 31-8220 was kindly donated by Dr G. Lawton, Roche Research Centre, Welwyn Garden City (UK).

N^G-monomethyl-L-arginine (L-NMMA) citrate was kindly donated by Dr Daryl Rees, Wellcome Research Laboratories, Kent (UK).

Dimethylthiourea, cycloheximide and sodium dithionite were obtained from Aldrich, Gillingham (UK).

SKF 94120, rolipram and zaprinast were obtained from Rhône Poulenc Rorer, Dagenham (UK).

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

2.3.3 Preparation of haemoglobin

Methaemoglobin (Fe^{3+}), the form of haemoglobin supplied by Sigma, requires a 10-fold molar excess of sodium dithionite (a reducing agent) for its conversion to haemoglobin (Fe^{2+}). The reduction was achieved as follows. Dialysis tubing was boiled in distilled water for 20 min. Haemoglobin (1.29 g) was dissolved in 20 ml water to give a 10^{-3} M solution. Sodium dithionite (34.8 mg) was dissolved in 100 µl to give a 2M solution which was added immediately to the haemoglobin solution with vigorous mixing. The haemoglobin-dithionite solution was then dialysed

against 5 litres of distilled water for 2-3 hours. The resulting solution of oxyhaemoglobin was either used immediately or frozen in aliquots and stored for use within 14 days.

2.3.4 Drug solutions

Sodium orthovanadate, LPS, L-NMMA, L-NAME, acetylcholine chloride and phenylephrine hydrochloride were dissolved in saline (0.9 %)

Cytochrome C, 8 bromo cyclic GMP, dibutyryl cyclic AMP, chelerythrine chloride, xanthine oxidase, mannitol, catalase, superoxide dismutase, cycloheximide and glyceryl trinitrate were dissolved in distilled water.

Hypoxanthine (10^{-2} M) was dissolved in 10^{-2} M NaOH and SKF 94120 (10^{-1} M) was dissolved in 1M NaOH and subsequent dilutions made using saline.

LY 83583 ($2x10^{-2}$ M), PMA (10^{-3} M), A23187 (10^{-2} M), rolipram (10^{-2} M) and zaprinast (10^{-2} M) were all prepared in 100% ethanol and subsequent dilutions made using saline.

Genistein (10^{-1} M), herbimycin A (1000 μg ml^{-1}) , Ro 31 8220 (10^{-2} M), staurosporine (10^{-3} M) and forskolin ($3x10^{-2}$ M) were all prepared in 100 % DMSO and subsequent dilutions made using saline.

In all studies, experiments were conducted to ensure that the solvents played no part in the responses seen. In some cases when a wide range of concentrations of a drug was used, high concentrations of solvent were able to affect the results, and this is stated in the corresponding Results section.

All drug solutions prepared or diluted in distilled water or saline were sterilised by passage through a Flowpore (ICN Biomedicals Ltd) filter pore size 0.22 μm.

Part 4
Statistical analysis

Results are expressed throughout as the mean ± the standard error of the mean for n separate experiments. Statistical significance was determined by one-way analysis of variance followed by Fisher's test, or by a 2-sample *t*-test, as appropriate, using the Minitab (version B) statistical software package on an IBM compatible P.C. A value of $P<0.05$ was considered significant.

RESULTS

CHAPTER 3

INVESTIGATION OF NITRITE PRODUCTION BY MACROPHAGE CELL LINES

Nitric oxide (NO) has a short half life of around 5 seconds under physiological conditions. It usually decays to the stable end products of nitrite and nitrate, with the majority in the form of nitrite *in vitro* (see Methods). Consequently, measuring nitrite accumulation into the medium bathing macrophage cell lines was chosen as a convenient way to measure NO production.

3.1 Investigation of nitrite accumulation stimulated by LPS

3.1.1 Effects of LPS on nitrite production by J774.7 cells

The concentration of nitrite accumulating into the medium bathing 10^6 unstimulated J774.7 cells during an 18 hour incubation was 5.1 ± 0.2 μM. This low basal level of nitrite accumulation was found in all experiments using the J774.7 macrophage cell line. The presence of bacterial endotoxin (lipopolysaccharide; LPS, phenol extracted from *Salmonella typhosa*) during the overnight incubation produced inconsistent effects. In one experiment, LPS (1-1000 ng ml^{-1}) produced a significant concentration-dependent rise in

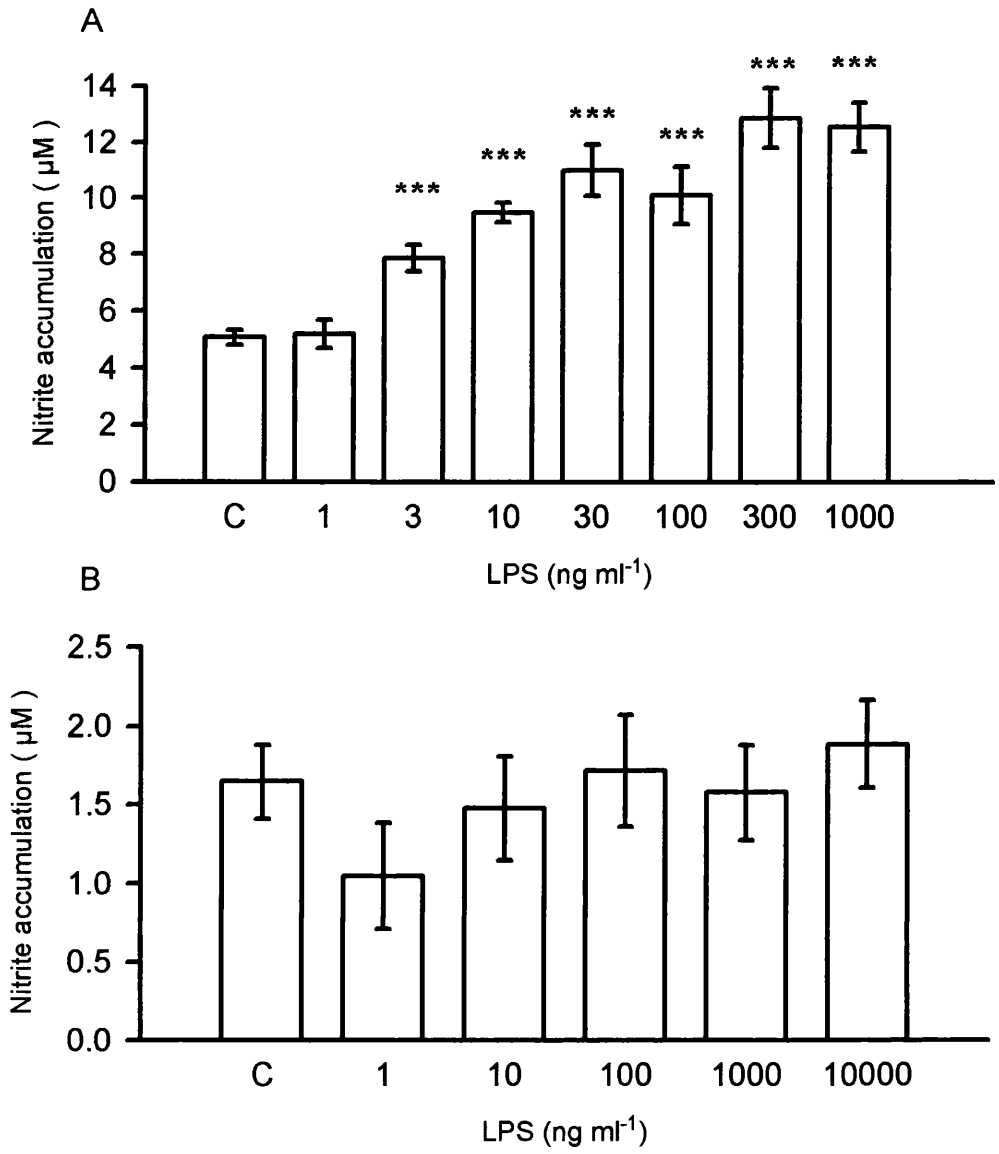


Figure 3.1 The inconsistent effects of lipopolysaccharide (LPS) from *Salmonella typhosa* (A) 1-1000 ng ml^{-1} and (B) 1-10000 ng ml^{-1} on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) over (A) 18 and (B) 22 hours. Some batches of cells responded to LPS (A) but others did not (B). Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ represents a significant increase from untreated control (C) cells.

nitrite accumulation over 18 hours, with a maximum 2.5-fold rise to 12.6 ± 0.9 μM at 300 ng ml^{-1} (Fig 3.1A). In a separate experiment, LPS (0.1-10000 ng ml^{-1}) produced no rise in nitrite accumulation (Fig 3.1B).

3.1.2 Effects of L-NMMA on basal and LPS-stimulated nitrite production

In order to determine if nitrite accumulating into the medium bathing J774.7 cells was derived from the L-arginine/NO pathway, cells were pretreated for 10 min with the NO synthase inhibitor, L-NMMA ($5x10^{-4}$ M), before addition of LPS (100 ng ml^{-1}), and the incubation was continued for a further 22 hours. L-NMMA significantly reduced basal nitrite accumulation by 68.9 ± 6.4 % (Fig 3.2). In this experiment, LPS induced a significant 2.1-fold rise in nitrite accumulation, reaching 8.0 ± 0.5 μM nitrite and L-NMMA also reduced this by 67.7 ± 1.9 %.

3.1.3 Effects of L-NAME on basal and LPS-stimulated nitrite production

Confirmation was sought that nitrite accumulation into the medium bathing J774.7 cells was derived from the L-arginine/NO pathway using another inhibitor of NO synthase, L-NAME. Cells were pretreated for 10 min with L-NAME ($5x10^{-4}$ M) before the addition of LPS (100 ng ml^{-1}), and the incubation was continued for a further 22 hours. L-NAME significantly reduced basal nitrite accumulation by 56.7 ± 7.7 % (Fig 3.3). LPS induced a significant rise in nitrite accumulation of 1.7-fold, and this too was blocked (49.9 ± 3.3 %) following pretreatment with L-NAME.

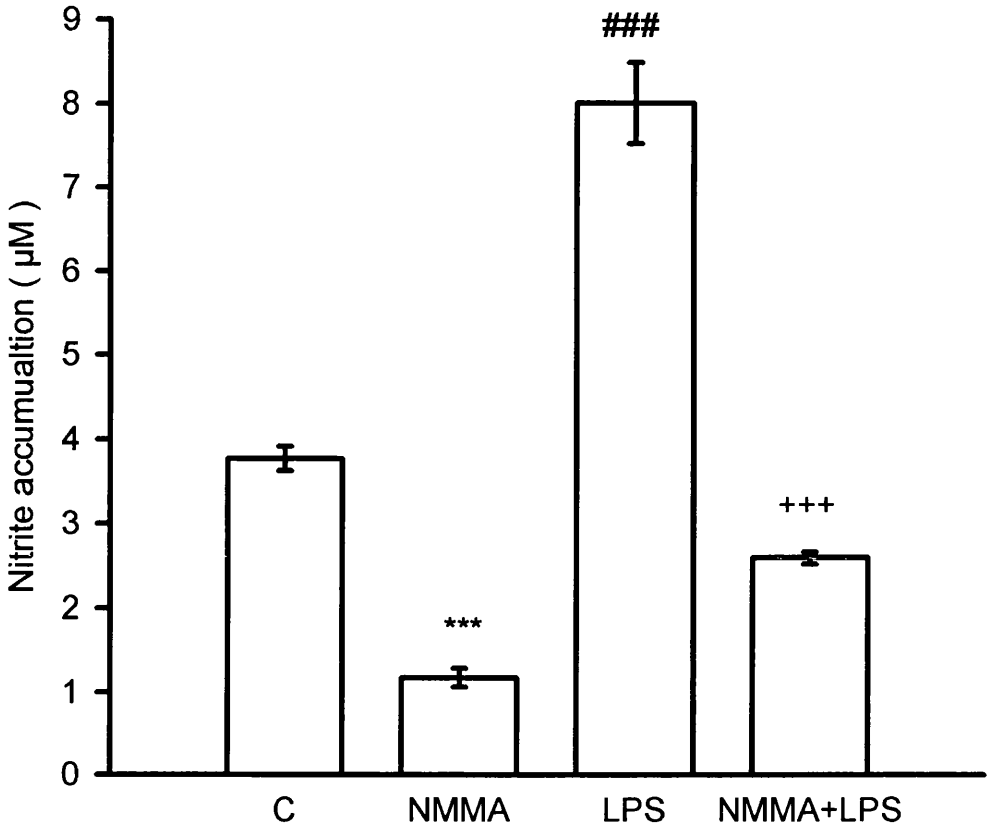


Figure 3.2 Effects of the nitric oxide synthase inhibitor, L-NMMA, on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following stimulation with lipopolysaccharide (LPS) from *Salmonella typhosa*. Cells were pretreated with L-NMMA (NMMA, $5x10^{-4}$ M) for 10 min before the addition of LPS (100 ng ml^{-1}) and the incubation was continued for a further 22 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant reduction from untreated control (C) cells. ### $P<0.001$ indicates a significant increase from untreated control (C) cells. +++ $P<0.001$ indicates a significant inhibition of LPS-stimulated nitrite levels by L-NMMA.

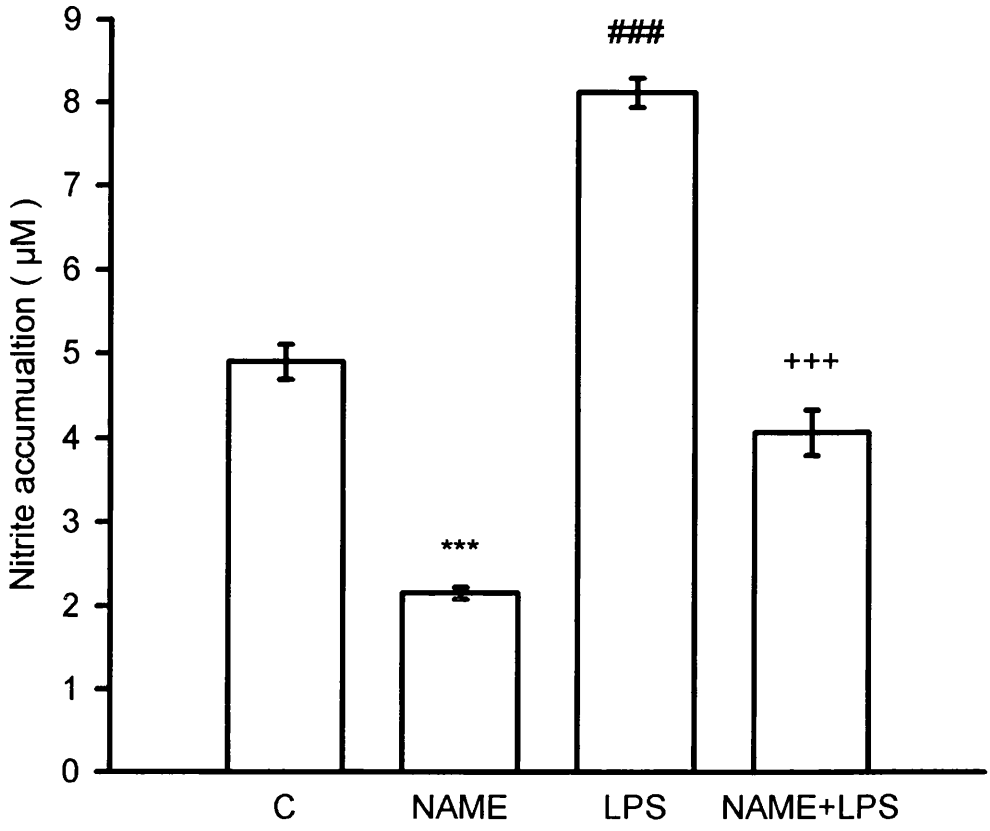


Figure 3.3 Effects of the nitric oxide synthase inhibitor, L-NAME, on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following stimulation with lipopolysaccharide (LPS) from *Salmonella typhosa*. Cells were pretreated with L-NAME (NAME, $5x10^{-4}$ M) for 10 min before the addition of LPS (100 ng ml^{-1}), and the incubation was continued for a further 22 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant reduction from untreated control (C) cells. ### $P<0.001$ indicates a significant increase from untreated cells. +++ $P<0.001$ indicates a significant inhibition of LPS-stimulated nitrite levels by L-NAME.

3.1.4 Effects of LPS on nitrite production by RAW 264 cells

Since LPS appeared to be a weak stimulus of nitrite production by J774.7 cells, the effects of this agent on a different macrophage cell line, the RAW 264 cell line, were examined. In this cell line, incubation for 24 hours with LPS (100 ng ml^{-1}) from *Salmonella typhosa* produced a maximum increase in nitrite production of around 2-fold (Fig 3.4 and 3.5).

The effects of the NO synthase inhibitors, L-NMMA and L-NAME, were assessed to determine if the nitrite produced was derived from the L-arginine/NO pathway. RAW 264 cells were pretreated with L-NMMA (10^{-5}-$5x10^{-4}$ M) and L-NAME (10^{-5}-$5x10^{-4}$ M) for 5 min before the addition of LPS (100 ng ml^{-1}) and the incubation continued for 24 hours in both cases. L-NMMA produced a concentration-dependent inhibition of nitrite accumulation, with a maximum inhibition of 92.4 ± 9.7 % at $5x10^{-4}$ M (Fig 3.4). L-NAME also produced a concentration-dependent inhibition, with a maximum inhibition of 98.6 ± 38.6 % at $5x10^{-4}$ M (Fig 3.5).

Thus, LPS seemed no more powerful a stimulus of nitrite production in RAW 264 cells than in the original J774.7 cell line. All subsequent experiments were therefore conducted using J774.7 cells.

3.1.5 Effects of polymyxin B on basal and LPS-stimulated nitrite production

The peptide polymyxin B binds and inactivates LPS (Lasfargues *et al.*, 1989). Polymyxin B would therefore be expected to inhibit the ability of

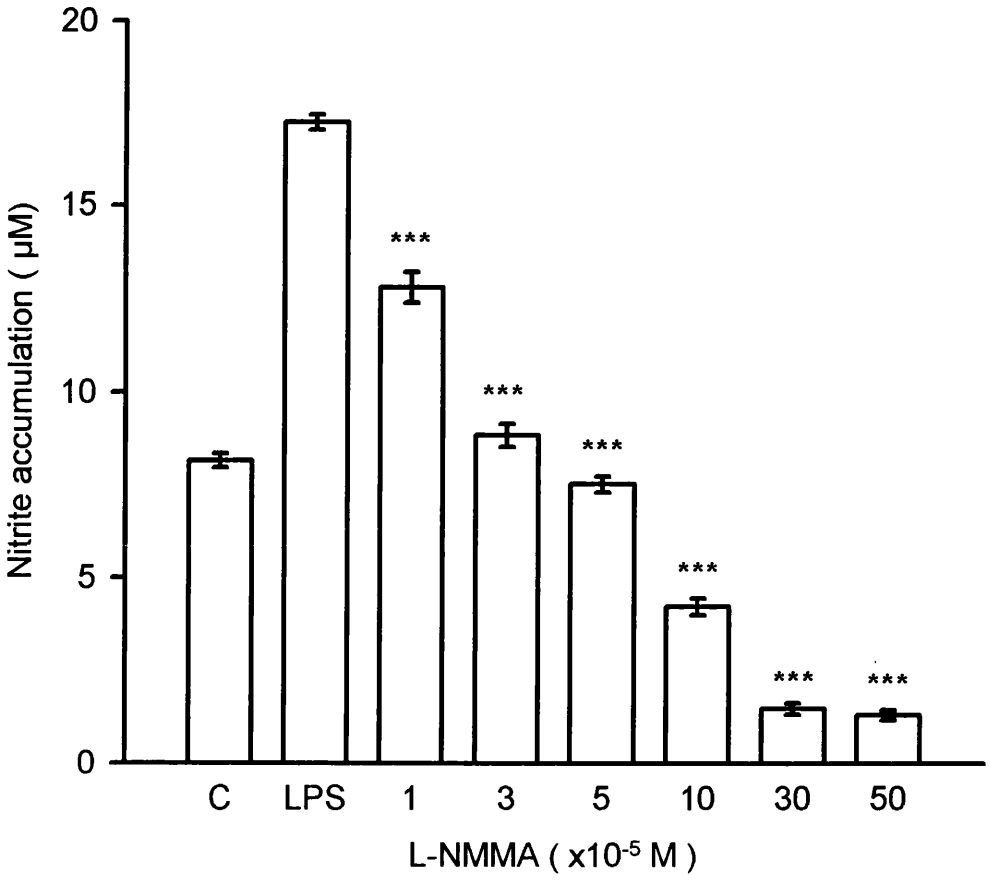


Figure 3.4 Effects of the inhibitor of nitric oxide synthase, L-NMMA, on nitrite accumulation into the medium bathing RAW 264 cells (10^6 cells ml^{-1}) following stimulation by lipopolysaccharide (LPS) from *Salmonella typhosa*. Cells were pretreated with L-NMMA (10^{-5}-$5x10^{-4}$ M) for 5 min before the addition of LPS (100 ng ml^{-1}) and the incubation was continued for a further 24 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant inhibition of LPS-stimulated nitrite levels by L-NMMA.

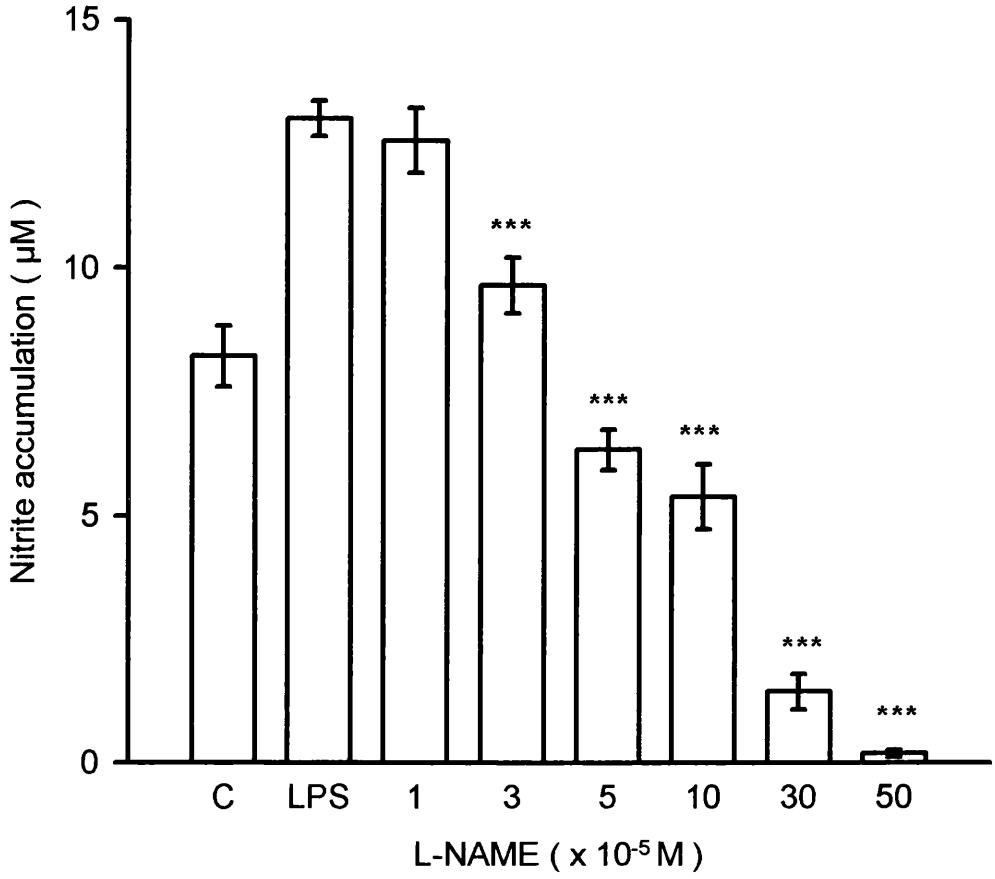


Figure 3.5 Effects of the inhibitor of nitric oxide synthase, L-NAME, on nitrite accumulation into the medium bathing RAW 264 cells (10^6 cells ml^{-1}) following stimulation by lipopolysaccharide from (LPS) *Salmonella typhosa*. Cells were pretreated with L-NAME (10^{-5}-$5x10^{-4}$ M) for 5 min before the addition of LPS (100 ng ml^{-1}) and the incubation was continued for a further 24 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant inhibition of LPS-stimulated nitrite levels by L-NAME.

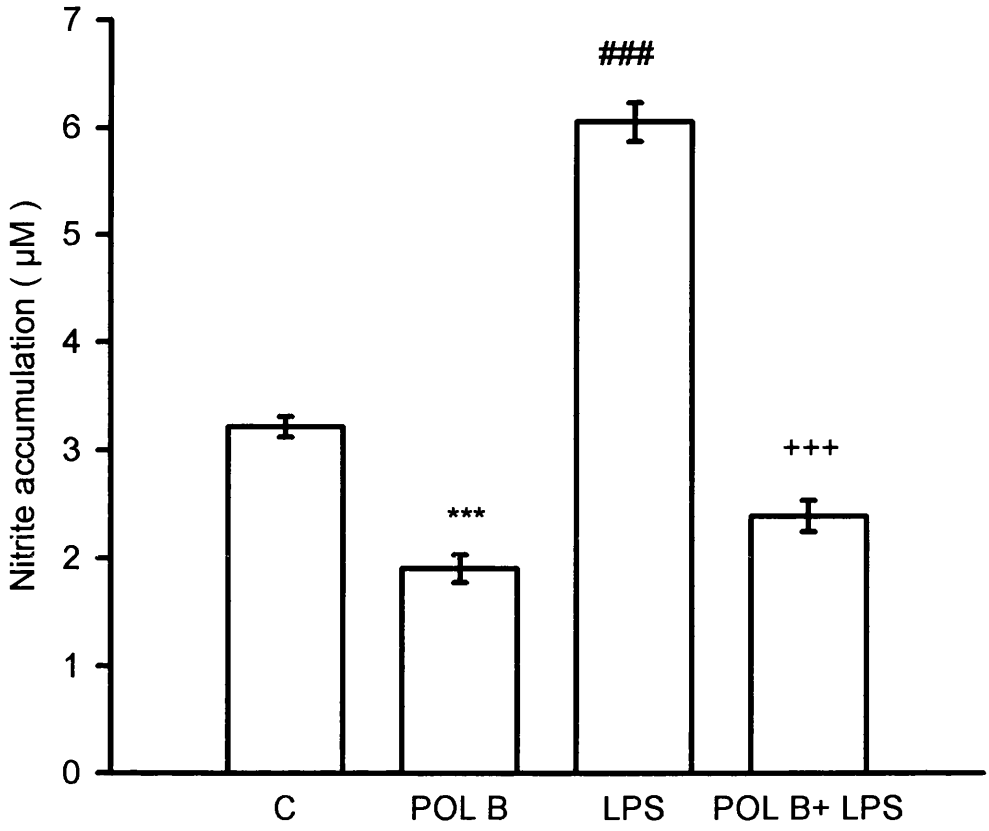


Figure 3.6 Effects of the peptide polymyxin B on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following stimulation with lipopolysaccharide (LPS) from *Salmonella typhosa*. Cells were pretreated with polymyxin B (POL B, 10 µg ml^{-1}) for 10 min before the addition of LPS (100 ng ml^{-1}) and the incubation was continued for a further 22 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant reduction from untreated control (C) cells. ### $P<0.001$ indicates a significant increase from untreated cells. +++ $P<0.001$ indicates a significant inhibition of LPS-stimulated nitrite levels by polymyxin B.

LPS to stimulate nitrite production. To test this, J774.7 cells were pretreated with polymyxin B (10 μg ml^{-1}) for 10 min before the addition of LPS (100 ng ml^{-1}) and the incubation was continued for a further 22 hours. Polymyxin B significantly reduced basal nitrite accumulation by 40.8 ± 2.8 %, perhaps suggesting the presence of a slight endotoxin contamination of the medium (Fig 3.6). LPS produced a 1.9-fold rise in nitrite accumulation and this was reduced by polymyxin B by 65.6 ± 3.8 %.

3.2 Investigation of nitrite accumulation stimulated by LPS and interferon-gamma (IFN-γ)

3.2.1 Effects of IFN-γ alone and in combination with LPS

Although LPS did stimulate nitrite accumulation in most experiments, it produced only very modest (2-3 fold) increases. An examination was therefore conducted into the ability of other agents to activate these cells.

Murine recombinant IFN-γ had inconsistent effects on nitrite production by J774.7 cells during an overnight incubation. In some experiments, IFN-γ (1-100 u ml^{-1}) produced small and inconsistent increases in nitrite production (Fig 3.7A), while in others, a concentration-dependent rise was seen (Fig 3.7B).

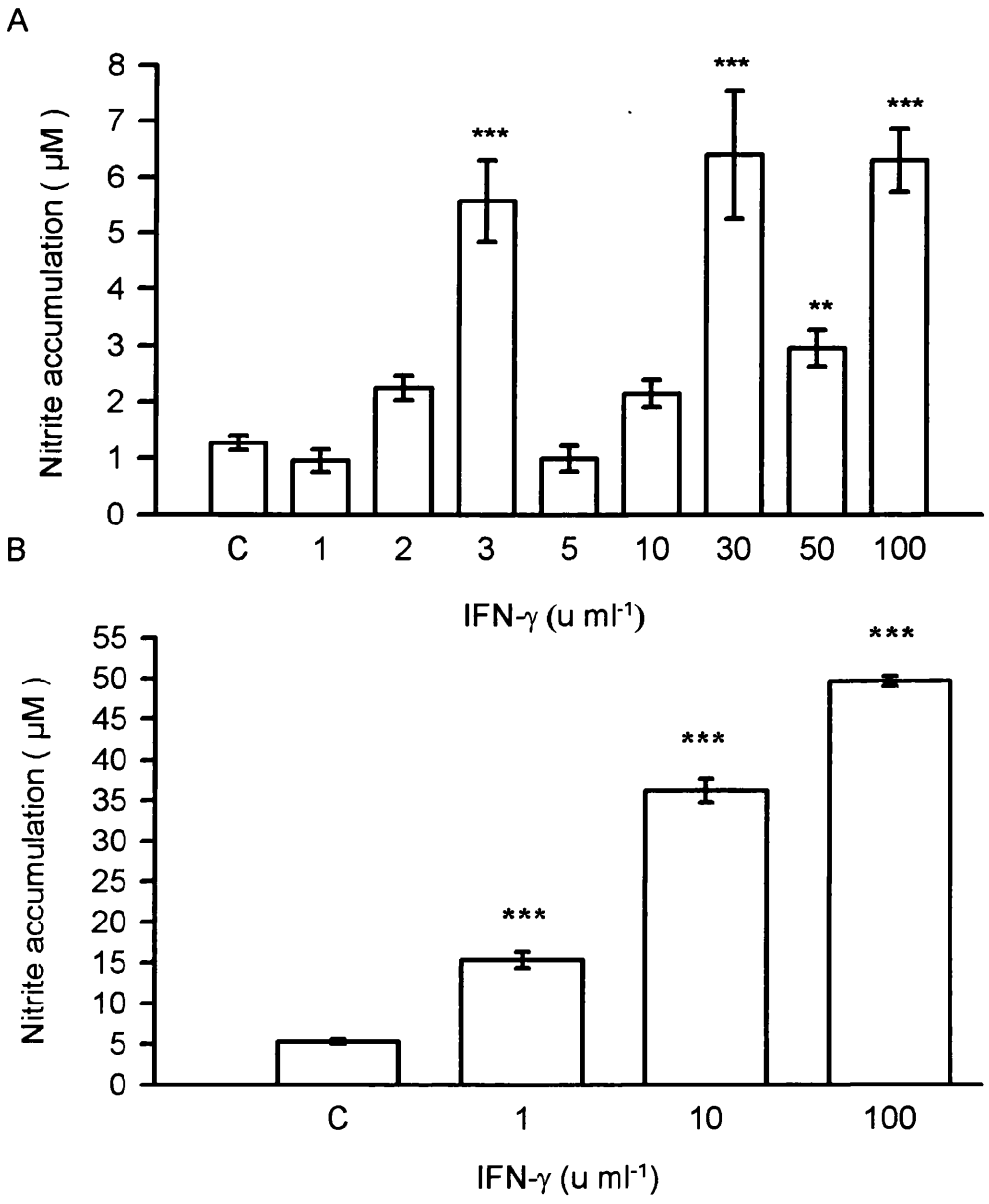


Figure 3.7 The inconsistent effects of interferon-gamma (IFN-γ, 1-100 u ml^{-1}) on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) over (A) 23 hours and (B) 18 hours. Each column represents the mean ± s.e. mean of 4-6 observations. In some experiments, a weak and inconsistent rise in nitrite accumulation was seen (A), whereas in others, a powerful, concentration-dependent rise was obtained (B). ** $P<0.01$ and *** $P<0.001$ represent a significant increase from untreated control (C) cells.

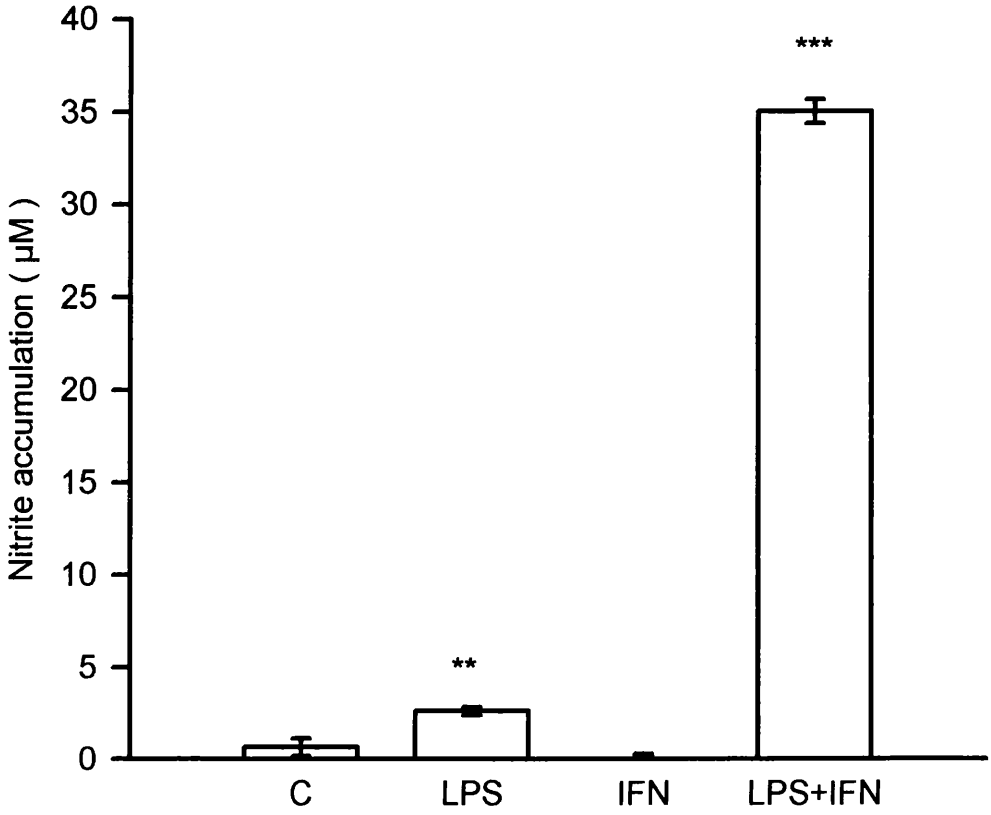


Figure 3.8 Effects of lipopolysaccharide (LPS) from *Salmonella typhosa* (100 ng ml^{-1}) and interferon-gamma (IFN, 10 u ml^{-1}) alone and in combination on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) over 22 hours. Each column represents the mean ± s.e. mean of 6 observations. ** $P<0.01$ and *** $P<0.001$ indicate a significant increase from untreated control (C) cells.

3.2.2 Effects of LPS and IFN-γ in combination

Having established that LPS and IFN-γ were each unable to stimulate reliably nitrite production by J774.7 cells during an overnight incubation, the effects of these stimuli in combination were examined. LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}) alone and in combination, were added to J774.7 cells and the incubation was continued for 22 hours. In this particular experiment, LPS produced a significant increase in nitrite accumulation of around 4-fold, but IFN-γ alone produced no rise (Fig 3.8). In contrast, the combined stimulus of LPS and IFN-γ produced a synergistic increase in nitrite accumulation of 50-fold, reaching 33.1 ± 0.6 μM.

3.2.3 Time course of nitrite accumulation

Having established that the combined stimulus of LPS and IFN-γ was much more effective than either stimulus on its own, the time course of this effect was studied over 48 hours. Untreated J774.7 cells (10^6 cells ml^{-1}) produced very little nitrite, with levels rising to only 8.1 ± 0.5 μM at 48 hours. Cells treated with LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}) produced significantly more nitrite than untreated cells: an increase was first seen at 12 hours (5.5 ± 0.3 μM), and production continued to rise to 77.1 ± 1.9 μM at 48 hours (Fig 3.9). The almost linear production of nitrite throughout this period suggested that the substrate for the production of NO, L-arginine, had not become rate-limiting.

3.2.4 Effects of L-NMMA on nitrite production

The inhibitor of NO synthase, L-NMMA, was used to assess whether or not nitrite produced by J774.7 cells during an overnight incubation with the

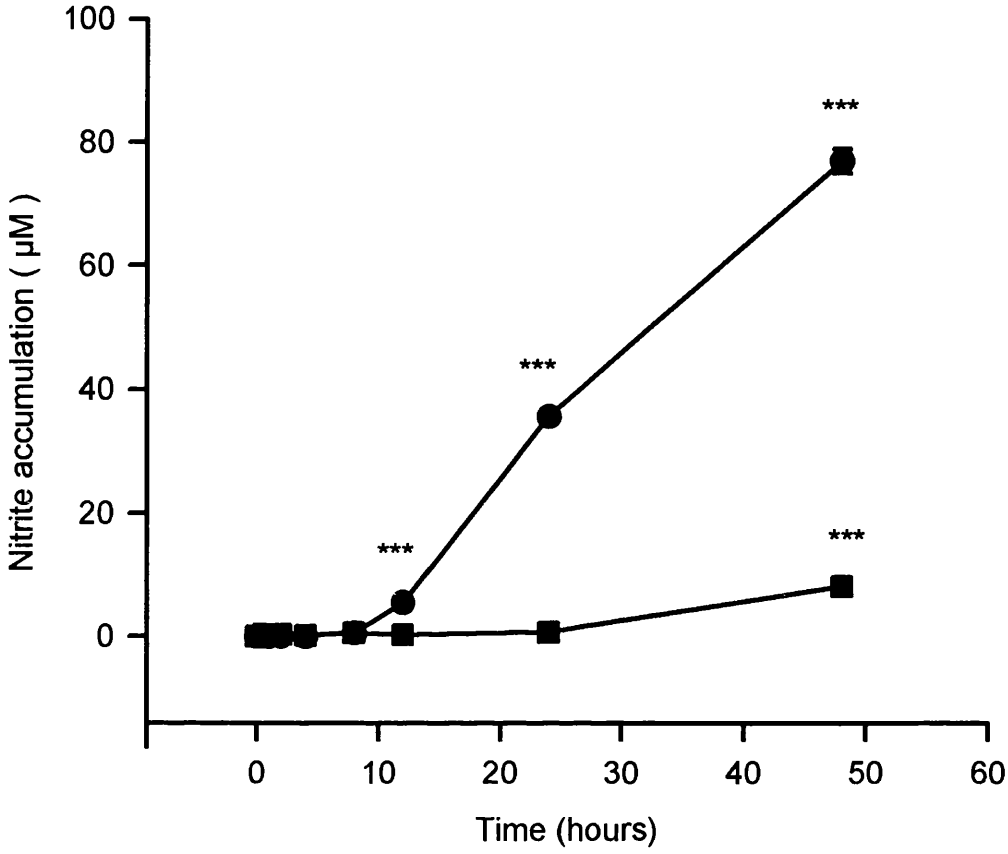


Figure 3.9 Time course of nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) over 48 hours. Cells were untreated (■) or treated with lipopolysaccharide (10 ng ml^{-1}) from *Salmonella typhosa* and interferon-gamma (2 u ml^{-1})(●). Each point represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant increase from nitrite accumulation at 0 time .

combined stimulus of LPS and IFN-γ was derived from the L-arginine/NO system. Cells were pretreated with L-NMMA for 10 min before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 22 hours. L-NMMA (10^{-4} M) produced a significant reduction (52.2 ± 7.8 %) of basal nitrite accumulation (Fig 3.10). L-NMMA (10^{-6}-$3x10^{-4}$ M) also produced a concentration-dependent inhibition of the increase in nitrite accumulation induced by the combined stimulus of LPS and IFN-γ: a maximum inhibition of 72.6 ± 3.4 % occurred at a concentration of $3x10^{-4}$ M (Fig 3.10).

3.2.5 Effects of L-NAME on nitrite production

The effects of a second inhibitor of NO synthase, L-NAME, were investigated on nitrite production by J774.7 cells in response to the combined stimulus of LPS and IFN-γ. Cells were pretreated with L-NAME for 20 min before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}) and the incubation was continued for a further 23 hours. In this experiment, L-NAME (10^{-4} M) had no effect on basal nitrite accumulation. L-NAME at concentrations of 10^{-6}-$3x10^{-5}$ M, also had no effect on nitrite accumulation stimulated by the combination of LPS and IFN-γ. It did, however, produce a slight concentration-dependent inhibition at concentrations of 10^{-4}-10^{-3} M: a maximum inhibition of 22.4 ± 0.7 % was achieved at 10^{-3} M L-NAME (Fig 3.11).

These findings indicate that, following stimulation of J774.7 cells with the combination of LPS and IFN-γ, L-NMMA is a much more effective inhibitor of nitrite production than L-NAME. This is in contrast to previous findings

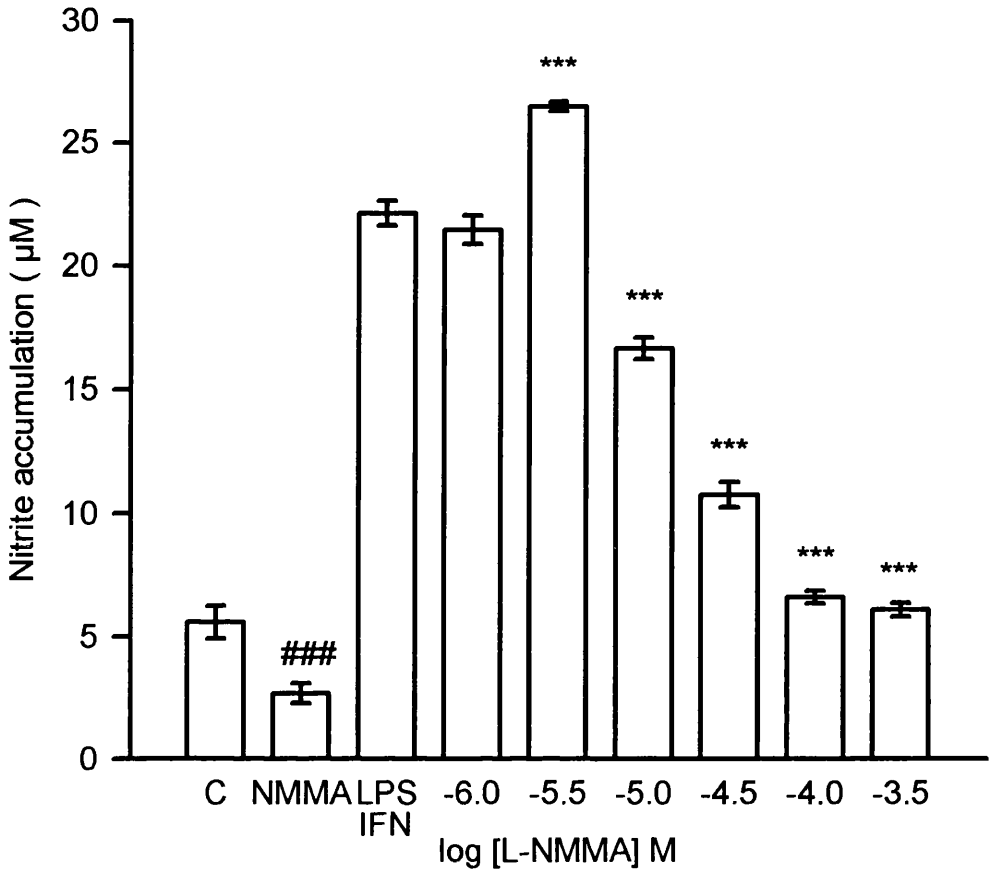


Figure 3.10 Effects of the inhibitor of nitric oxide synthase, L-NMMA, on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) from *Salmonella typhosa* and interferon-gamma (IFN-γ). Cells were pretreated with L-NMMA (10^{-6}-$3x10^{-4}$ M) for 10 min before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}) and the incubation was continued for a further 22 hours. L-NMMA (NMMA, 10^{-4} M) was also added to cells not stimulated with LPS and IFN-γ. Each column represents the mean ± s.e. mean of 6 observations. ### $P<0.001$ indicates a significant decrease from control (C) cells. *** $P<0.001$ indicates a significant change by L-NMMA of nitrite levels stimulated by LPS and IFN-γ.

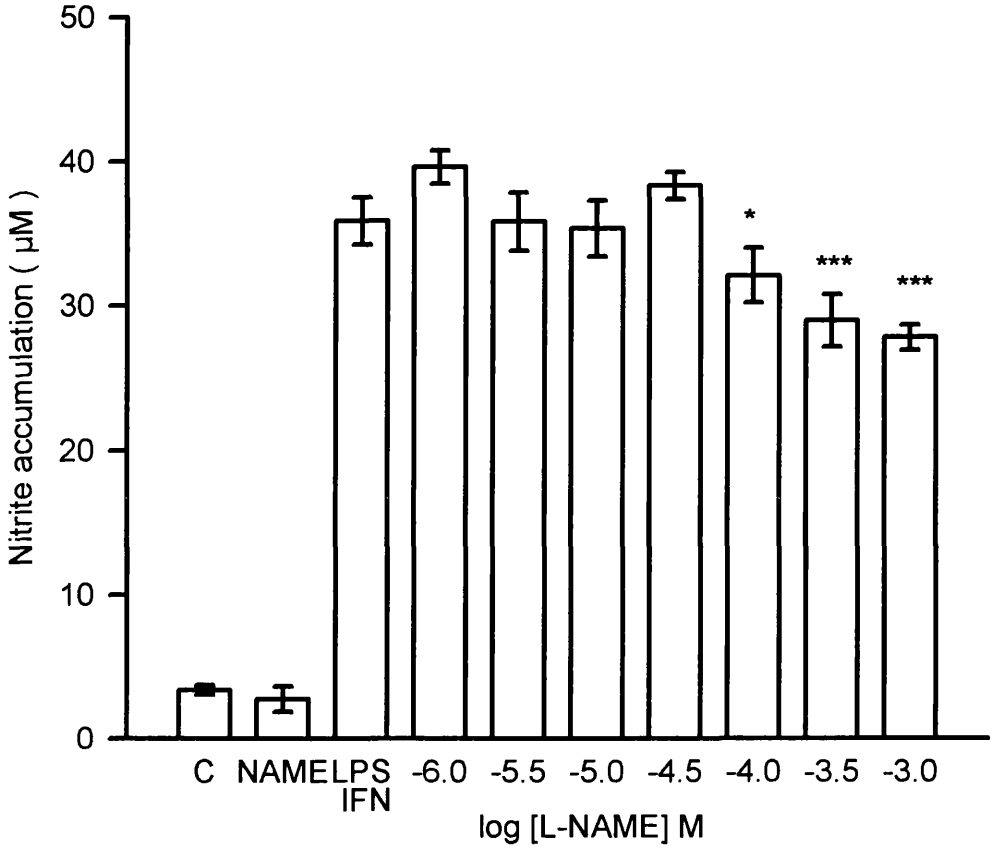


Figure 3.11 Effects of the inhibitor of nitric oxide synthase, L-NAME, on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) from *Salmonella typhosa* and interferon-gamma (IFN-γ). Cells were pretreated with L-NAME (10^{-6}-10^{-3} M) for 20 min before addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}) and the incubation was continued for a further 23 hours. L-NAME (NAME, 10^{-4} M) was also added to cells not stimulated with LPS and IFN-γ. Each column represents the mean ± s.e. mean of 6 observations. * $P<0.05$ and ***$P<0.001$ indicate a significant inhibition by L-NAME of nitrite levels stimulated by LPS and IFN-γ.

with J774.7 and RAW 264 cells, where L-NMMA and L-NAME were equally effective at inhibiting nitrite accumulation stimulated by LPS alone.

3.2.6 Effects of polymyxin B on nitrite production

Polymyxin B binds and inactivates LPS. It would therefore be expected to inhibit selectively the effects of LPS but not of IFN-γ on nitrite production by J774.7 cells. Cells were pretreated with polymyxin B (10 μg ml^{-1}) before addition of LPS (100 ng ml^{-1}), IFN-γ (10 u ml^{-1}) or a combination of the two, and the incubation was continued for a further 22 hours. Polymyxin B had no effect on basal nitrite accumulation by the cells in this particular experiment (Fig 3.12). LPS alone produced a small rise (5.4-fold) in nitrite accumulation and this was inhibited by 88.8 ± 45.9 % by polymyxin B. IFN-γ alone also produced a significant rise (15-fold) in nitrite accumulation and this too was inhibited by 49.7 ± 2.7 % by polymyxin B. The combined stimulus of LPS and IFN-γ produced around a 60-fold rise in nitrite accumulation and this was inhibited by 79.2 ± 3.7 % by polymyxin B (Fig 3.12).

3.2.7 Effects of dexamethasone on nitrite production

Dexamethasone inhibits the expression of inducible nitric oxide synthase (Di Rosa *et al.*, 1990). It would therefore be expected to inhibit nitrite production by J774.7 cells stimulated by LPS and IFN-γ. Cells were pretreated with dexamethasone (10^{-8}-$3x10^{-6}$ M) for 1 hour before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. Dexamethasone (10^{-6} M) had no effect on basal production of nitrite (Fig 3.13). The effects on production stimulated

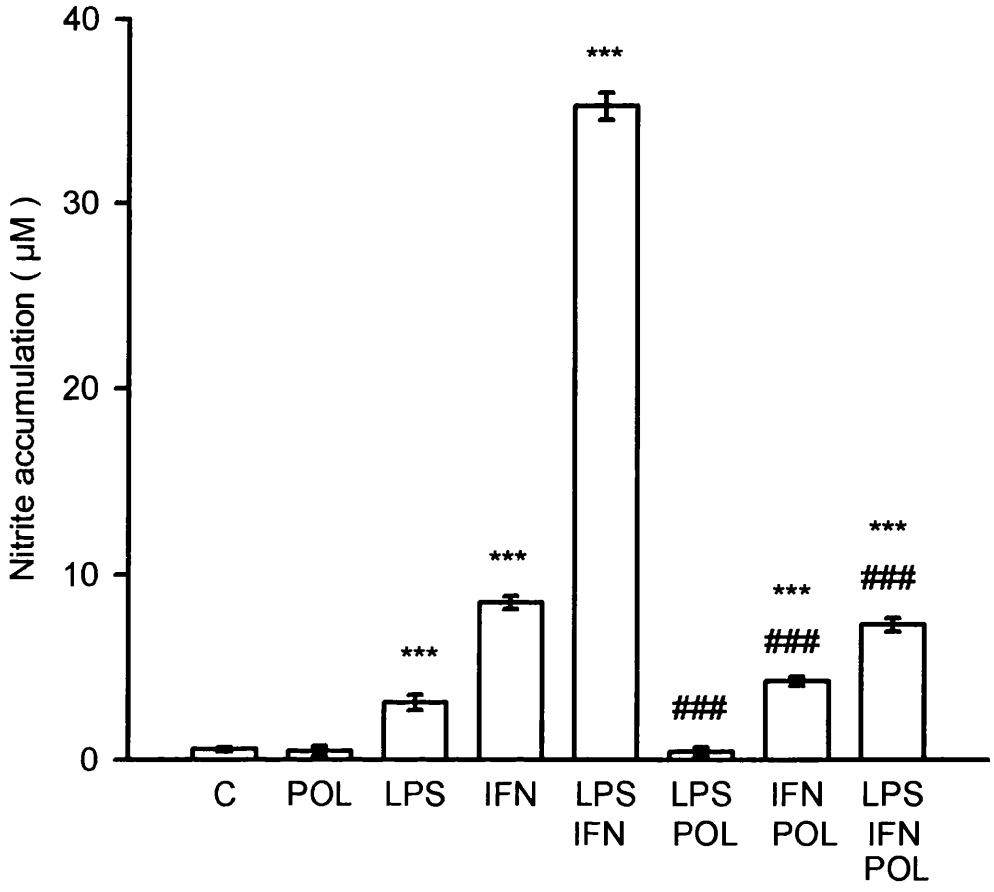


Figure 3.12 Effects of the peptide, polymyxin B, on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the addition of lipopolysaccharide (LPS) from *Salmonella typhosa*, interferon-gamma (IFN-γ) or a combination of the two. Cells were pretreated with polymyxin B (POL, 10 µg ml^{-1}) for 20 min before addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}) and the incubation was continued for a further 22 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant increase from untreated control (C) cells. ### $P<0.001$ indicates a significant inhibition by polymyxin B.

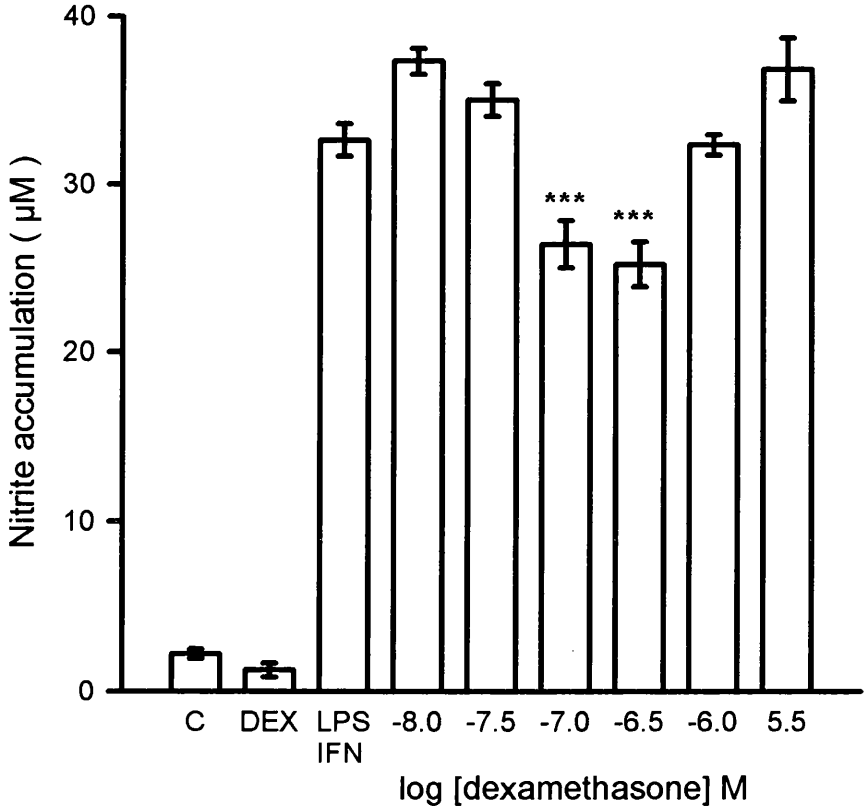


Figure 3.13 Effects of dexamethasone on basal nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) and accumulation following the combined stimulus of lipopolysaccharide (LPS) from *Salmonella typhosa* and interferon-gamma (IFN-γ). Cells were pretreated with dexamethasone (10^{-8}-$3x10^{-6}$ M) for 1 hour before the addition of LPS (100 ng ml^{-1}) and IFN-γ (2 u ml^{-1}) and the incubation was continued for a further 24 hours. Dexamethasone (DEX, 10^{-6} M) was also added to cells not stimulated with LPS and IFN-γ. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant inhibition by dexamethasone of nitrite levels stimulated by LPS and IFN-γ.

by the combination of LPS and IFN-γ were complex: inhibition was obtained at 10^{-7} M and $3x10^{-7}$ M, but higher concentrations of dexamethasone produced no inhibition; the maximum inhibition obtained was 21.8 ± 1.2 % at $3x10^{-7}$ M.

The effects of dexamethasone were also examined on cells stimulated with LPS or IFN-γ alone. Cells were pretreated with dexamethasone (10^{-6} M) for 1 hour before the addition of LPS (100 ng ml^{-1}) or IFN-γ (2 u ml^{-1}) and the incubation was continued for a further 24 hours. Dexamethasone significantly reduced by 45.4 ± 1.7 % the small (6.8-fold) increase in nitrite accumulation stimulated by LPS (Fig 3.14). IFN-γ alone had no effect on nitrite accumulation, and this was unaffected by dexamethasone. As before, dexamethasone (10^{-6} M) had only a slight inhibitory effect (14.0 ± 0.1 %) on nitrite accumulation induced by the combined stimulus of LPS and IFN-γ.

3.2.8 Effects of hydrocortisone on nitrite production

Due to the poor ability of dexamethasone to inhibit nitrite production stimulated by LPS and IFN-γ in J774.7 cells, the effects of the corticosteroid, hydrocortisone, were investigated. Cells were pretreated with hydrocortisone (10^{-6} M) for 1 hour before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 22 hours. Hydrocortisone had no effect on nitrite accumulation stimulated by the combination of LPS and IFN-γ but it did reduce basal accumulation (Fig 3.15).

3.2.9 Effects of cycloheximide on nitrite production

Cycloheximide, which inhibits protein synthesis, has previously been shown

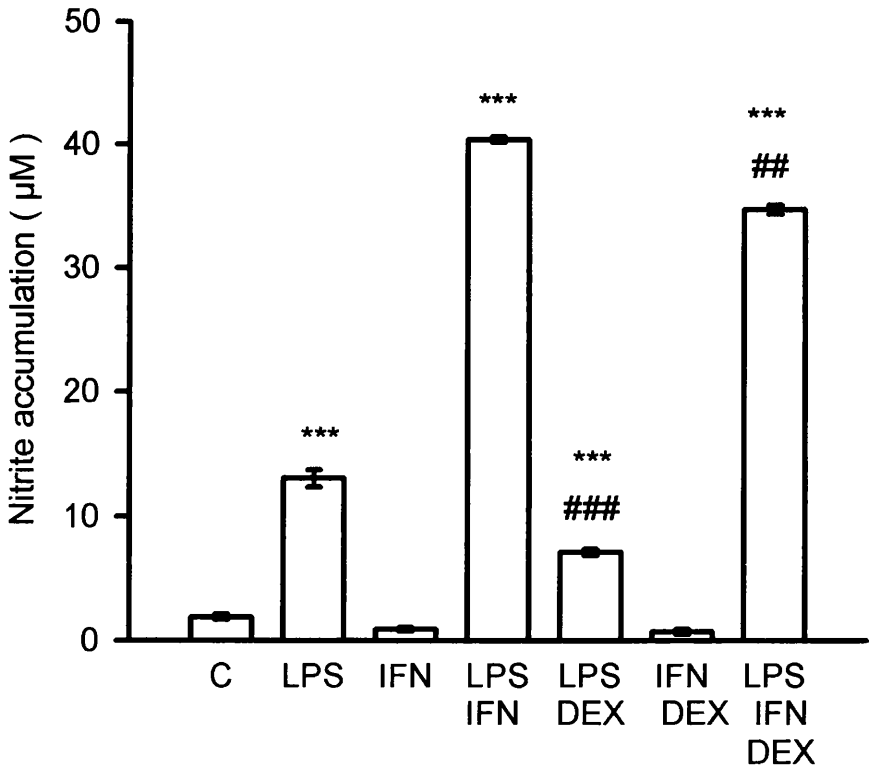


Figure 3.14 Effects of dexamethasone on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following stimulation with lipopolysaccharide (LPS) from *Salmonella typhosa*, interferon-gamma (IFN-γ) or the combined stimulus of LPS and IFN-γ. Cells were pretreated for 1 hour with dexamethasone (DEX, 10^{-6} M) before the addition of LPS (100 ng ml^{-1}), IFN-γ (2 u ml^{-1}) or a combination of both, and incubation was continued for a further 24 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant increase from untreated control (C) cells. ## $P<0.01$ and ### $P<0.001$ indicate a significant inhibition by dexamethasone.

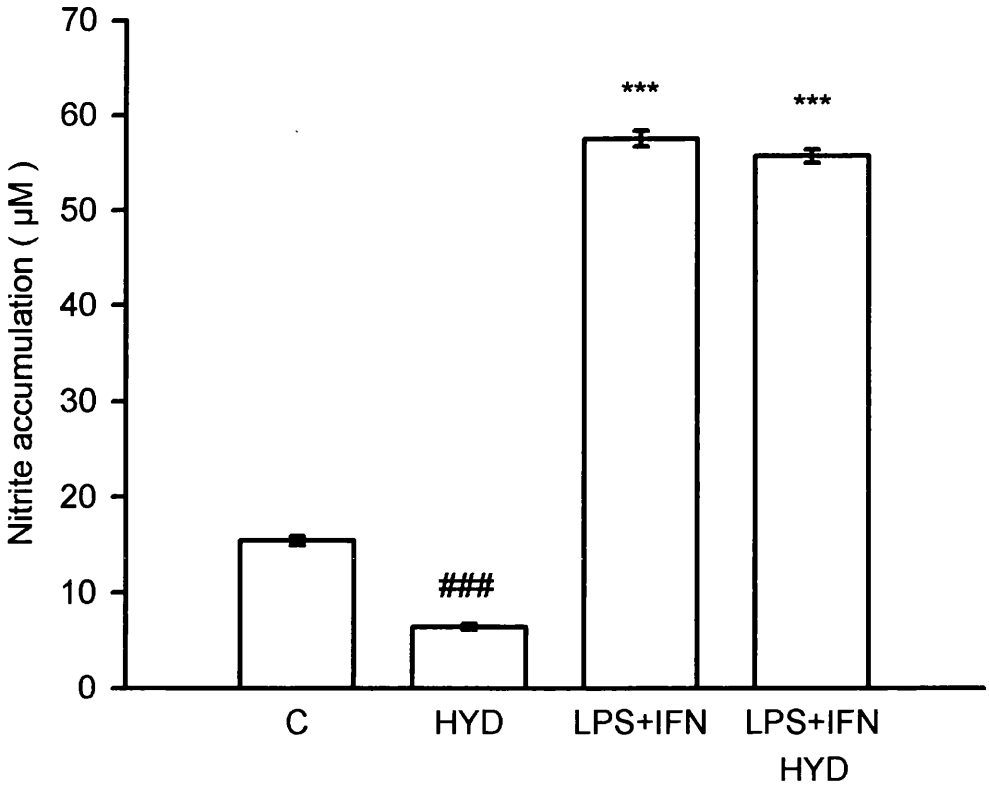


Figure 3.15 Effects of the corticosteroid, hydrocortisone, on basal accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) and accumulation following the combined stimulus of lipopolysaccharide (LPS) from *Salmonella typhosa* and interferon-gamma (IFN-γ). Cells were pretreated with hydrocortisone (HYD, 10^{-6} M) for 1 hour before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 22 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant increase from untreated control (C) cells and ### indicates a significant decrease.

to inhibit the expression of NO synthase (Oguchi *et al.*, 1994). It was therefore expected that this agent would inhibit the production of nitrite following stimulation of J774.7 cells. Cells were pretreated with cycloheximide for 2 hours before the addition of LPS (100 ng ml^{-1}), IFN-γ (10 u ml^{-1}) or a combination of the two, and the incubation was continued for a further 23 hours. Cycloheximide had no effect on basal accumulation of nitrite but decreased the 3-fold and the 14-fold increases in nitrite accumulation stimulated by IFN-γ alone and the combination of LPS and IFN-γ, respectively (Fig 3.16). In this particular experiment, LPS alone did not affect the accumulation of nitrite. It was clear in this experiment, however, that cycloheximide was exerting a cytotoxic action, since all cells treated with this agent had detached from the tissue culture plate.

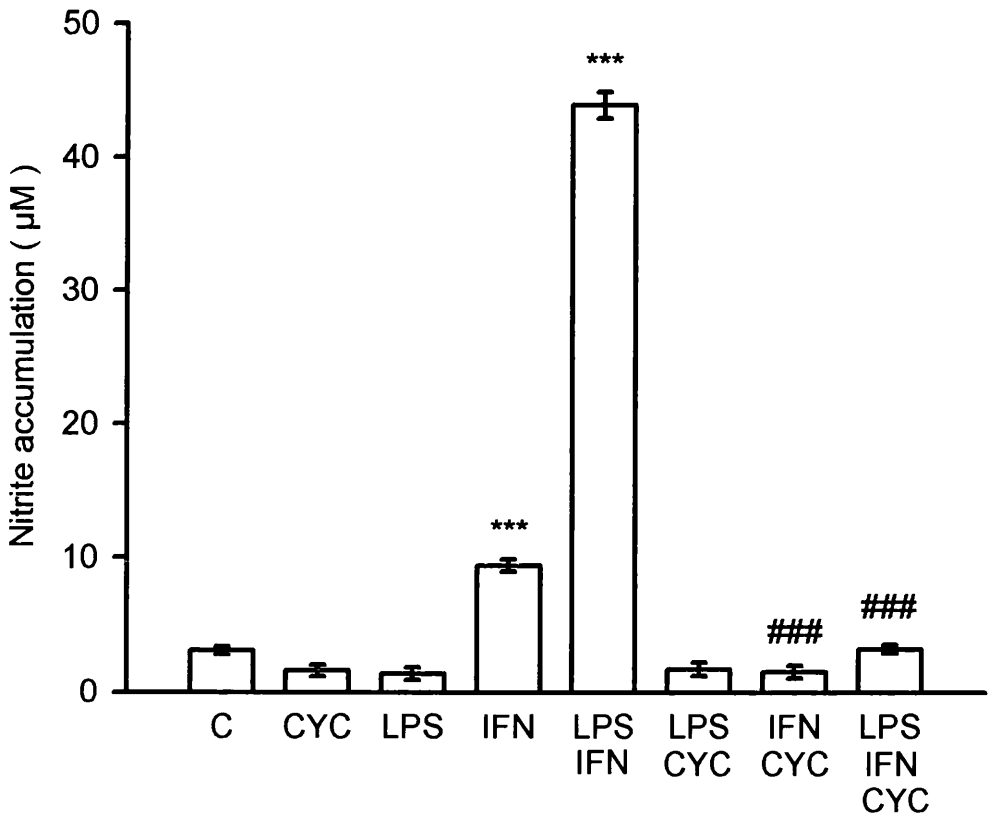


Figure 3.16 Effects of the inhibitor of protein synthesis, cycloheximide, on basal accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) and accumulation following stimulation with lipopolysaccharide (LPS) from *Salmonella typhosa*, interferon-gamma (IFN-γ) or a combination of the two. Cells were pretreated with cycloheximide (CYC, 10 µg ml^{-1}) for 2 hours before the addition of LPS (100 ng ml^{-1}), IFN-γ (10 u ml^{-1}) or a combination of both, and the incubation was continued for a further 23 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant stimulation of nitrite accumulation and ### indicates inhibition by cycloheximide of nitrite levels stimulated by LPS and IFN-γ.

CHAPTER 4

INVESTIGATION OF SECOND MESSENGER SYSTEMS REGULATING NITRITE PRODUCTION BY THE J774.7 MACROPHAGE CELL LINE

Regulation of nitrite accumulation by cyclic nucleotides

4.1 Effects of cyclic AMP on nitrite production

Cyclic AMP is well known to suppress activation of cells involved in inflammatory processes (Renz *et al.*, 1988). Since induction of NO synthase is one of the mechanisms by which macrophages contribute to the development of inflammation, the possibility of regulation of induction of NO synthase by modulating cyclic AMP levels was therefore investigated.

4.1.1 Effects of dibutyryl cyclic AMP on nitrite production

The effects of dibutyryl cyclic AMP, a membrane permeable analogue of cyclic AMP capable of mimicking the effects of this second

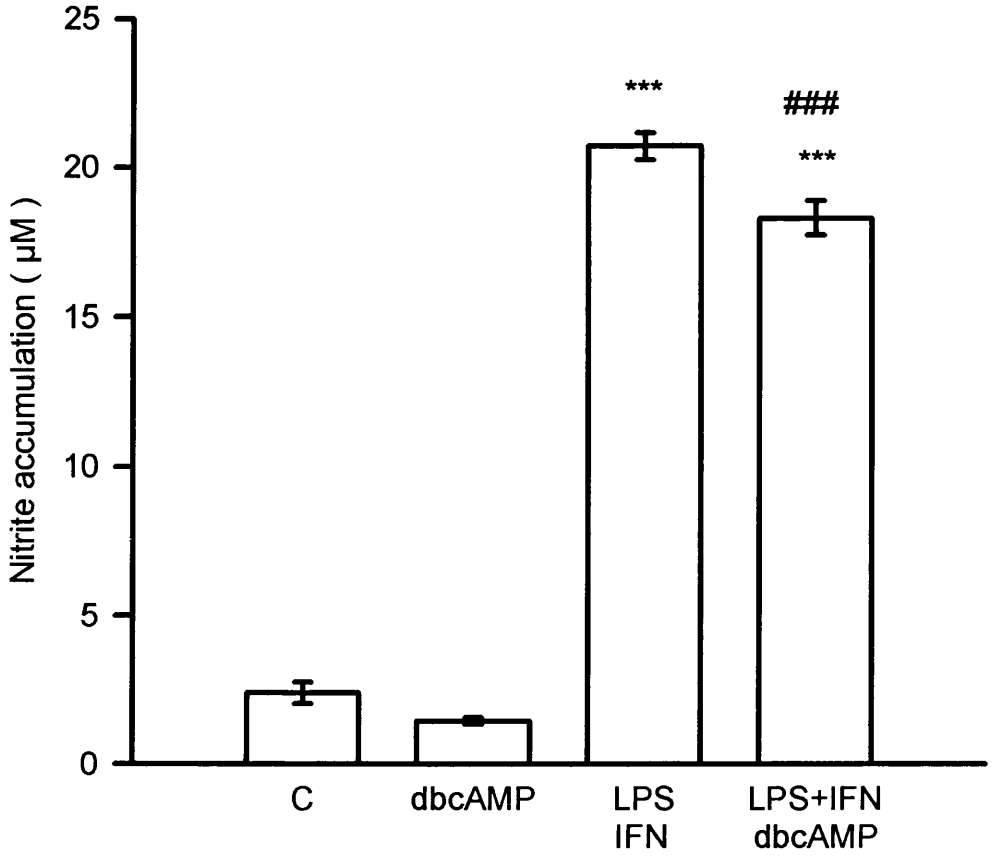


Figure 4.1 Effects of dibutyryl cyclic AMP on basal nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) and accumulation following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with dibutyryl cyclic AMP (dbcAMP, 10^{-3} M) for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 22 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant increase from untreated control (C) cells. ### $P<0.001$ indicates a significant decrease by dibutyryl cyclic AMP of nitrite levels stimulated by LPS and IFN-γ.

messenger, were investigated on nitrite production by J774.7 cells. Cells were pretreated with dibutyryl cyclic AMP (10^{-3} M) for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}) and the incubation was continued for a further 22 hours. Dibutyryl cyclic AMP had no effect on basal (3.7 ± 0.4 μM) accumulation of nitrite, but inhibited that stimulated by the combination of LPS and IFN-γ by 11.7 ± 2.8 % (Fig 4.1).

Elevation of cyclic AMP levels is known to suppress the induction of NO synthase at the level of transcription and/or translation (Marotta *et al.*, 1992; Bulut *et al.*, 1993). However, it is known that inducible NO synthase has a consensus sequence for phosphorylation by protein kinase A (Lowenstein *et al.*, 1992), and agents which elevate cyclic AMP levels may therefore have an effect on enzyme activity by activating PKA. An attempt was therefore made to determine if dibutyryl cyclic AMP could affect activity of the already induced NO synthase, and this too was assessed by measuring nitrite accumulation. Cells were treated with LPS and IFN-γ for 12 hours to induce the enzyme. After 12 hours the activating stimuli were removed and the cells were treated with dibutyryl cyclic AMP (10^{-3} M) and the incubation was continued for a further 24 hours. Dibutyryl cyclic AMP produced a significant inhibition (9.9 ± 0.8 %, n=6) of nitrite accumulation which did not significantly differ (P= 0.56) from that occurring (11.7 ± 2.7 %) when dibutyryl cyclic AMP was added to the cells prior to LPS and IFN-γ (Fig 4.1).

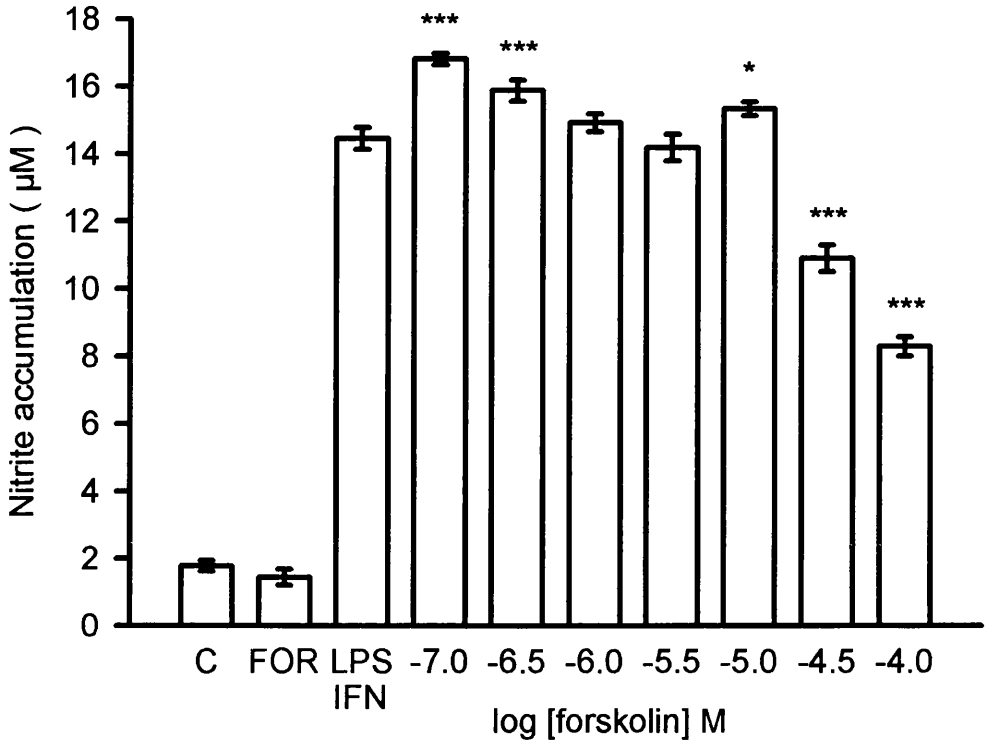


Figure 4.2 Effects of forskolin on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with forskolin (10^{-7}-10^{-4} M) for 10 min before the addition of LPS (100 ng ml^{-1}) and IFN-γ (3 u ml^{-1}), and the incubation was continued for a further 24 hours. Forskolin (FOR, $3x10^{-6}$ M) was also added to cells not stimulated with LPS and IFN-γ. Each column represents the mean ± s.e. mean of 6 observations. * $P<0.05$ and *** $P<0.001$ indicate a significant difference by forskolin of nitrite levels stimulated by LPS and IFN-γ.

4.1.2 Effects of forskolin on nitrite production

Forskolin activates the catalytic subunit of adenylate cyclase (Seamon & Daly, 1981), thus leading to elevated cyclic AMP levels. The effects of forskolin on nitrite production by J774.7 cells were therefore investigated. Cells were pretreated with forskolin (10^{-7}-10^{-4} M) for 10 min before the addition of LPS (100 ng ml^{-1}) and IFN-γ (3 u ml^{-1}) and the incubation was continued for a further 24 hours. Forskolin ($3x10^{-6}$ M) had no effect on basal nitrite accumulation. Forskolin had no consistent effect on nitrite accumulation at concentrations of 10^{-7}-10^{-5} M, but higher concentrations were inhibitory: the maximum inhibition produced by forskolin was 42.6 ± 1.4 % at 10^{-4} M (Fig 4.2).

The vehicle used to dissolve forskolin was DMSO and this reached a concentration of 0.3 % when the highest concentration of forskolin (10^{-4} M) was used. The solvent alone at this concentration slightly (12.7 ± 5.7 %) reduced the accumulation of nitrite stimulated by LPS and IFN-γ.

To assess its actions on nitrite accumulation when given after the activating stimulus, forskolin ($3x10^{-5}$ M) was added to cells after an initial 12 hour stimulation by LPS and IFN-γ and the incubation was continued for a further 24 hours. In contrast to its effects when given before LPS and IFN-γ, forskolin produced a significant (4.3 ± 1.5 %, n=6) increase in nitrite accumulation, which significantly differed ($P<0.001$) from the inhibition that occurred (24.5 ± 2.7 %) when it was added to the cells prior to LPS and IFN-γ (Fig 4.2).

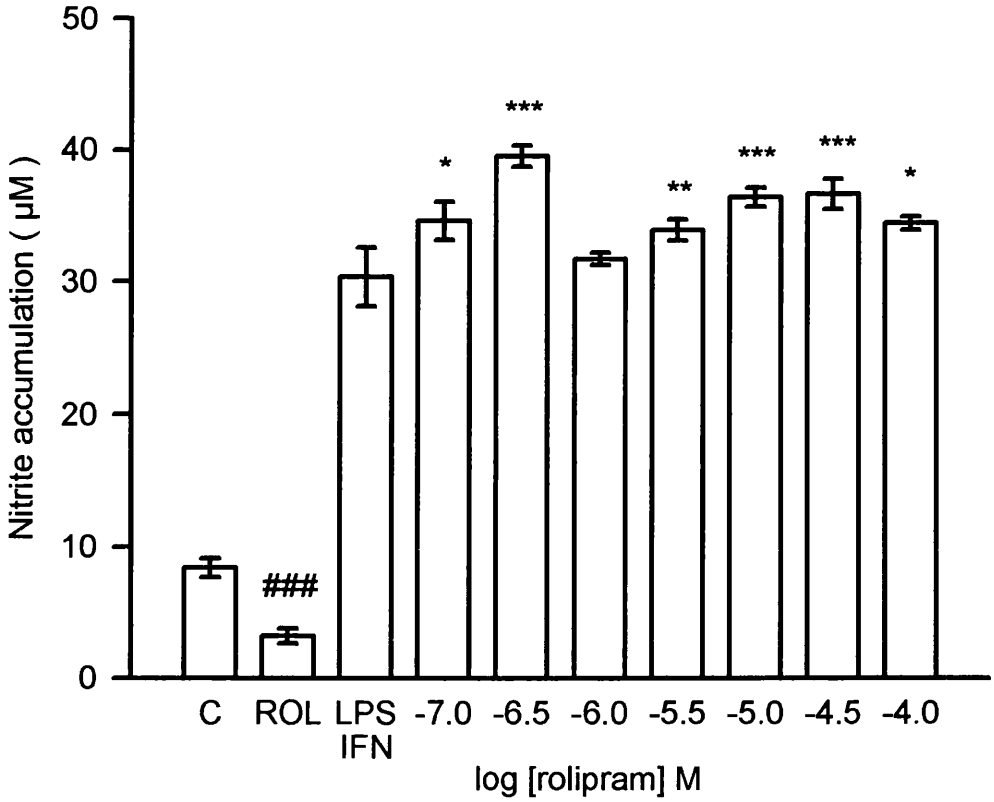


Figure 4.3 Effects of rolipram on basal accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) and accumulation following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with rolipram (10^{-7}-10^{-4} M) for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 24 hours. Rolipram (ROL, 10^{-4} M) was also added to cells not stimulated with LPS and IFN-γ. Each column represents the mean ± s.e. mean of 6 observations. ### $P<0.001$ indicates a significant decrease from untreated control (C) cells. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ indicate a significant increase by rolipram of nitrite levels stimulated by LPS and IFN-γ.

4.1.3 Effects of rolipram on nitrite production

Rolipram inhibits type IV phosphodiesterase (Beavo & Reifsnyder, 1990), a cyclic AMP-selective isoform. The effects of rolipram on nitrite production by J774.7 cells were therefore investigated. Cells were pretreated with rolipram (10^{-7}-10^{-4} M) for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}) and the incubation was continued for a further 24 hours. Rolipram reduced basal accumulation of nitrite but had no consistent effect on nitrite accumulation induced by the combination of LPS and IFN-γ (Fig 4.3). At some concentrations it produced an increase in nitrite accumulation, with a maximum increase of 30.4 ± 0.6 % at $3x10^{-7}$ M.

To assess its actions on nitrite accumulation when given after the activating stimulus, rolipram ($3x10^{-6}$ M) was added to cells after an initial 12 hour stimulation by LPS and IFN-γ and the incubation was continued for a further 24 hours. In contrast to the 11.6 ± 2.6 % increase in nitrite accumulation when given before LPS and IFN-γ (Fig 4.3), rolipram had no significant effect on nitrite accumulation when added 12 hours after stimulation with LPS and IFN-γ.

4.1.4 Effects of SKF 94120 on nitrite production

SKF 94120 inhibits type III phosphodiesterase (Beavo & Reifsnyder, 1990), another cyclic AMP selective isoform. The effects of SKF 94120 on nitrite production by J774.7 cells were therefore investigated. Cells were pretreated with SKF 94120 (10^{-7}-10^{-4} M) for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 24 hours. SKF 94120 (10^{-4} M) had no effect on basal nitrite production (Fig 4.4). It also had inconsistent effects on nitrite accumulation stimulated by LPS and IFN-γ: it produced a slight increase (37.5 ± 3.1 %) at

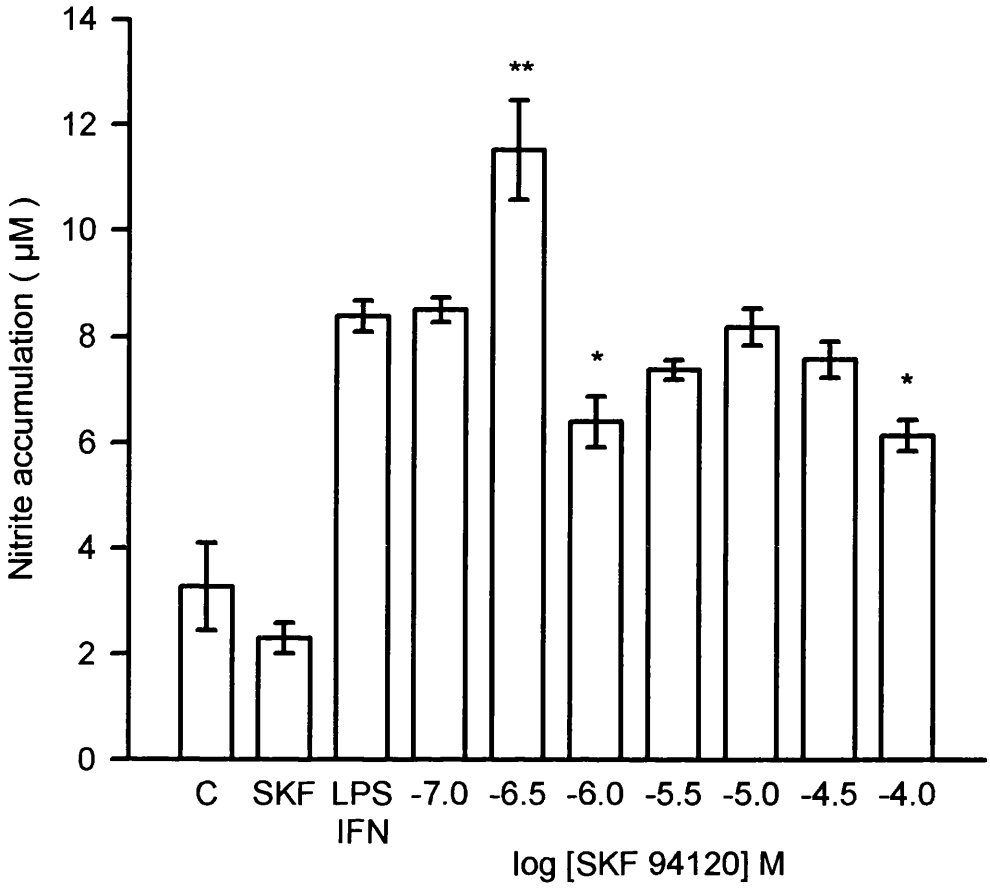


Figure 4.4 Effects of SKF 94120 on basal accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) and accumulation following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with SKF 94120 (10^{-7}-10^{-4} M) for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 24 hours. SKF 94120 (SKF, 10^{-4} M) had no effect on basal nitrite accumulation. Each column represents the mean ± s.e. mean of 6 observations.* $P<0.05$ and ** $P<0.01$ indicate a significant difference by SKF 94120 of nitrite levels stimulated by LPS and IFN-γ.

$3x10^{-7}$ M, whereas at 10^{-6} and 10^{-4} M it produced a slight decrease with a maximum of 26.8 ± 1.3 % at 10^{-4} M.

To assess its actions on nitrite accumulation when given after the activating stimulus, SKF 94120 (10^{-6} M) was added to cells after an initial 12 hour stimulation by LPS and IFN-γ and the incubation was continued for a further 24 hours. SKF 94120 had no effect (2.4 ± 0.8 % inhibition, n=6) on nitrite accumulation, which differed significantly ($P<0.05$) from the inhibition occurring (23.8 ± 5.7 %) when SKF 94120 was added to the cells prior to LPS and IFN-γ (Fig 4.4).

4.1.5 Effects of the combination of forskolin and rolipram on nitrite production

Although forskolin, which activates adenylate cyclase, consistently inhibited nitrite production by the combined stimulus of LPS and IFN-γ, the magnitude of the inhibition was low. An attempt was therefore made to determine if the magnitude of the inhibition could be increased by combining forskolin with the type IV phosphodiesterase inhibitor, rolipram. Cells were pretreated with forskolin ($3x10^{-5}$ M) and rolipram ($3x10^{-6}$ M) for 30 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 22 hours. When used alone, forskolin produced 12.8 ± 0.4 % inhibition of nitrite accumulation, and rolipram 23.6 ± 1.0 % inhibition (Fig 4.5). When used in combination, the inhibition (27.5 ± 0.9 %) was no greater than with rolipram alone.

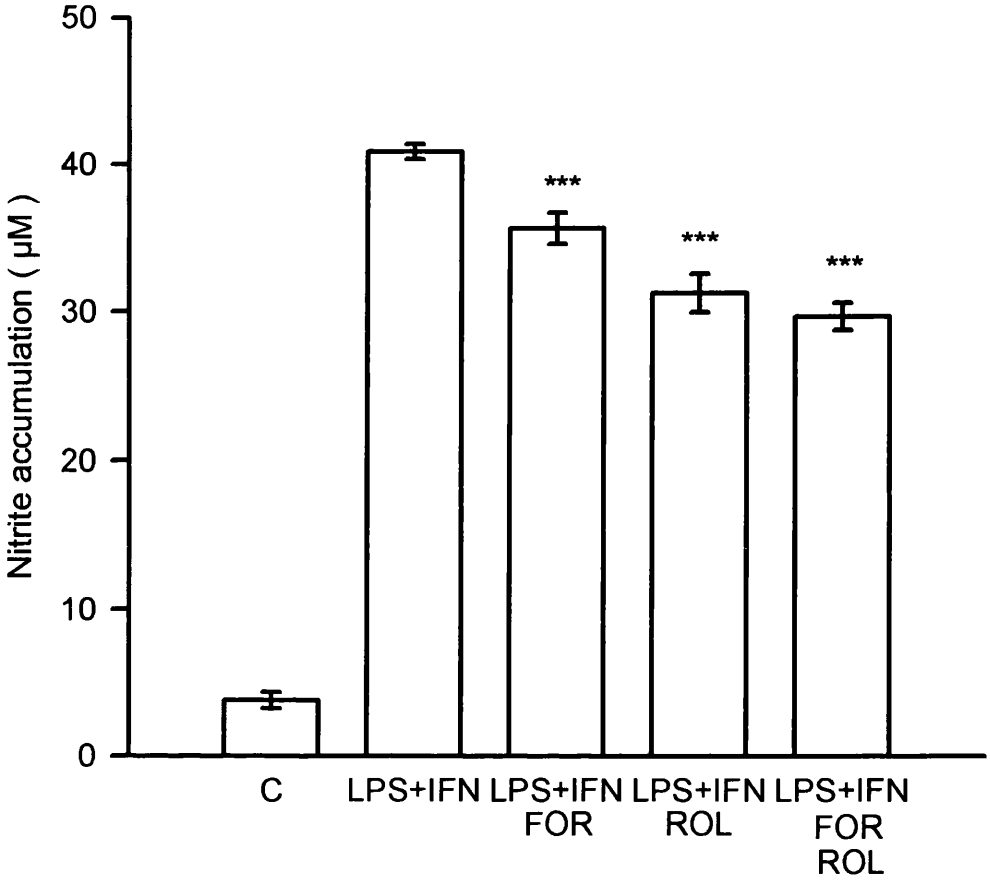


Figure 4.5 Effects of forskolin and rolipram alone and in combination on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with forskolin (FOR, $3x10^{-5}$ M) and rolipram (ROL, $3x10^{-6}$ M) alone and in combination for 30 min before the addition of LPS (10 ng m^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 22 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant decrease by forskolin and rolipram, alone and in combination, of nitrite levels stimulated by LPS and IFN-γ.

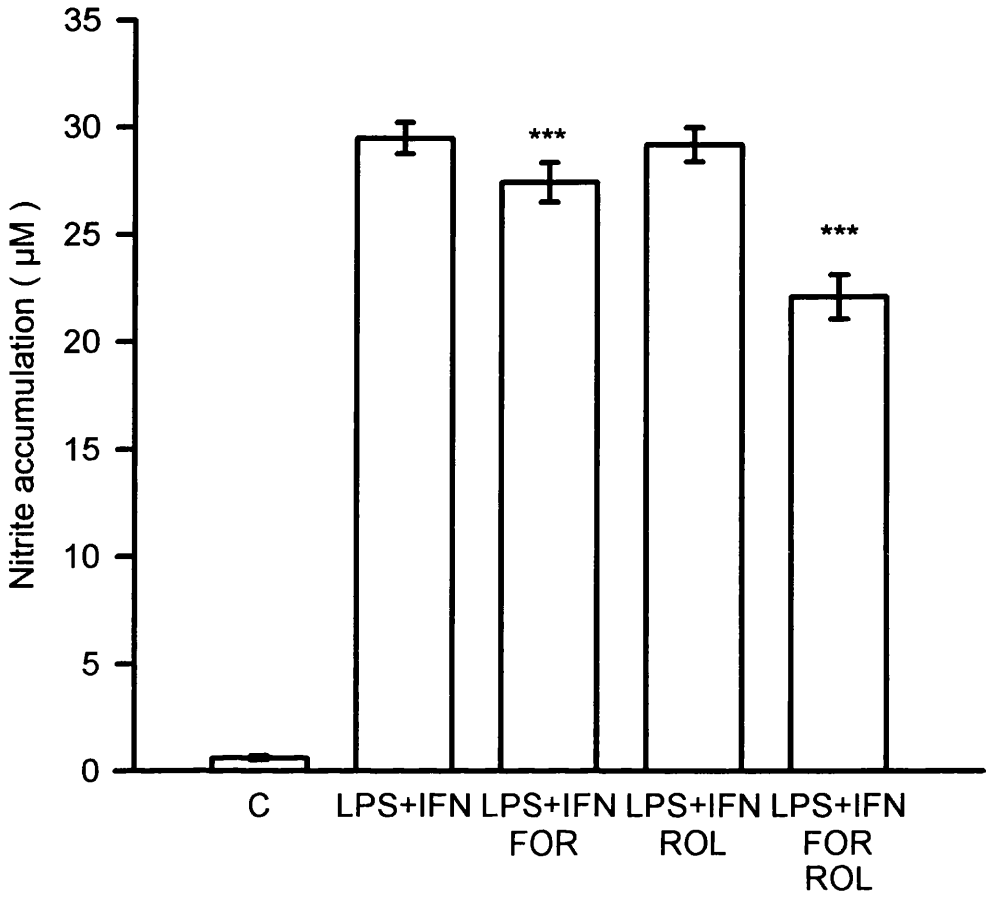


Figure 4.6 Effects of forskolin and rolipram alone and in combination on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with forskolin (FOR, $3x10^{-6}$ M) and rolipram (ROL, $3x10^{-7}$ M) alone and in combination for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 23 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant decrease by forskolin alone, and in combination with rolipram, of nitrite levels stimulated by LPS and IFN-γ.

In view of the lack of additivity of forskolin and rolipram, a further experiment was conducted using sub-maximal concentrations of both drugs in an attempt to determine if additivity could be achieved. Cells were pretreated with forskolin ($3x10^{-6}$ M) and rolipram ($3x10^{-7}$ M) for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 23 hours. Forskolin alone produced 11.0 ± 0.4 % inhibition of nitrite accumulation, whereas rolipram alone had no inhibitory effect. The combination of the two drugs produced an additive inhibitory effect of 25.1 ± 1.2 % (Fig 4.6).

4.1.6 Effects of the combination of forskolin and SKF 94120 on nitrite production

A series of experiments was conducted to determine if the ability of forskolin to inhibit the accumulation of nitrite stimulated by LPS and IFN-γ in J774.7 cells could be potentiated by the type III phosphodiesterase inhibitor, SKF 94120. Cells were pretreated with forskolin ($3x10^{-5}$ M) and SKF 94120 (10^{-6} M) alone or in combination for 30 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 24 hours. When used alone, forskolin significantly reduced nitrite accumulation by 30.5 ± 1.9 %, and SKF 94120 by 20.7 ± 0.8 % (Fig 4.7). When the drugs were used in combination, the inhibition obtained (32.6 ± 1.5 %) was no greater than that obtained by forskolin alone.

In view of the lack of additivity of forskolin and SKF 94120, a further experiment was conducted using sub-maximal concentrations of both

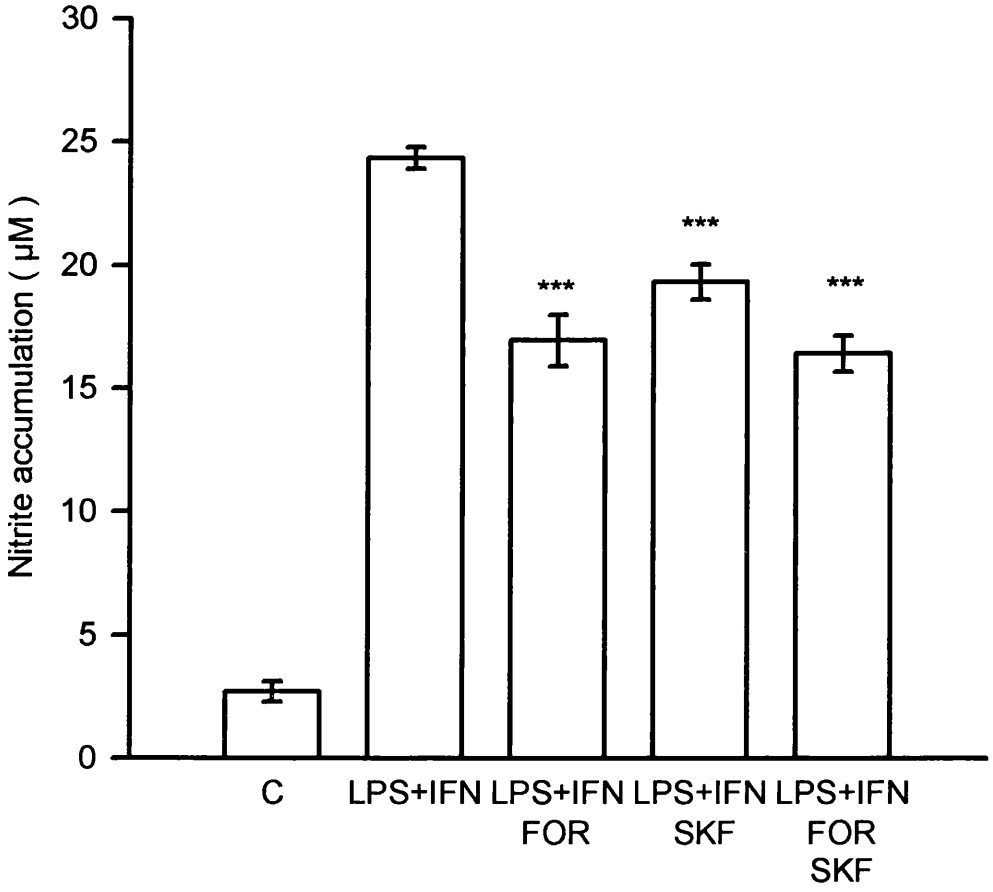


Figure 4.7 Effects of forskolin and SKF 94120 alone and in combination on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with forskolin (FOR, $3x10^{-5}$ M) and SKF 94120 (SKF, 10^{-6} M) alone and in combination for 30 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 24 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant decrease by forskolin and SKF 94120, alone and in combination, of nitrite levels stimulated by LPS and IFN-γ.

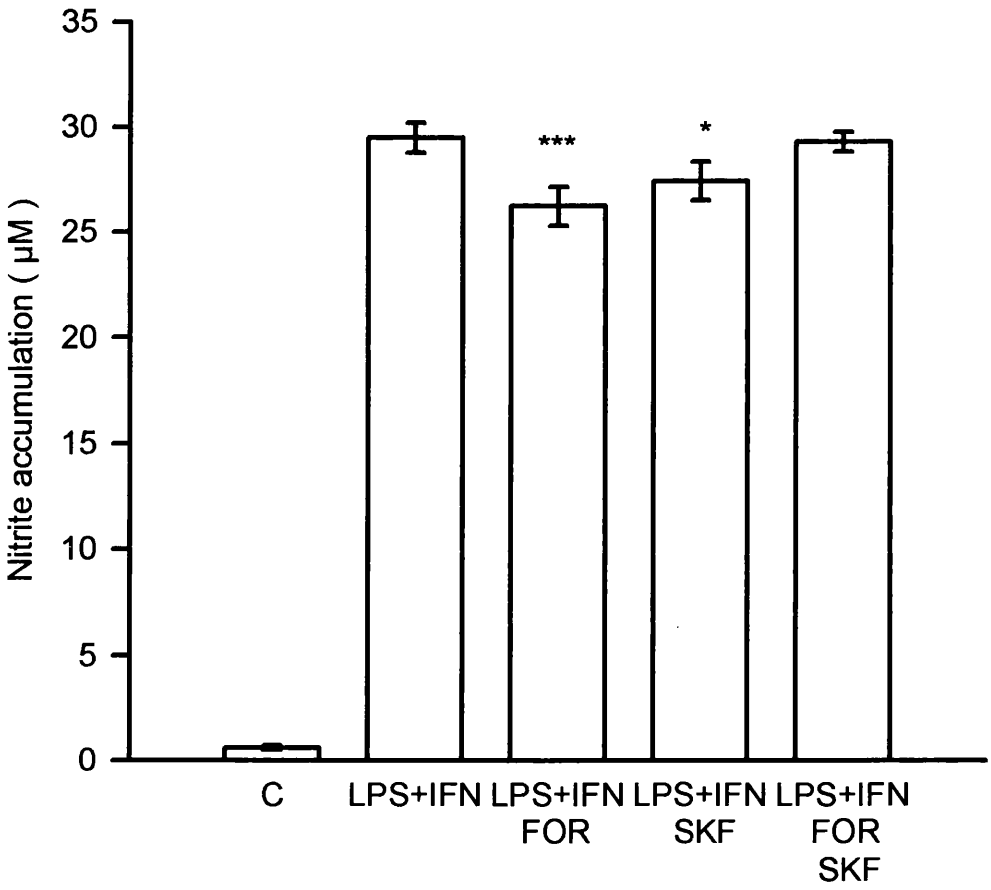


Figure 4.8 Effects of forskolin and SKF 94120 alone and in combination on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with forskolin (FOR, $3x10^{-6}$ M) and SKF 94120 (SKF, 10^{-7} M) alone and in combination for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for 23 hours. Each column represents the mean ± s.e. mean of 6 observations. * $P<0.05$ and *** $P<0.001$ indicate a significant decrease by forskolin and SKF 94120 alone, of nitrite levels stimulated by LPS and IFN-γ.

drugs in an attempt to determine if additivity could be achieved. Cells were pretreated with SKF 94120 (10^{-7} M) and forskolin ($3x10^{-6}$ M) for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 23 hours. When used alone, forskolin produced 11.0 ± 0.4 % inhibition of nitrite accumulation, whereas SKF 94120 produced 7.0 ± 0.2 % (Fig 4.8). When used in combination, the drugs produced no additive inhibitory effect.

4.2 Effects of cyclic GMP on nitrite production

A number of reports have suggested that NO can exert a negative feedback role in controlling its own production (Assreuy *et al.*, 1993; Buga *et al.*, 1993; Griscavage *et al.*, 1993). Since NO leads to elevation of cyclic GMP levels via activation of soluble guanylate cyclase, the possibility that cyclic GMP may have a regulatory role in the induction of NO synthase was investigated.

4.2.1 Effects of 8-bromo-cyclic GMP on nitrite production

The effects of 8-bromo-cyclic GMP, a membrane permeant nucleotide analogue of cyclic GMP capable of mimicking the actions of this second messenger, were investigated on nitrite production by J774.7 cells. Cells were pretreated with 8-bromo-cyclic GMP ($3x10^{-4}$ M) for 15 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (5 u ml^{-1}) and the incubation was continued for a further 24 hours. 8-bromo-cyclic GMP had no effect on basal accumulation of nitrite by the cells, but slightly increased (8.8 ± 0.2 %) that induced by the combination of LPS and IFN-γ (Fig 4.9).

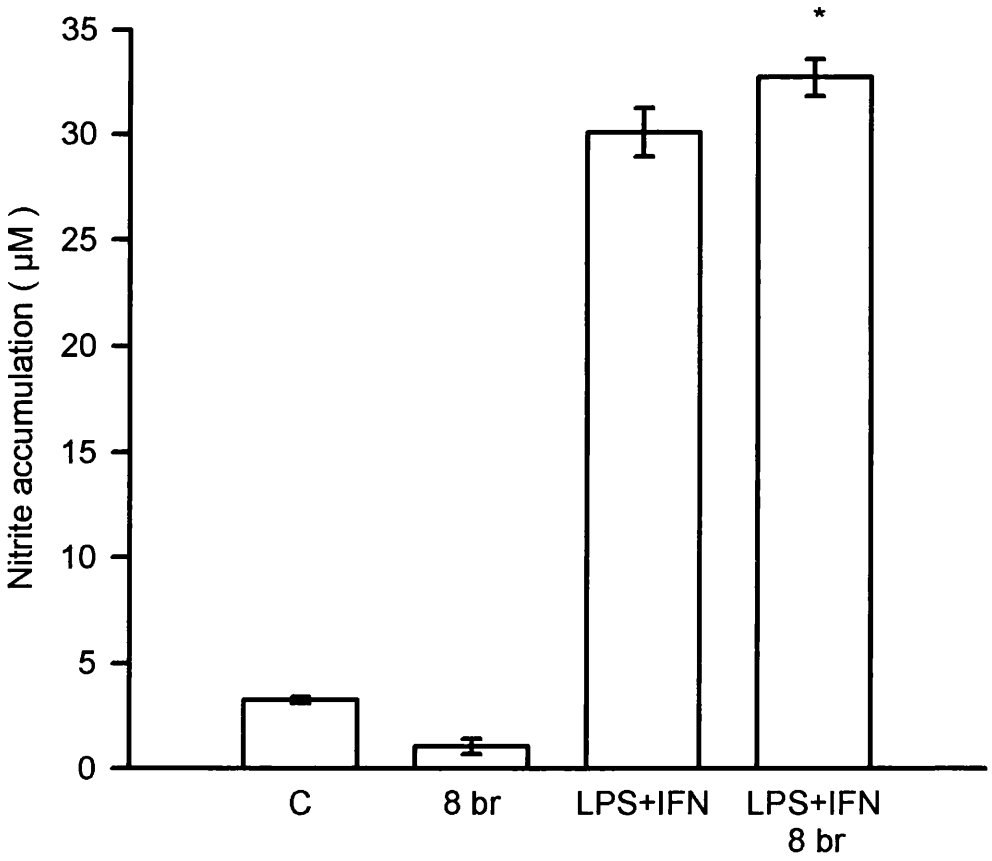


Figure 4.9 Effects of 8-bromo-cyclic GMP on basal nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) and accumulation following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated for 15 min with 8-bromo-cyclic GMP (8 br, $3x10^{-4}$ M) before the addition of LPS (10 ng ml^{-1}) and IFN-γ (5 u ml^{-1}), and the incubation was continued for a further 24 hours. Each column represents the mean ± s.e. mean of 6 observations. * $P<0.05$ indicates a significant increase by 8-bromo-cyclic GMP of nitrite levels stimulated by LPS and IFN-γ.

4.2.2 Effects of glyceryl trinitrate on nitrite production

The effects of glyceryl trinitrate (GTN), which is converted to NO by reductive enzymes located inside cells leading to elevation of cyclic GMP levels (Armstrong *et al.*, 1980; Feelisch, 1991; Schröder, 1992), were investigated on nitrite production by J774.7 cells. Cells were pretreated with GTN (10^{-8}-10^{-5} M) for 10 min before the addition of LPS (100 ng ml^{-1}) and IFN-γ (3 u ml^{-1}), and the incubation was continued for a further 23 hours. GTN (10^{-6} M) had no effect on basal nitrite production. GTN produced a biphasic effect: at concentrations of $3x10^{-7}$ M and 10^{-6} M, it inhibited nitrite accumulation by 10.8 ± 0.7 and 18.6 ± 0.6 %, respectively (Fig 4.10). However, GTN increased nitrite accumulation by 20.5 ± 1.5 % at a concentration of 10^{-5} M.

4.2.3 Effects of zaprinast on nitrite production

Zaprinast, which inhibits type I and type V phosphodiesterase isoenzymes (Beavo & Reifsnyder, 1990), would be expected to lead to elevation of cyclic GMP levels. The effects of this agent were therefore investigated on nitrite production by J774.7 cells. Cells were pretreated with zaprinast (10^{-7}-10^{-4} M) for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 23 hours. Zaprinast (10^{-4} M) had no effect on basal levels accumulation of nitrite (Fig 4.11). It did, however, at concentrations of 10^{-6} M or more, inhibit nitrite accumulation stimulated by LPS and IFN-γ, with a maximum inhibition of 33.0 ± 2.4 % at $3x10^{-5}$ M.

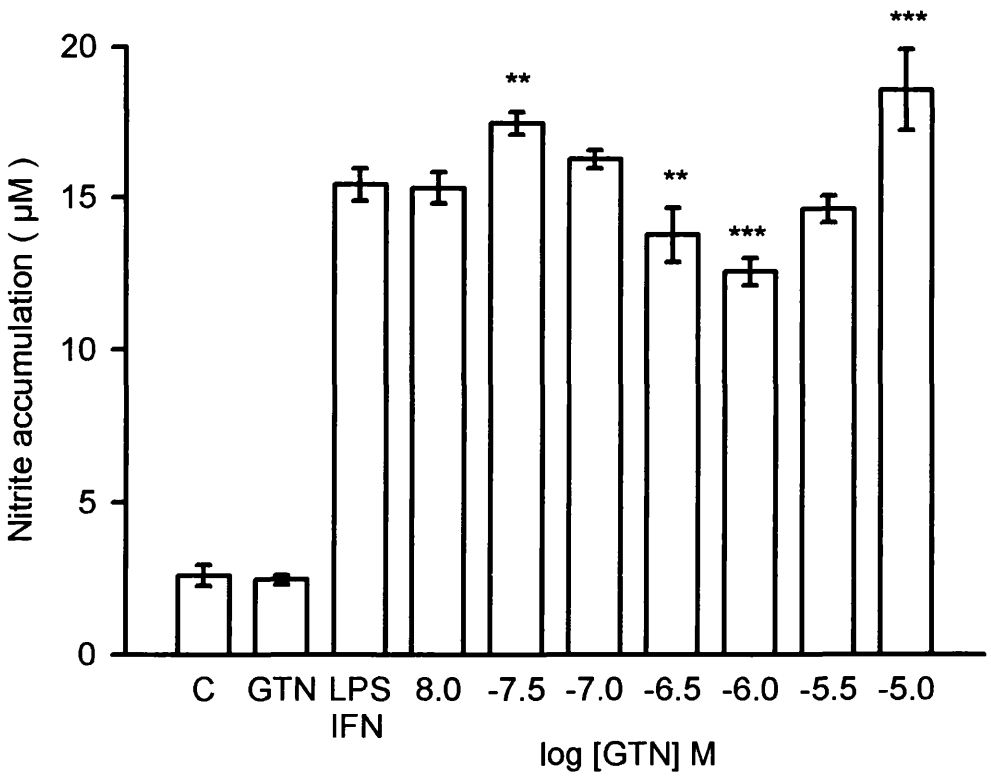


Figure 4.10 Effects of glyceryl trinitrate on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with glyceryl trinitrate (GTN, 10^{-8}-10^{-5} M) for 10 min before the addition of LPS (100 ng ml^{-1}) and IFN-γ (3 u ml^{-1}), and the incubation was continued for a further 23 hours. GTN (10^{-6} M) was also added to cells not stimulated with LPS and IFN-γ. Each column represents the mean ± s.e. mean of 6 observations. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ indicate a significant difference by GTN of nitrite levels stimulated by LPS and IFN-γ.

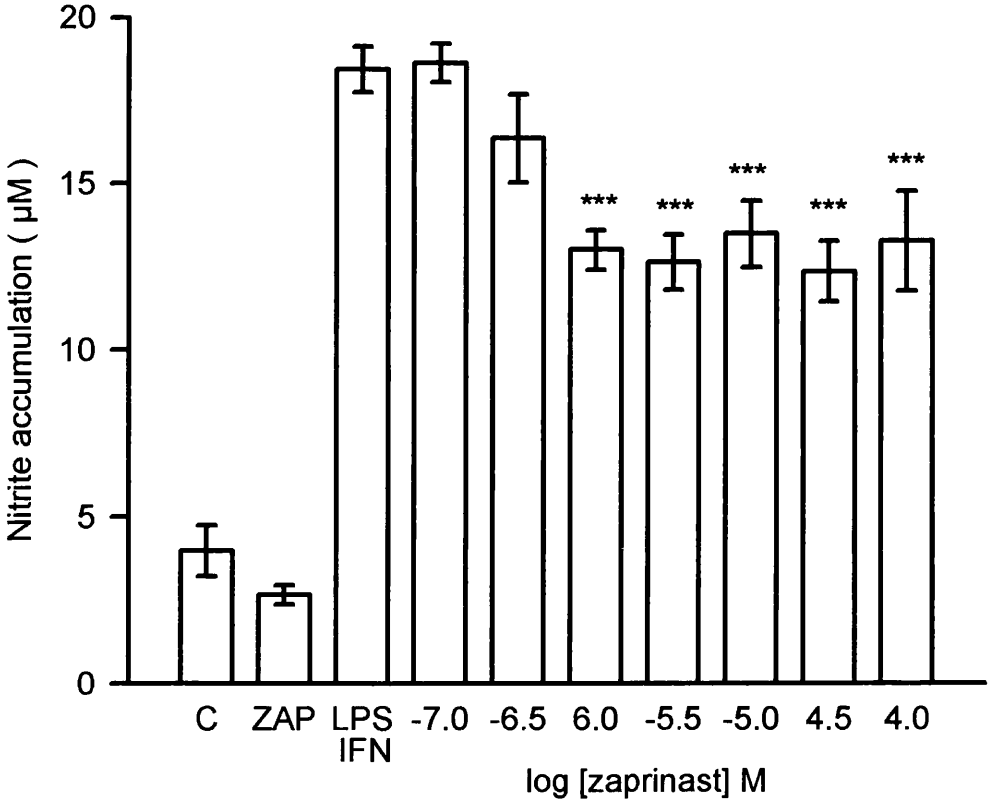


Figure 4.11 Effects of zaprinast on nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with zaprinast (10^{-7}-10^{-4} M) for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u m^{-1}), and the incubation was continued for a further 23 hours. Zaprinast (ZAP, 10^{-4} M) was also added to cells not stimulated with LPS and IFN-γ. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant decrease by zaprinast of nitrite levels stimulated by LPS and IFN-γ.

4.2.4 Effects of the combination of glyceryl trinitrate and zaprinast on nitrite production

The ability of zaprinast to inhibit type I and V phosphodiesterases should enable it to potentiate the actions of GTN mediated through formation of cyclic GMP. The effects of the combining zaprinast and GTN were therefore investigated on nitrite production by J774.7 cells. Cells were pretreated with zaprinast (10^{-5} M) and GTN (10^{-6} M), alone or in combination, before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 24 hours. Neither zaprinast nor GTN had any effect on basal nitrite production (Fig 4.12). When used alone on cells stimulated with LPS and IFN-γ, zaprinast produced a 17.4 ± 0.6 % inhibition of nitrite accumulation, whereas GTN produced 18.5 ± 1.1 % inhibition. The combination of zaprinast and GTN produced a similar degree of inhibition (16.0 ± 0.7 %) to that obtained with each drug alone.

Regulation of nitrite accumulation by calcium and protein kinase C

4.3 Stimulatory effects of PMA and A23187

Stimulation of protein kinase C is one of the proposed second messenger pathways involved in the induction of NO synthase (Hortelano *et al.*, 1992; Severn *et al.*, 1992). The phorbol ester, phorbol 12-myristate 13-acetate (PMA) is known to activate PKC, and elevation of calcium levels with the

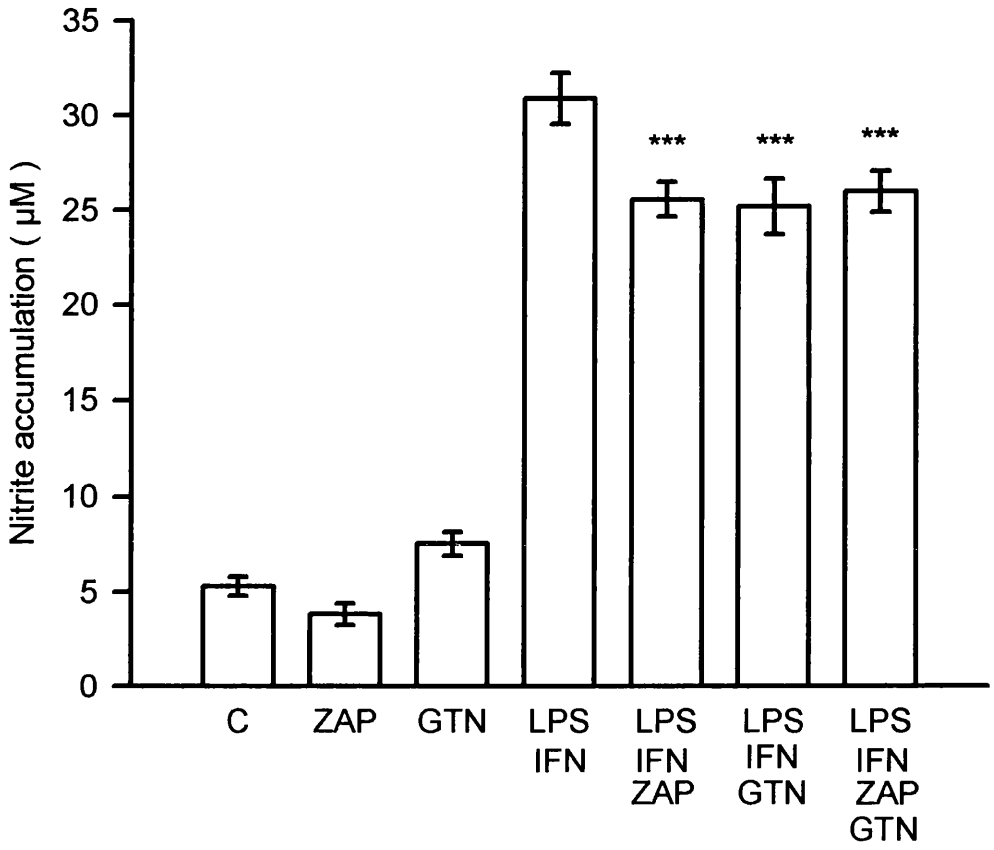


Figure 4.12 Effects of glyceryl trinitrate and zaprinast on basal nitrite accumulation into the medium bathing J774.7 cells (10^6 cells ml^{-1}) and accumulation following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with zaprinast (ZAP, 10^{-5} M) and glyceryl trinitrate (GTN, 10^{-6} M) alone or in combination for 20 min before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 24 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant decrease by zaprinast and GTN, alone and in combination, of nitrite levels stimulated by LPS and IFN-γ.

calcium ionophore, A23187, also leads to activation of PKC, as well as of calcium-calmodulin-dependent protein kinase. This section is concerned with investigating if treatment with PMA and A23187 is sufficient to induce NO synthase.

4.3.1 Effects of A23187 and PMA on nitrite production

The effects of the calcium ionophore, A23187 and the phorbol ester, PMA, an agent which stimulates PKC, were investigated on nitrite accumulation by J774.7 cells. Cells were treated with A23187 (10^{-6} M) and PMA (10^{-6} M) alone and in combination, and also with LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}) in combination for 24 hours. When used alone, A23187 and PMA had no effect on basal nitrite accumulation (Fig 4.13). The combination of A23187 and PMA produced a small rise from 3.2 ± 0.2 to 5.4 ± 0.4 μM. However, this combination did not mimic the powerful stimulation of the cells induced by the combination of LPS and IFN-γ (45.3 ± 0.9 μM).

4.3.2 Comparison of the effects of PMA and A23187 with those of IFN-γ in enhancing the effects of LPS

A23187 and PMA together have the ability to mimic the effect of IFN-γ in priming the induction of tumouricidal activity by LPS in macrophages (Celada & Schreiber, 1986; Somers *et al.*, 1986). The effects of pretreatment with A23187 and PMA were therefore compared with those of IFN-γ on nitrite production stimulated by LPS in J774.7 cells. Cells were pretreated with A23187 (10^{-6} M) and PMA (10^{-8} M) alone and in combination for 4 hours before the addition of LPS (10 ng ml^{-1}). Separate aliquots of cells received the standard stimulus of LPS (10 ng ml^{-1}) and IFN-

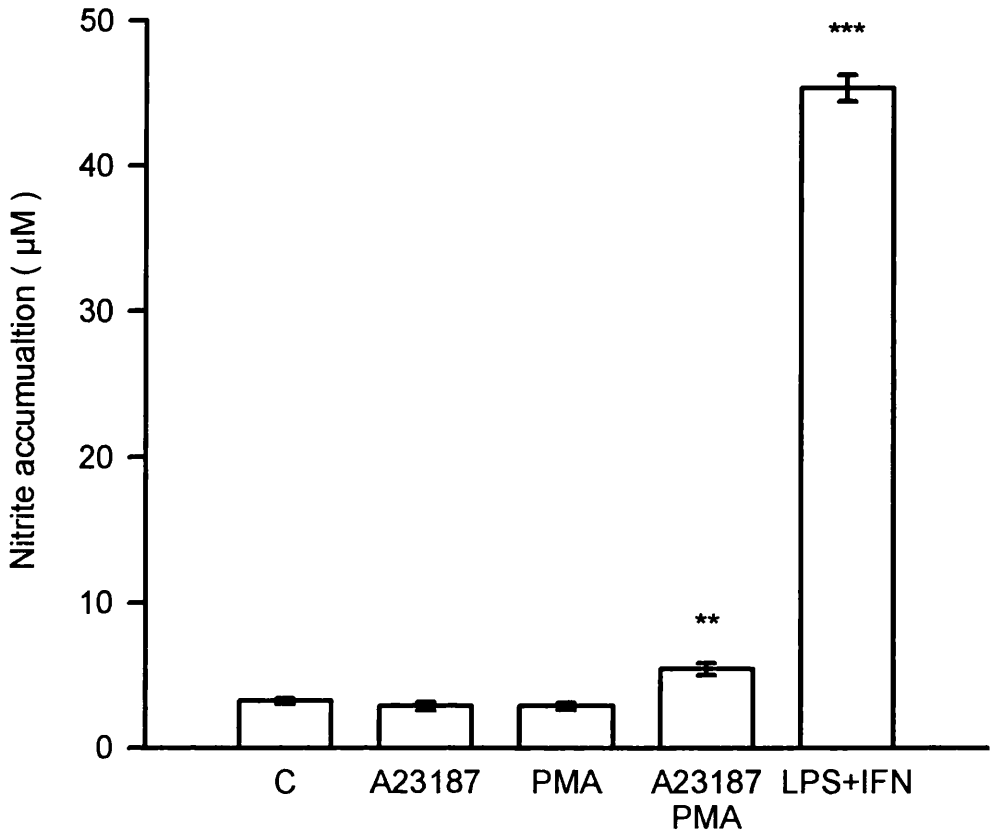


Figure 4.13 Effects of the calcium ionophore, A23187, and the phorbol ester, phorbol 12-myristate 13-acetate (PMA) alone and in combination on accumulation of nitrite into the medium bathing J774.7 cells and a comparison of their effects with those of the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were treated with A23187 (10^{-6} M) and PMA (10^{-6} M) alone and in combination and also with the combination of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}) and the incubation was continued for 24 hours. Each column represents the mean ± s.e. mean of 6 observations. ** $P<0.01$ and *** $P<0.001$ indicate a significant increase in nitrite levels from untreated control (C) cells.

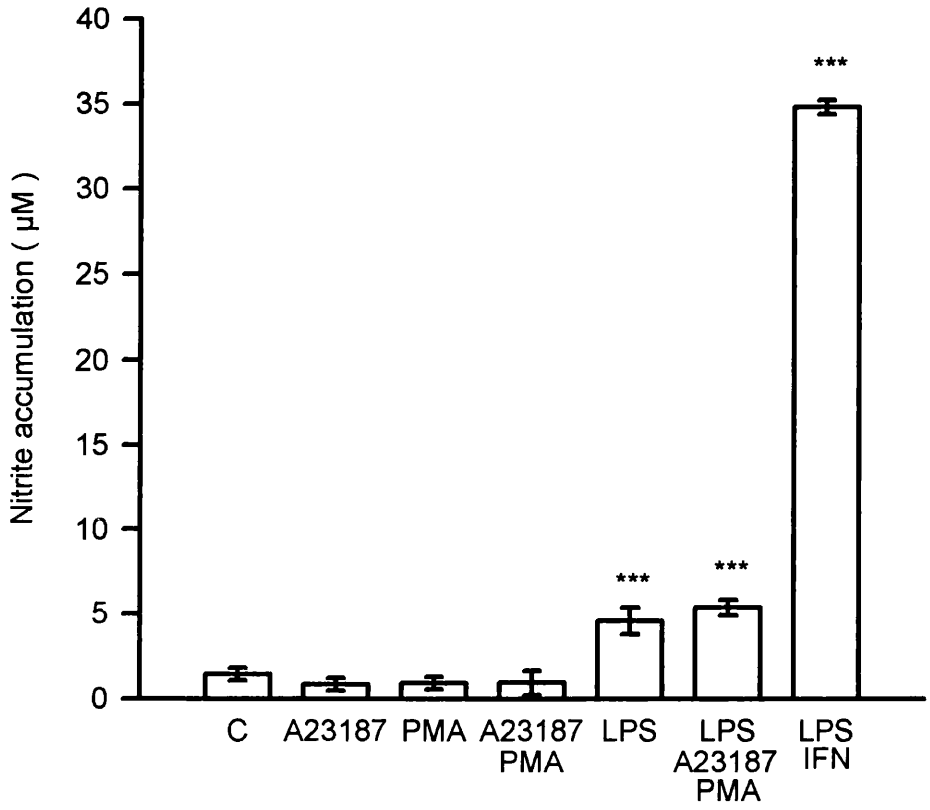


Figure 4.14 A comparison of the effects of A23187 and phorbol 12-myristate 13-acetate (PMA), alone and in combination, with those of IFN-γ in enhancing the ability of LPS to stimulate the accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}). Cells were pretreated with A23187 (10^{-6} M) and PMA (10^{-8} M) alone and in combination for 4 hours before the addition of lipopolysaccharide (LPS, 100 ng ml^{-1}), and the incubation was continued for a further 24 hours. Separate aliquots of cells received the standard stimulus of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}). Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant increase in nitrite levels from untreated control (C) cells.

γ (2 u ml^{-1}), and the incubation was continued for a further 24 hours. In this particular experiment, when used alone or in combination, A23187 and PMA had no effect on basal accumulation of nitrite (1.4 ± 0.4 μM)(Fig 4.14). LPS induced a significant rise in nitrite accumulation to 4.6 ± 0.8 μM. Pretreatment of cells with the combination of A23187 and PMA followed by exposure to LPS had no greater effect on nitrite accumulation (5.4 ± 0.4 μM) than LPS alone. Thus, the combination of A23187 and PMA with LPS did not mimic the powerful stimulation of the action obtained with the combination of LPS and IFN-γ (34.8 ± 0.4 μM).

4.3.3 Effects of a wide range of concentrations of PMA on basal nitrite production

The effects of a wide range of concentrations of PMA were investigated on basal production of nitrite by J774.7 cells as a prelude to establishing the effects of this agent on nitrite production stimulated by the combination of LPS and IFN-γ. Cells were treated with PMA ($3x10^{-10}$-$3x10^{-6}$ M) and the incubation was continued for 22 hours. PMA had no consistent effect on accumulation of nitrite into the medium bathing the cells, with only a small increase arising due to experimental variability at 10^{-9} M (Fig 4.15).

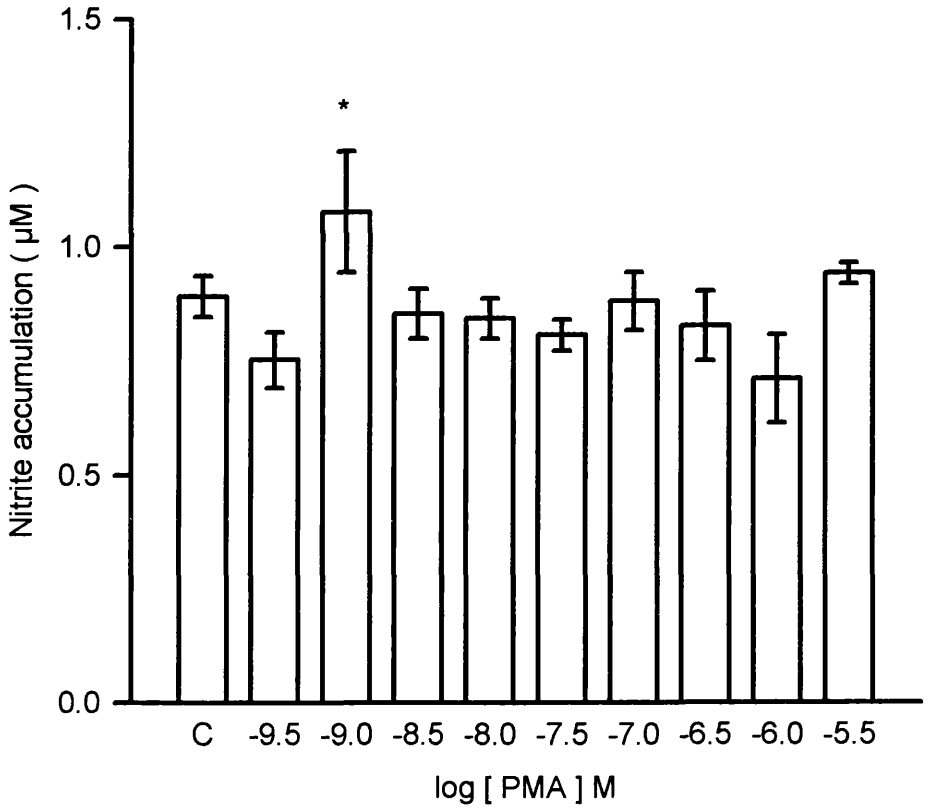


Figure 4.15 Effects of phorbol 12-myristate 13-acetate (PMA) on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}). Cells were treated with PMA ($3x10^{-10}$-$3x10^{-6}$ M) and the incubation was continued for 22 hours. Each column represents the mean ± s.e. mean of 6 observations. * $P<0.05$ indicates a significant increase in nitrite levels from untreated control (C) cells.

4.3.4 Effects of PMA on nitrite production stimulated by LPS and IFN-γ

Although PMA had no effect on basal accumulation of nitrite by J774.7 cells, the effects of PMA were investigated on cells stimulated with the combination of LPS and IFN-γ. Cells were pretreated with PMA (10^{-9}-$3x10^{-6}$ M) for 1 hour before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 23 hours. PMA produced a concentration-dependent inhibition of nitrite accumulation, with a maximum inhibition of 27.3 ± 0.8 % at 10^{-8} M (Fig 4.16).

This inhibition could have been due either to inhibition of induction or activity of inducible NO synthase. The latter was investigated by examining the actions of PMA (10^{-7} M) 12 hours after stimulation with LPS and IFN-γ. The incubation was continued for a further 24 hours and nitrite accumulation was then measured. In contrast to its inhibitory action when given before LPS and IFN-γ (Fig 4.16), PMA produced a significant increase (58.8 ± 2.4 %, n=6) in nitrite accumulation when added 12 hours after stimulation.

4.4 Inhibition of PKC

Although PMA inhibited nitrite production stimulated by LPS and IFN-γ, it was not clear whether this was a result of stimulation or down-regulation of PKC.

In order to investigate further the involvement of PKC in the induction of nitrite production by J774.7 cells, the effects of inhibition of this enzyme were investigated.

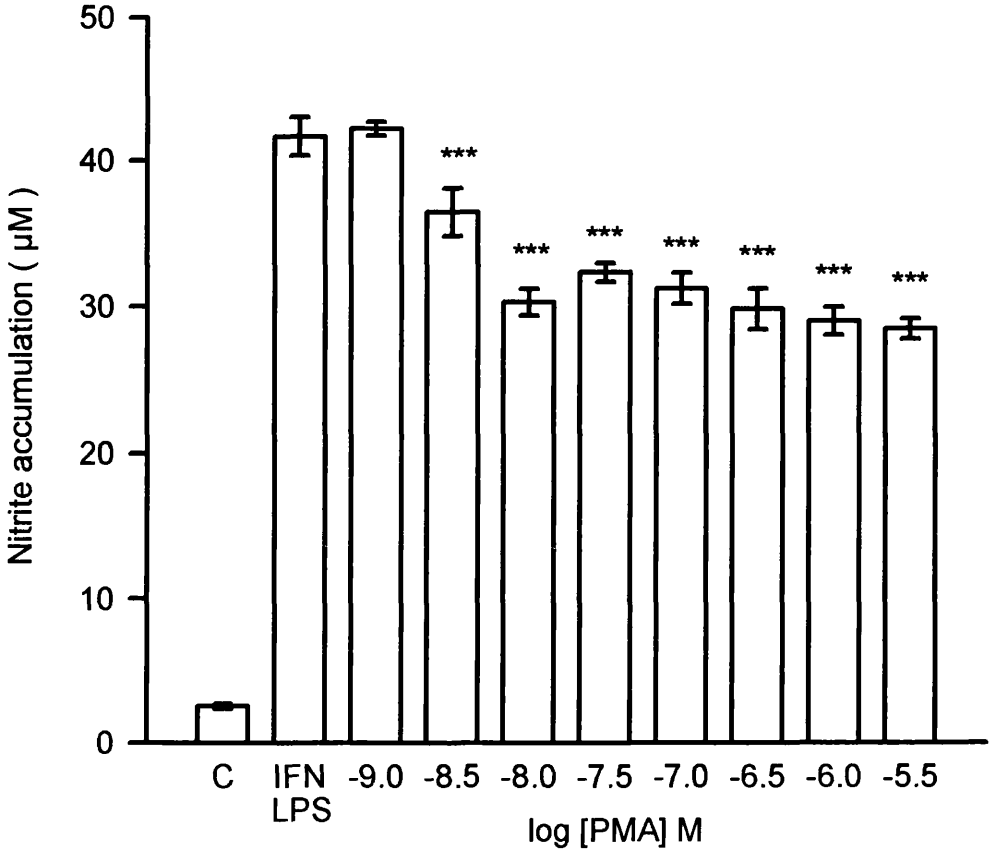


Figure 4.16 Effects of phorbol 12-myristate 13-acetate (PMA) on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with PMA (10^{-9}-$3x10^{-6}$ M) for 1 hour before the addition of LPS (10 ng ml^{-1}) and IFN-γ (2 u ml^{-1}), and the incubation was continued for a further 23 hours. Each observation represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant decrease by PMA of nitrite levels stimulated by LPS and IFN-γ.

4.4.1 Effects of staurosporine on nitrite production

Staurosporine inhibits PKC with a relatively low specificity, since it is also able to inhibit other protein kinases, e.g. protein kinase A (PKA) and myosin light chain kinase (MLCK)(Tamaoki *et al.*, 1986). Nevertheless, the effects of this commonly used inhibitor of PKC were examined on nitrite production by J774.7 cells. Cells were pretreated with staurosporine (10^{-9}-$3x10^{-6}$ M) for 2 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 22 hours. Staurosporine produced a concentration-dependent inhibition of nitrite accumulation induced by the combination of LPS and IFN-γ, with a maximum inhibition of 99.9 ± 44.0 % at $3x10^{-6}$ M (Fig 4.17). However, concentrations of $3x10^{-7}$ M and above appeared to exert a toxic action since they induced detachment of cells from the tissue culture plastic. Thus, the maximum inhibition of nitrite accumulation induced by the highest concentration of staurosporine (10^{-7} M) which did not induce detachment was 57.7 ± 1.1 %.

In order to determine whether the inhibitory effects of staurosporine resulted from inhibition of induction or activity of inducible NO synthase, it was necessary to assess the actions of the drug after stimulation with LPS and IFN-γ. Cells were therefore treated with staurosporine (10^{-7} M) after an initial 12 hour stimulation with LPS and IFN-γ and the incubation was continued for a further 24 hours. Staurosporine produced a significant (12.9 ± 1.5 %, n=6) inhibition of nitrite accumulation, but this was much smaller than ($P<0.05$) that occurring (57.7 ± 1.1 %) when it was added to the cells prior to LPS and IFN-γ (Fig 4.17).

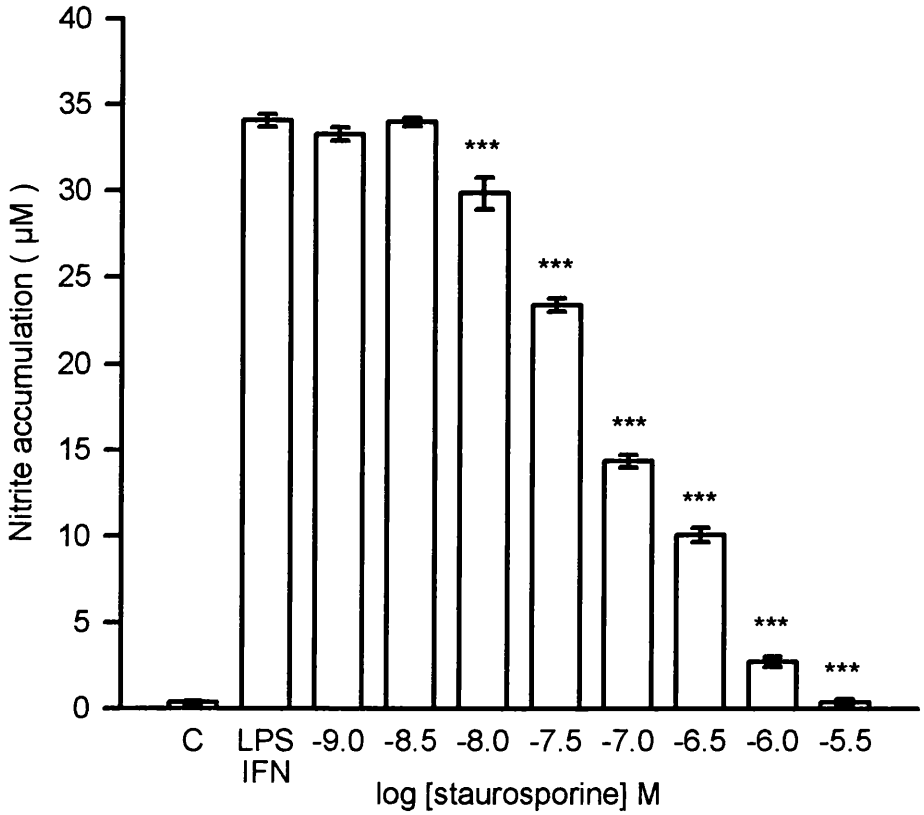


Figure 4.17 Effects of staurosporine on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with staurosporine (10^{-9}-$3x10^{-5}$ M) for 2 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 22 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant decrease by staurosporine of nitrite levels stimulated by LPS and IFN-γ.

4.4.2 Effects of Ro 31-8220 on nitrite production

The effects of Ro 31-8220, known to be a more selective inhibitor of PKC than staurosporine (Keller & Niggli, 1993), were investigated on nitrite production by J774.7 cells. Cells were pretreated with Ro 31-8220 ($3x10^{-9}$-10^{-5} M) for 2 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. Ro 31-8220 produced inconsistent effects when used in the concentration range $3x10^{-9}$-$3x10^{-7}$ M, but concentrations of 10^{-6} and higher produced concentration-dependent inhibition of nitrite accumulation, with a maximum of 73.2 ±14.3 % at 10^{-5} M (Fig 4.18). In contrast to staurosporine, Ro 31-8220 did not induce cell detachment at any concentration used.

It was important to establish if the inhibitory actions of Ro 31-8220 occurred as a consequence of inhibition of induction or activity of inducible NO synthase. Cells were therefore treated with Ro 31-8220 (10^{-5} M) after an initial 12 hour stimulation by LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}) and the incubation was continued for a further 24 hours. Ro 31-8220 produced a significant (11.7 ± 2.0 %, n=6) inhibition of nitrite accumulation, but this was much smaller ($P<0.05$) than that occurring (70.9 ± 5.7 %) when it was added to the cells prior to LPS and IFN-γ (Fig 4.18).

4.4.3 Effects of chelerythrine chloride on nitrite production

Chelerythrine chloride is known to be a highly selective inhibitor of PKC (Herbert *et al.*, 1990; Barg *et al.*, 1992). The effects of chelerythrine chloride on nitrite production by J774.7 cells were therefore investigated.

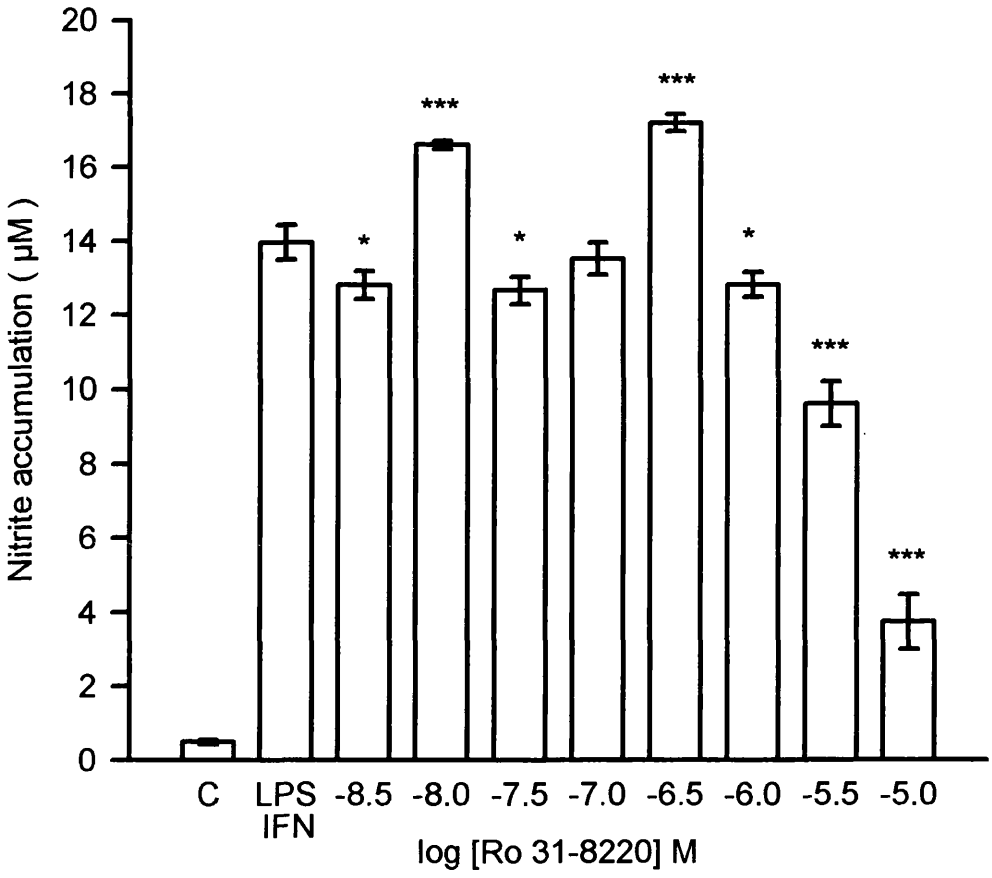


Figure 4.18 Effects of Ro 31-8220 on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with Ro 31-8220 ($3x10^{-9}$-10^{-5} M) for 2 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. Each column represents the mean ± s.e. mean of 6 observations. * $P<0.05$ and *** $P<0.001$ indicate a significant change by Ro 31-8220 of nitrite levels stimulated by LPS and IFN-γ.

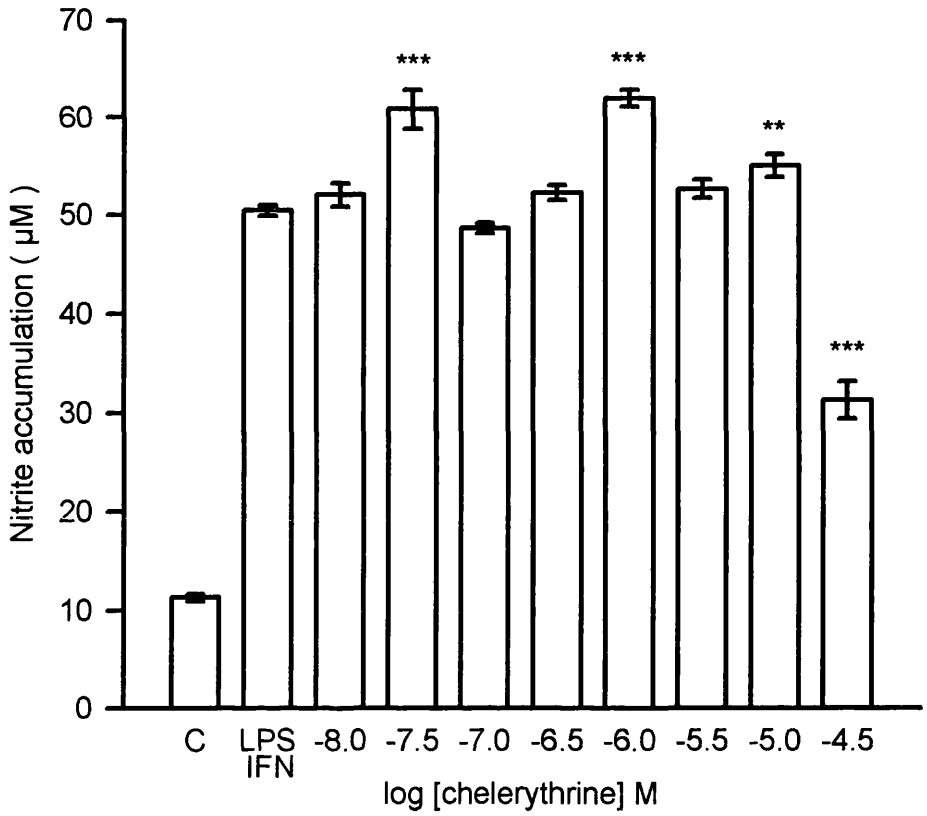


Figure 4.19 Effects of chelerythrine chloride on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated for 2 hours with chelerythrine chloride (10^{-8}-$3x10^{-5}$ M) before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. Each column represents the mean ± s.e. mean of 6 observations. ** $P<0.01$ and *** $P<0.001$ indicate a significant change by chelerythrine chloride of nitrite levels stimulated by LPS and IFN-γ.

Cells were pretreated with chelerythrine chloride (10^{-8}-$3x10^{-5}$ M) for 2 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. Chelerythrine chloride had no consistent effect on nitrite accumulation, except at a concentration of $3x10^{-5}$ M where it also induced detachment of cells (Fig 4.19).

To assess its actions on nitrite accumulation when given after the activating stimulus, chelerythrine chloride (10^{-5} M) was added to cells after an initial 12 hour stimulation by LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}) and the incubation was continued for a further 24 hours. Chelerythrine produced a significant (17.2 ± 2.3 %, n=6) increase in nitrite accumulation which did not significantly differ (P= 0.79) from that occurring (13.1 ± 1.7 %) when chelerythrine was added to the cells prior to LPS and IFN-γ (Fig 4.19).

4.4.4 Effects of the combination of staurosporine and PMA on nitrite production

Due to the uncertainty as to which action of PMA mediates the inhibition of nitrite accumulation, the effects of PKC inhibitors in combination with PMA were examined. This combination may clarify whether the effects resulted from stimulation or down-regulation. If it resulted from the former, it should be blocked by inhibitors of PKC. Alternatively, if it resulted from down-regulation of PKC, then inhibitors of this enzyme should mimic this action

Cells were pretreated with staurosporine (10^{-8} M) and PMA (10^{-8} M) for 2 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 22 hours. When used alone, staurosporine produced no inhibition of nitrite accumulation stimulated by

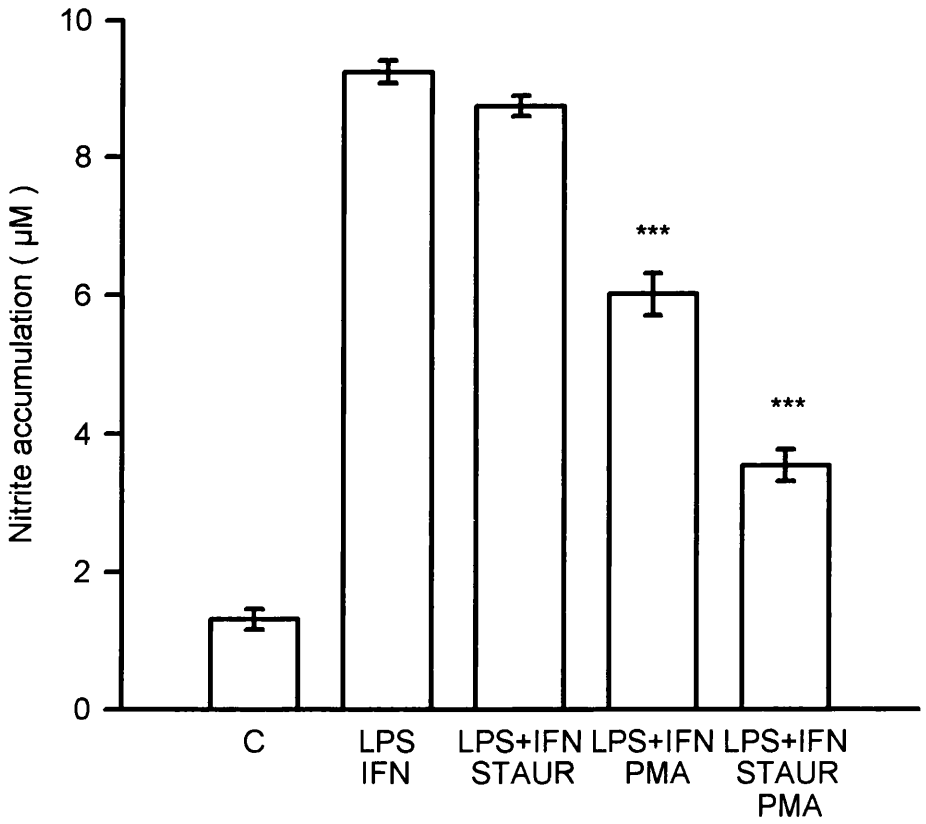


Figure 4.20 Effects of staurosporine and phorbol 12-myristate 13-acetate (PMA) on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with staurosporine (STAUR, 10^{-8} M) and PMA (10^{-8} M) alone and in combination for 2 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 22 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant decrease by PMA and the combination of PMA and staurosporine of nitrite levels stimulated by LPS and IFN-γ.

LPS and IFN-γ, whereas PMA produced a 34.9 ± 1.8 % inhibition (Fig 4.20). The combination of PMA and staurosporine produced a further degree of inhibition of 61.6 ± 4.0 %, demonstrating an additive inhibitory effect.

4.4.5 Effects of the combination of PMA and Ro 31-8220 on nitrite production

The effects of the combination of PMA and Ro 31-8220 on nitrite production by J774.7 cells were investigated. Cells were pretreated with PMA ($3x10^{-9}$ M) and Ro 31-8220 (10^{-6} M) alone or in combination for 2 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 22 hours. When used alone, PMA produced a 30.7 ± 0.5 % inhibition of nitrite accumulation stimulated by LPS and IFN-γ and Ro 31-8220 produced an inhibition of 18.8 ± 0.3 % (Fig 4.21). When PMA and Ro 31-8220 were used in combination the degree of inhibition was no greater than with either drug alone.

Involvement of the tyrosine kinase pathway

Protein tyrosine kinases play an important role in signal transduction pathways that regulate cell proliferation and differentiation. They are also thought to play a part in the pathway involved in the induction of NO synthase by cytokines (Marczin *et al.*, 1993; Feinstein *et al.*, 1994). This section outlines an investigation of the involvement of tyrosine kinase in the induction of nitrite production in J774.7 cells stimulated by LPS and IFN-γ.

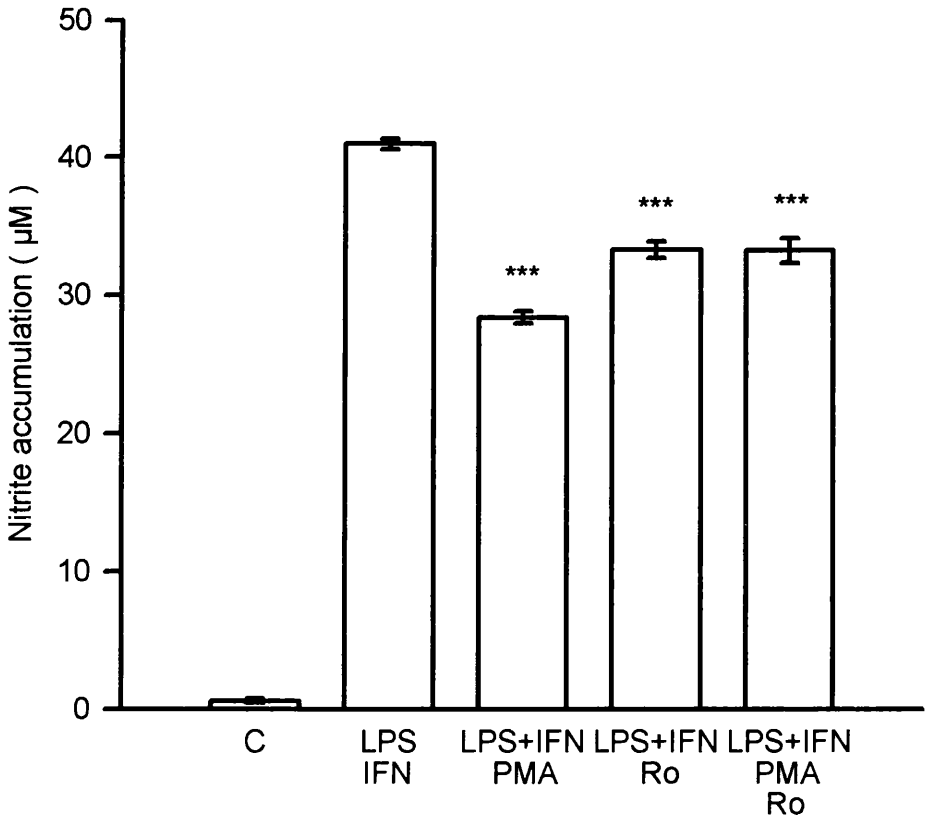


Figure 4.21 Effects of Ro 31-8220 and phorbol 12-myristate 13-acetate (PMA) on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with PMA ($3x10^{-9}$ M) and Ro 31-8220 (Ro, 10^{-6} M) alone and in combination for 2 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 22 hours. Each column represents the mean ± s.e. mean of 6 observations . *** $P<0.001$ indicates a significant decrease by PMA and Ro 31-8220 alone and in combination of nitrite levels stimulated by LPS and IFN-γ.

4.5 Inhibition of tyrosine kinase

4.5.1 Effects of herbimycin A on nitrite production

The effects of herbimycin A, a selective inhibitor of tyrosine kinase (Satoh *et al.* 1992; Dong *et al.*, 1993c), were investigated on nitrite production by J774.7 cells. Cells were pretreated with herbimycin A (0.003-1 μg ml^{-1}) for 4 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23.5 hours. Herbimycin A produced a concentration-dependent inhibition of nitrite accumulation stimulated by LPS and IFN-γ, with a maximum of 96.1 ± 10.3 % at 1 μg ml^{-1} (Fig 4.22). This agent did not produce detachment of cells at any of the concentrations used.

It was important to establish if the inhibitory actions of herbimycin A occurred as a consequence of inhibition of induction or activity of inducible NO synthase. To assess its actions on nitrite accumulation when given after the activating stimulus, herbimycin A (0.3 μg ml^{-1}) was added to cells after an initial 12 hour stimulation with LPS and IFN-γ and the incubation was continued for a further 24 hours. Herbimycin A produced a significant (24.5 ± 2.0 %, n=6) inhibition of nitrite accumulation when added 12 hours following LPS and IFN-γ, but the magnitude of this was much smaller ($P<0.05$) than that occurring (62.8 ± 1.0 %) when it was added to the cells prior to LPS and IFN-γ (Fig 4.22).

4.5.2 Effects of genistein on nitrite production

The effects of genistein, another inhibitor of tyrosine kinase, were investigated on nitrite production by J774.7 cells. Cells were pretreated with genistein (10^{-7}-10^{-4} M) for 4 hours before the addition of LPS (10 ng ml^{-1})

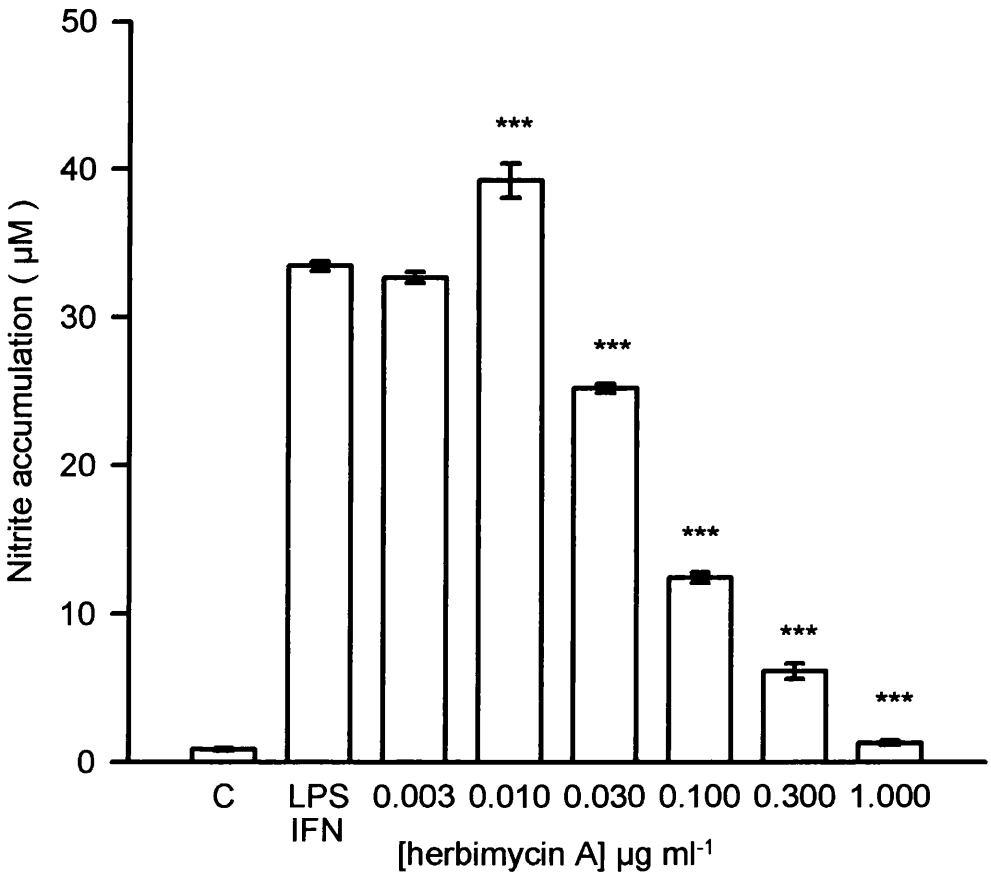


Figure 4.22 Effects of herbimycin A on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with herbimycin A (0.003-1 μg ml^{-1}) for 4 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23.5 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant change by herbimycin A of nitrite levels stimulated by LPS and IFN-γ.

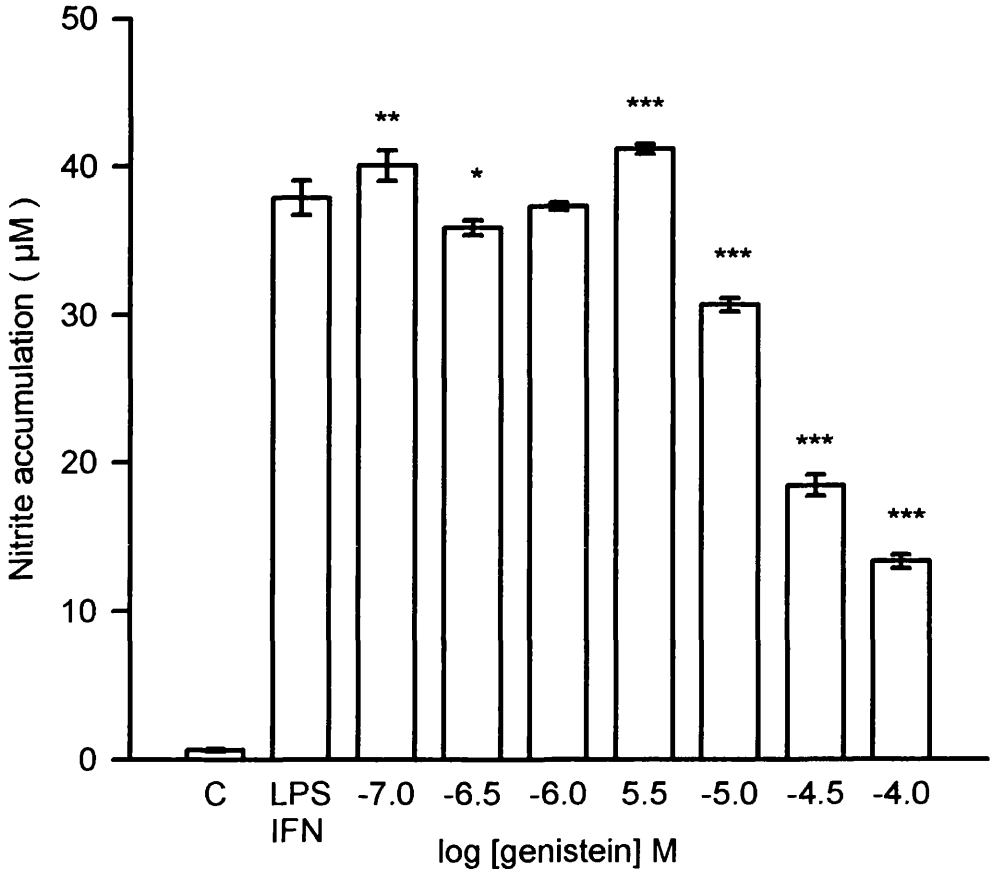


Figure 4.23 Effects of genistein on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with genistein (10^{-7}-10^{-4} M) for 4 hours before the addition of LPS (10 ng ml^{-1}) and IFN-γ (5 u ml^{-1}), and the incubation was continued for a further 22 hours. Each column represents the mean ± s.e. mean of 6 observations. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ indicate a significant change by genistein of nitrite levels stimulated by LPS and IFN-γ.

and IFN-γ (5 u ml^{-1}), and the incubation was continued for a further 22 hours. Genistein, in concentrations up to $3x10^{-6}$ M had no effect, but higher concentrations produced a concentration-dependent inhibition of nitrite accumulation stimulated by LPS and IFN-γ. Cell detachment was observed with genistein at a concentration of 10^{-4} M, but lower concentrations did not produce this effect. The maximum inhibition of nitrite accumulation produced by genistein at a concentration which did not induce detachment ($3x10^{-5}$ M) was 51.2 ± 1.9 % (Fig 4.23).

The inhibitory effects of herbimycin A and genistein on nitrite accumulation stimulated by LPS and IFN-γ were directly compared in a single log concentration-response graph (Fig 4.24). Although both drugs were effective in producing inhibition of nitrite accumulation, herbimycin A was more potent and more effective.

It was important to establish if the inhibitory actions of genistein occurred as a consequence of inhibition of induction or activity of inducible NO synthase. To assess its actions on nitrite accumulation when given after the activating stimulus, genistein ($3x10^{-5}$ M) was added to cells after an initial 12 hour stimulation with LPS and IFN-γ and the incubation was continued for a further 24 hours. Genistein had no effects (0.6 ± 2.3 % inhibition, n=6) on nitrite accumulation when added 12 hours after stimulation with LPS and IFN-γ, which differed significantly ($P<0.05$) from the inhibition (51.2 ± 1.9 %) obtained when it was added to the cells prior to LPS and IFN-γ (Fig 4.23).

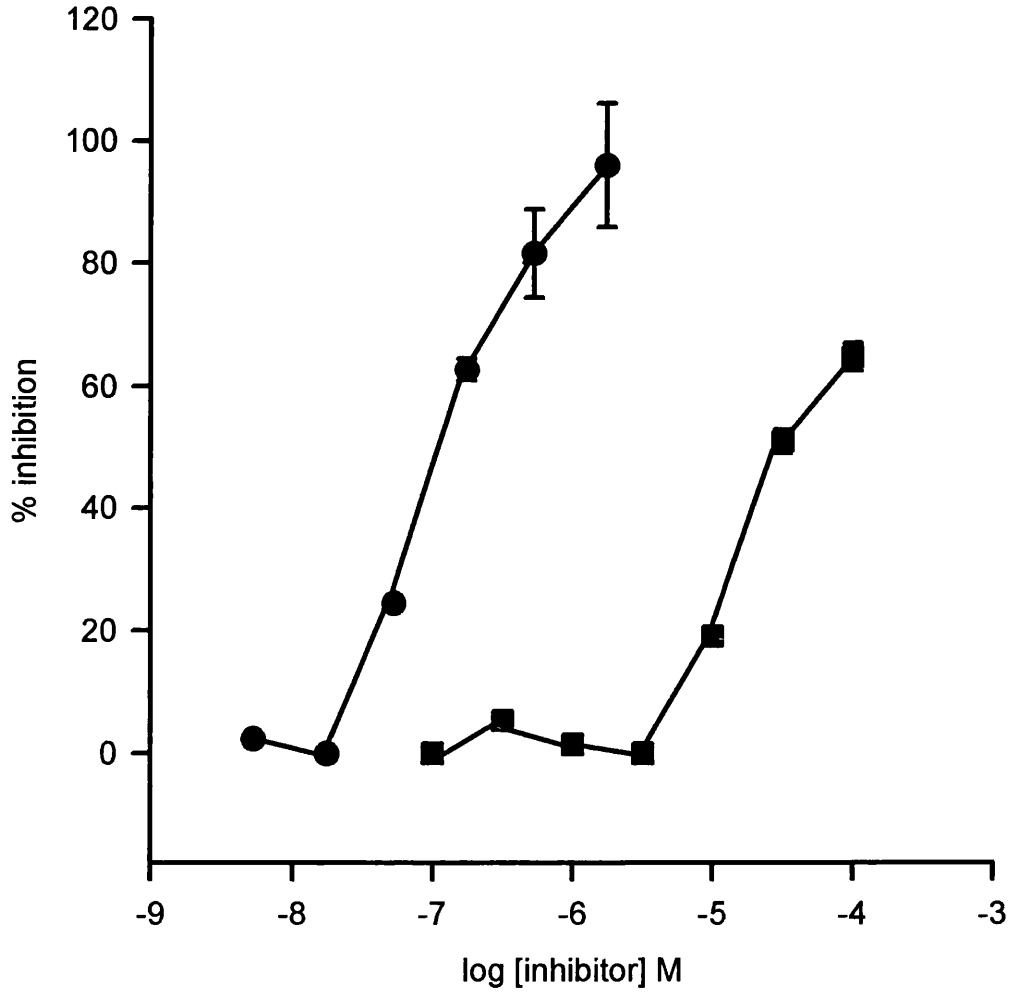


Figure 4.24 Comparison of the inhibitory effects of herbimycin A and genistein on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with genistein (■) or herbimycin A (●) for 4 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. Each point represents the mean ± s.e mean of 6 observations.

4.5.3 Effects of sodium orthovanadate on nitrite production

An alternative way to investigate the involvement of tyrosine kinases in the induction process is to inhibit tyrosine phosphatases, thus prolonging the duration of phosphorylation. The effects of sodium orthovanadate, an inhibitor of tyrosine phosphatase (Swarup *et al.*, 1982), were therefore investigated on nitrite production by J774.7 cells. Cells were pretreated with sodium orthovanadate (10^{-8}-$3x10^{-5}$ M) for 3 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. Sodium orthovanadate produced no consistent effect on nitrite accumulation induced by the combination of LPS and IFN-γ (Fig 4.25).

4.6 Combined inhibition of protein kinase C and tyrosine kinase

The ability of LPS and IFN-γ to induce a synergistic rise in nitrite production by J774.7 cells suggests that the two drugs act via distinct effector pathways. The experiments thus far have indicated a possible role for PKC and tyrosine kinase in the induction of nitrite accumulation. This section describes experiments in which the effects of combined blockade of tyrosine kinase and protein kinase C were investigated. In these experiments, herbimycin A was chosen as the common inhibitor of tyrosine kinase, and the effects of this together with three inhibitors of PKC, i.e. staurosporine, Ro 31-8220 and chelerythrine chloride were examined. In addition, since PMA may down-regulate PKC after an initial stimulation, experiments were

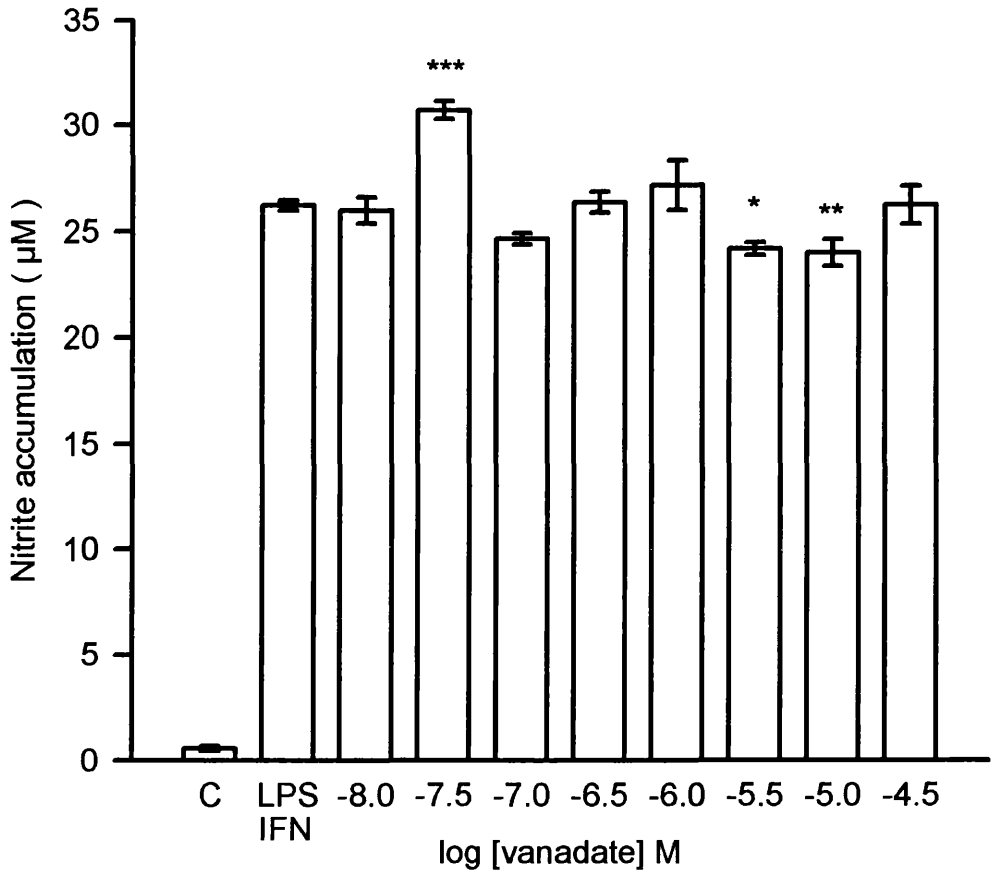


Figure 4.25 Effects of sodium orthovanadate (vanadate) on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated with vanadate (10^{-8}-$3x10^{-5}$ M) for 3 hours before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. Each column represents the mean ± s.e. mean of 6 observations. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ indicate a significant change by orthovanadate of nitrite levels stimulated by LPS and IFN-γ.

also carried out with this agent in combination with herbimycin A.

4.6.1 Effects of the combination of herbimycin and staurosporine on nitrite production

Cells were pretreated for 4 hours with herbimycin A (0.1 μg ml^{-1}) and for 2 hours with staurosporine ($3x10^{-8}$ M), alone and in combination before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. When used alone, herbimycin A produced a 30.3 ± 1.1 % inhibition of nitrite accumulation induced by the combination of LPS and IFN-γ and staurosporine produced an inhibition of 33.6 ± 1.3 % (Fig 4.26). The combination of the two drugs produced a greater degree of inhibition of nitrite accumulation (67.1 ± 4.5 %) than either drug on its own.

4.6.2 Effects of the combination of herbimycin A and Ro 31-8220 on nitrite production

The combined actions of effective concentrations of Ro 31-8220 and herbimycin A were then investigated on nitrite production by J774.7 cells. Cells were pretreated for 4 hours with herbimycin A (0.1 μg ml^{-1}) and for 2 hours with Ro 31-8220 ($3x10^{-6}$ M) alone and in combination before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. When used alone, herbimycin A produced a 43.9 ± 2.2 % inhibition of nitrite accumulation stimulated by the combination of LPS and IFN-γ, and Ro 31-8220 produced an inhibition of 35.7 ± 1.6 % (Fig 4.27). The combination of the two drugs produced a slight but significantly greater (51.1 ± 3.4 %) inhibition of nitrite accumulation than either on its own.

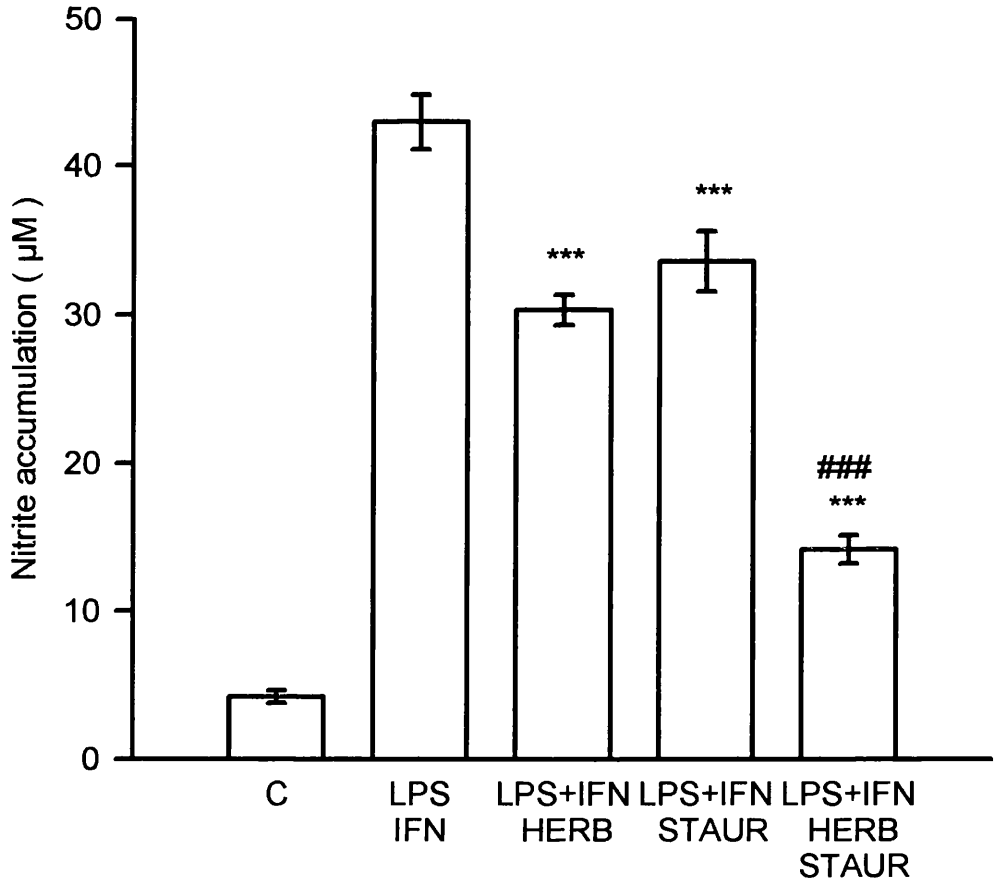


Figure 4.26 Effects of herbimycin A and staurosporine on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated for 4 hours with herbimycin A (HERB, 0.1 μg ml^{-1}) and for 2 hours with staurosporine (STAUR, $3x10^{-6}$) alone and in combination before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant decrease by herbimycin A and staurosporine alone and in combination of nitrite levels stimulated by LPS and IFN-γ. ### $P<0.001$ indicates a significantly greater inhibition with the combination of herbimycin A and staurosporine than with either on its own.

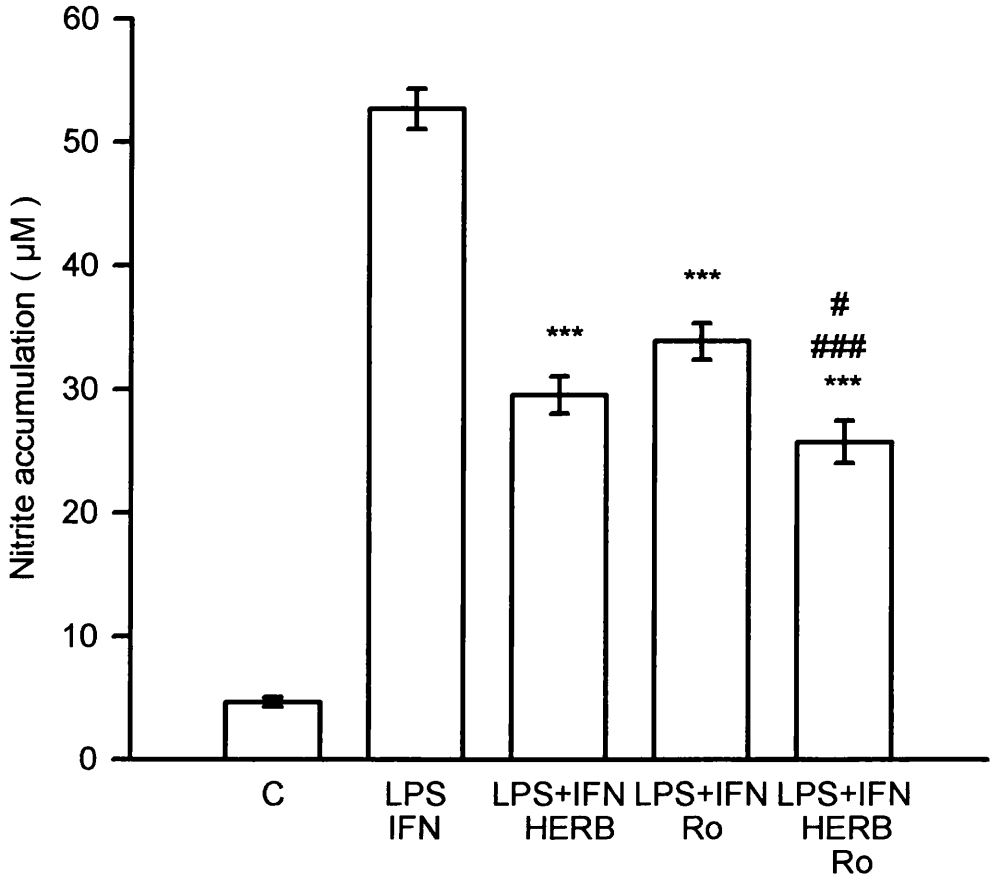


Figure 4.27 Effects of herbimycin A and Ro 31-8220 on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated for 4 hours with herbimycin A (HERB, 0.1 μg ml^{-1}) and for 2 hours with Ro 31-8220 (Ro, $3x10^{-6}$ M) alone and in combination before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. Each column represents the mean ± s.e. mean of 6 observations.*** $P<0.001$ indicates a significant decrease by herbimycin A and staurosporine alone and in combination of nitrite levels stimulated by LPS and IFN-γ. # $P<0.05$ indicates a significant difference in the inhibition obtained between herbimycin A alone and herbimycin A in combination with Ro 31-8220. ### $P<0.001$ indicates a significant difference in the inhibition obtained between Ro 31-8220 alone and Ro 31-8220 in combination with herbimycin A.

4.6.3 Effects of the combination of herbimycin A and chelerythrine chloride on nitrite production

J774.7 cells were pretreated for 4 hours with herbimycin A (0.1 μg ml^{-1}) and for 2 hours with chelerythrine chloride (10^{-5} M) alone and in combination before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 24 hours. When used alone, herbimycin A produced a 38.9 ± 0.4 % inhibition of nitrite accumulation stimulated by LPS and IFN-γ, whereas chelerythrine chloride produced an inhibition of 13.3 ± 0.2 % (Fig 4.28). The combination of the two drugs produced no greater inhibition (31.81 ± 0.36 %) than with herbimycin A alone.

4.6.4 Effects of the combination of herbimycin A and PMA on nitrite production

The effects of down-regulation of PKC with PMA together with inhibition of tyrosine kinase with herbimycin A were investigated on nitrite production by J774.7 cells. Cells were pretreated for 4 hours with herbimycin A (0.03 μg ml^{-1}) and for 2 hours with PMA (10^{-8} M) alone and in combination before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 23 hours. When used alone, herbimycin A produced a 44.3 ± 1.7 % inhibition of nitrite accumulation stimulated by LPS and IFN-γ, whereas PMA produced an inhibition of 39.4 ± 1.2 % (Fig 4.29). The combination of the two drugs produced no greater inhibition (43.7 ± 2.9 %) than with either alone.

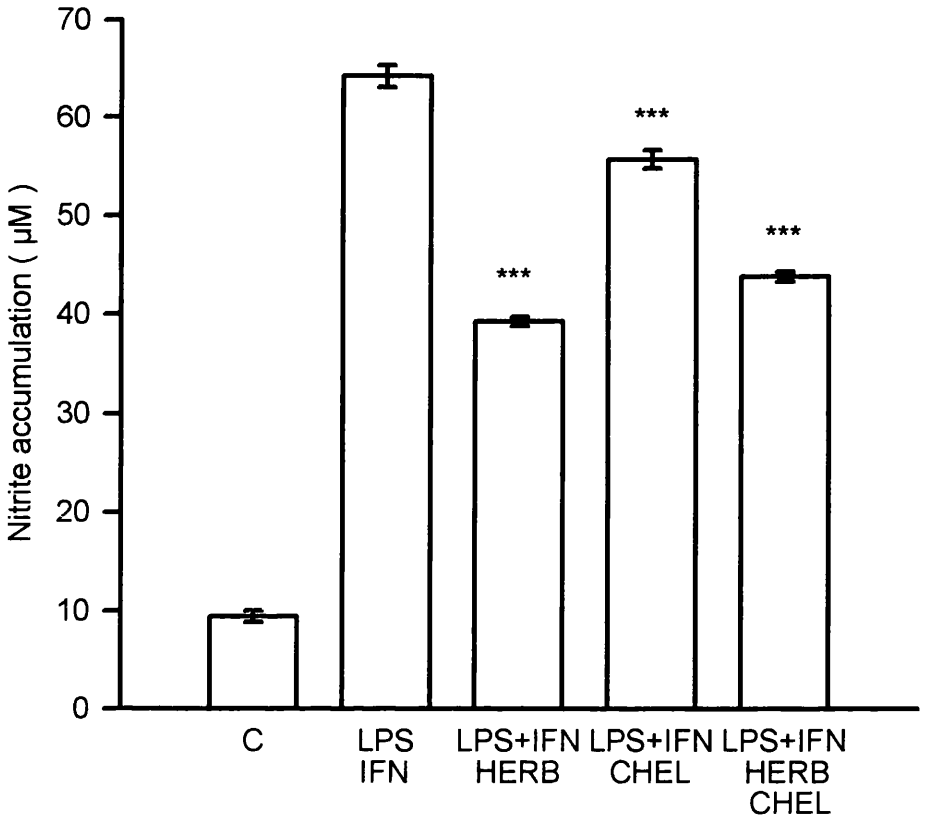


Figure 4.28 Effects of herbimycin A and chelerythrine chloride on accumulation of nitrite into the medium bathing J774 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated for 4 hours with herbimycin A (HERB, 0.1 µg ml^{-1}) and for 2 hours with chelerythrine chloride (CHEL, 10^{-5} M) alone or in combination before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for a further 24 hours. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant decrease by herbimycin A and chelerythrine chloride alone and in combination of nitrite levels stimulated by LPS and IFN-γ.

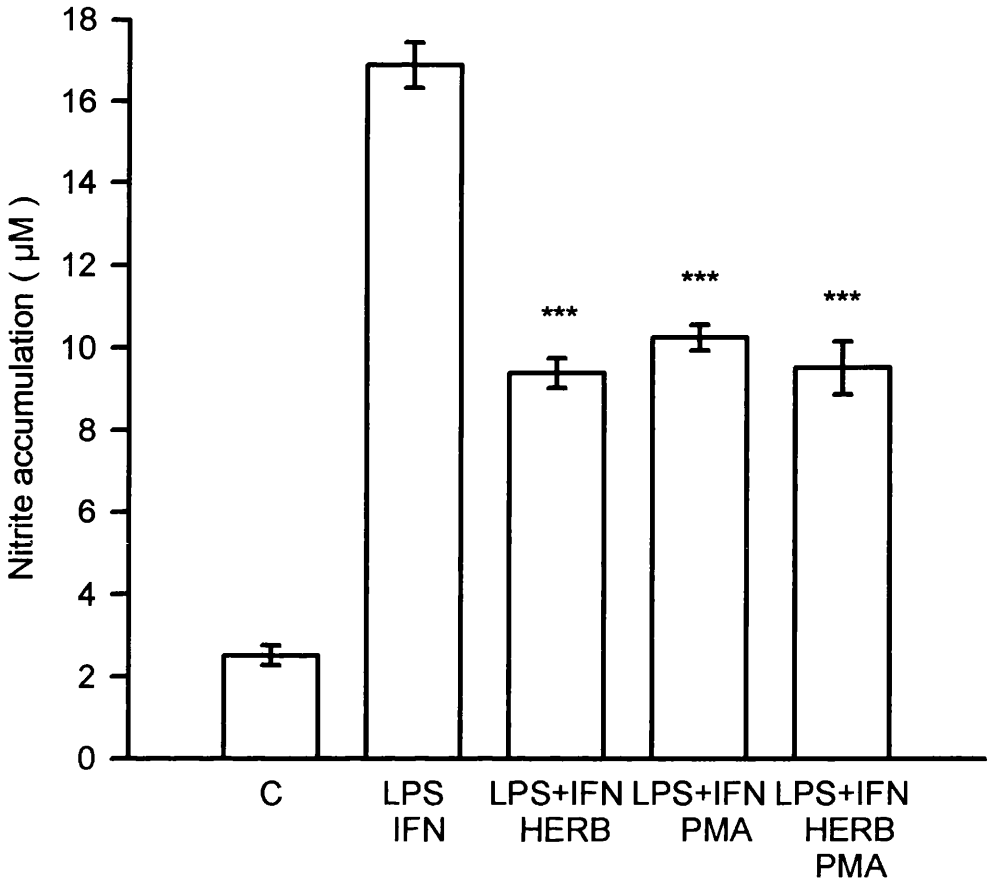


Figure 4.29 Effects of herbimycin A and phorbol 12-myristate 13-acetate (PMA) on accumulation of nitrite into the medium bathing J774.7 cells (10^6 cells ml^{-1}) following the combined stimulus of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Cells were pretreated for 4 hours with herbimycin A (HERB, 0.03 µg ml^{-1}) and for 2 hours with PMA (10^{-8} M) alone and in combination before the addition of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), and the incubation was continued for 23 hours. Each column represents the mean s.e. mean of 6 observations. *** $P<0.001$ indicates a significant decrease by herbimycin A and PMA alone and in combination of nitrite levels stimulated by LPS and IFN-γ.

CHAPTER 5

EFFECTS OF J774.7 MACROPHAGES ON TONE IN RAT AORTIC RINGS

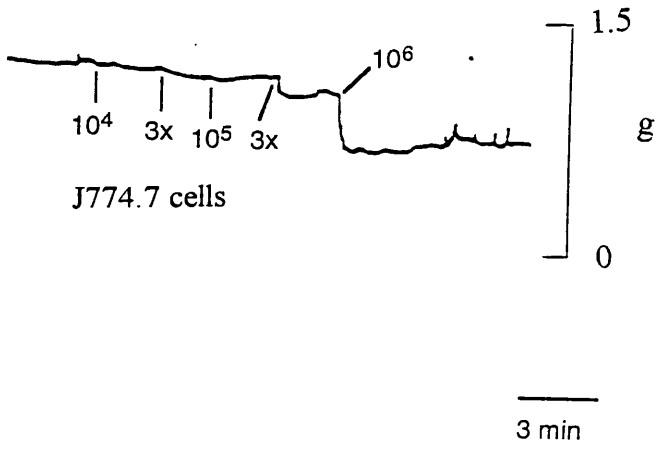
In previous chapters, NO release from J774.7 macrophages was assessed by measuring the accumulation of its stable product, nitrite, into the medium bathing the cells. In this chapter, an alternative way to assess NO production was sought by examining the effects of activated J774.7 cells on the tone of rings of rat aorta suspended in organ baths.

Since macrophages produce large quantities of NO which are clearly cytotoxic or cytostatic to other cells, it is possible that the macrophage has evolved mechanisms to protect itself from these actions. One possible protective action is for the macrophage to bind NO, thus preventing it from interfering with important cellular processes. Consequently, a number of pharmacological tools were used to determine if the relaxant produced by macrophages was NO *per se* or an NO-releasing molecule.

5.1 Effects of activated and unactivated J774.7 macrophages on phenylephrine-induced tone in rat aortic rings

Sub-maximal phenylephrine-induced tone (0.7 ± 0.1 g) was generated in endothelium-denuded rings of rat aorta using a concentration range of 10^{-7}-$3x10^{-7}$ M. When tone had stabilised, J774.7 cells, which were either

Activated



Unactivated

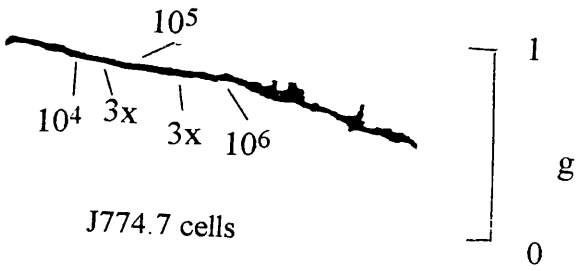


Figure 5.1a Traces demonstrating typical effects of addition of activated and unactivated J774.7 cells on phenylephrine-induced tone in rat aortic rings. Macrophages were treated overnight for approximately 18 hours with lipopolysaccharide (LPS, 100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}). They were then re-suspended in Krebs and added to the organ baths cumulatively. Concentrations of cells are given per ml of solution. Rings were contracted with phenylephrine (PE, $3x10^{-8}$ M) before the addition of cells.

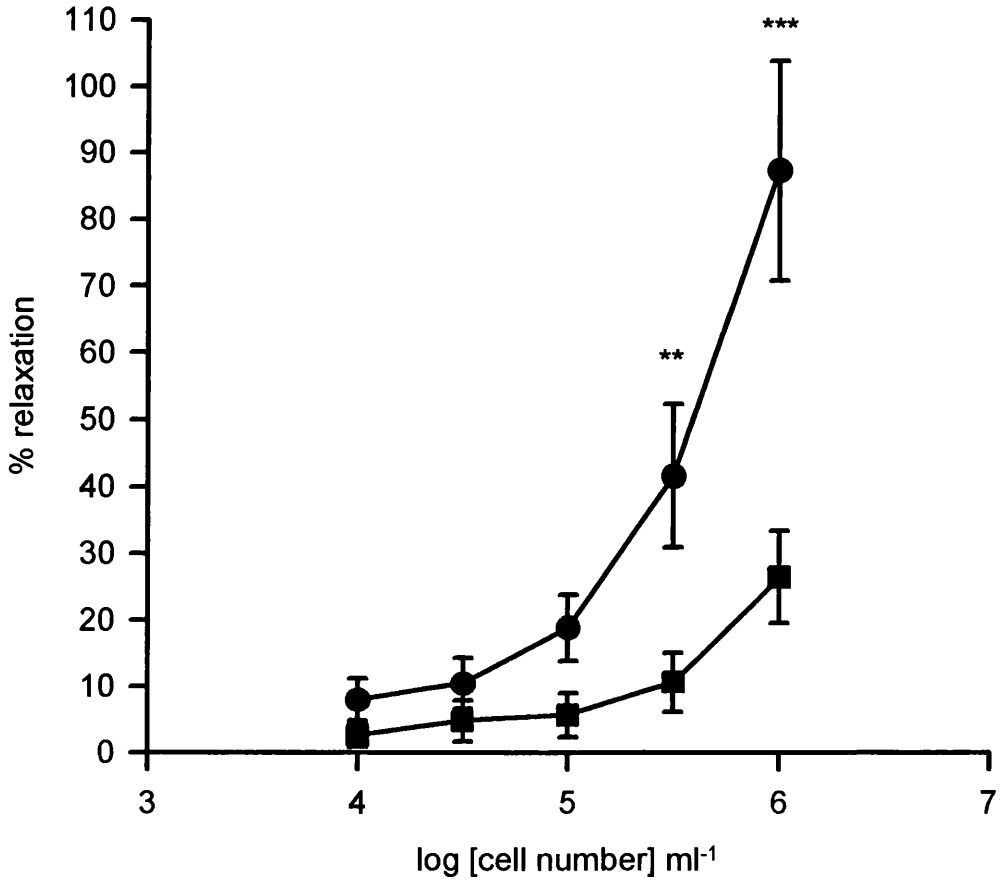


Figure 5.1b Effects of addition of unactivated and activated J774.7 cells on phenylephrine-induced tone in rat aortic rings. Macrophages were treated overnight with lipopolysaccharide (LPS, 100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}) to induce activation, were suspended in Krebs and were added to the organ baths cumulatively. Rings were contracted with phenylephrine before the addition of stimulated (●) or unstimulated (■) cells. Each point represents the mean ± s.e. mean of 6 observations. ** $P<0.01$ and *** $P<0.001$ indicate a significant difference between the relaxation induced with stimulated and unstimulated cells.

unactivated or had been activated approx. 18 hours previously with the combined stimulus of LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}), were added cumulatively and the effects on tone assessed. Activated J774.7 cells (10^4-10^6 cells ml^{-1} final concentration) produced a rapid, cell number-dependent relaxation of phenylephrine-induced tone, reaching a maximum of 87.4 ± 16.6 % with 10^6 cells ml^{-1} (Fig 5.1a & 5.1b). In contrast, unactivated cells (10^4-10^6 cells ml^{-1}) produced a far lesser degree of relaxation, and this was much slower to develop reaching a maximum of only 26.3 ± 6.9 % with 10^6 cells ml^{-1}.

It is apparent therefore that J774.7 cells when activated by LPS and IFN-γ produce a factor which induces powerful relaxation (Fig 5.1b).

5.2 Effects of superoxide dismutase on relaxation of phenylephrine-induced tone by activated J774.7 macrophages

Superoxide dismutase (SOD), by removing destructive superoxide anions potentiates the vasodilator actions of NO (Gryglewski *et al.*, 1986b). If activated J774.7 cells induced relaxation of rat aortic rings by releasing NO, then SOD should potentiate this action. The effects of SOD were therefore investigated on macrophage-induced relaxation of rat aortic rings. In the absence of SOD, J774.7 cells (10^4-10^6 cells ml^{-1} final concentration) activated by LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}) for approx. 18 hours, produced a cell number-dependent relaxation, with a maximum relaxation of 73.2 ± 13.9 % at 10^6 cells ml^{-1} (Fig 5.2a & 5.2b). When SOD (250 u ml^{-1}) was added to the tissue baths, the relaxation induced by activated J774.7 cells was potentiated with a maximum relaxation of 100.0 ± 4.6 %

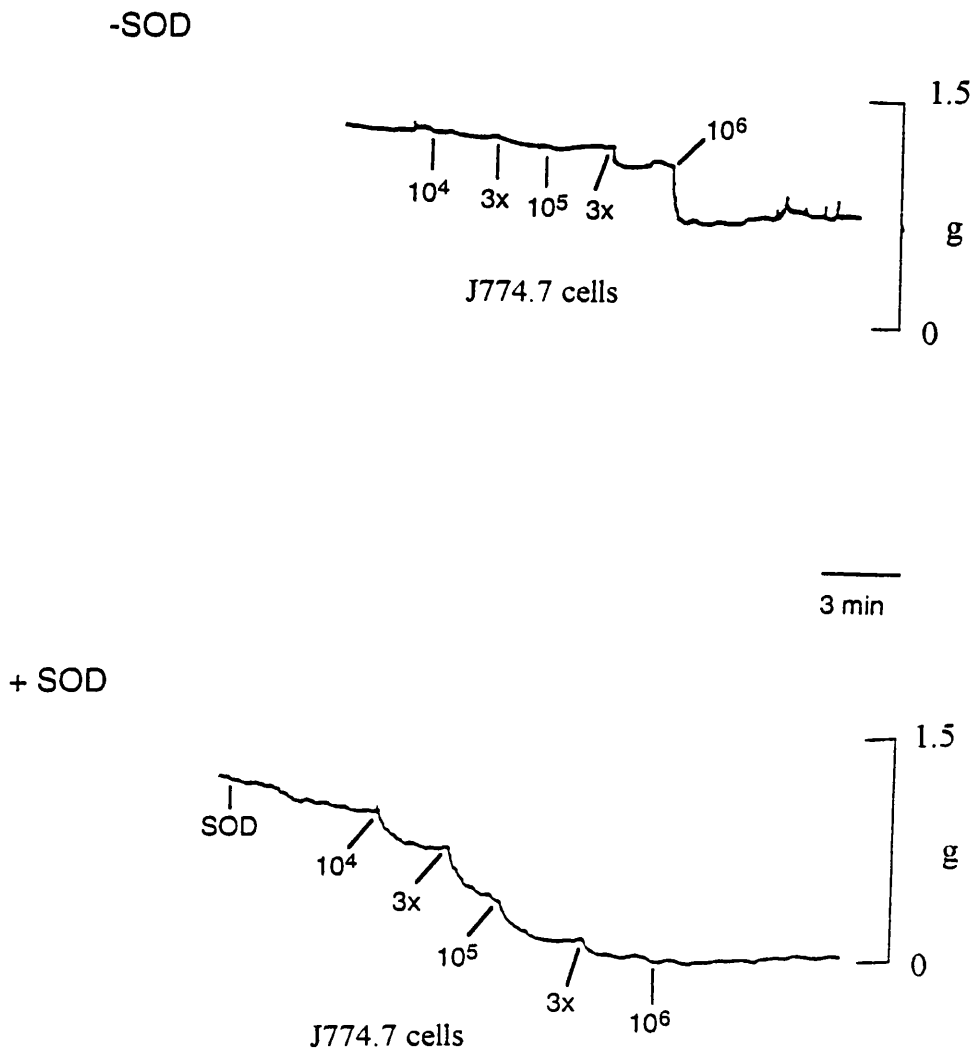


Figure 5.2a Traces demonstrating the ability of superoxide dismutase (SOD) to potentiate the relaxation induced by activated J774.7 cells on phenylephrine-contracted rings of rat aorta. Cells were treated overnight for approximately 18 hours with lipopolysaccharide (LPS, 100 ng ml^{-1}) and interferon-gamma (IFN-γ, 10 u ml^{-1}). They were then re-suspended in Krebs and added to the organ baths cumulatively. Concentrations of cells are given per ml of solution. Rings were contracted with phenylephrine (PE, $3x10^{-8}$ M) and cells were added in the absence or presence of SOD (250 u ml^{-1}).

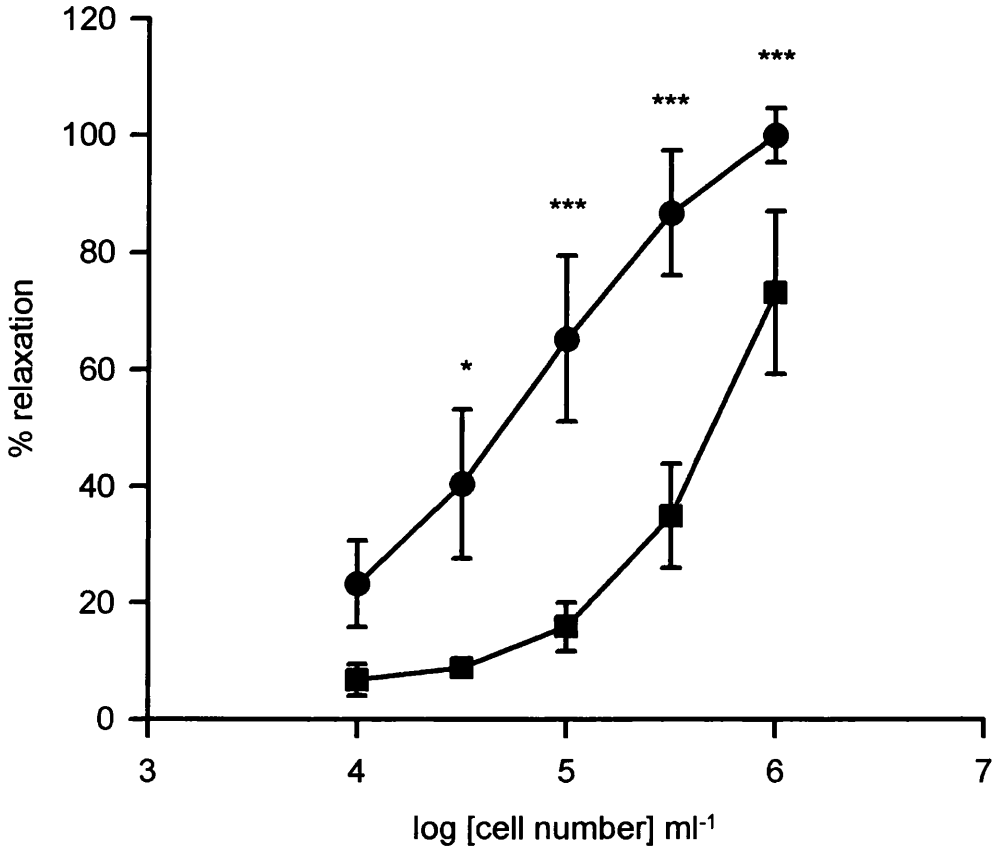


Figure 5.2b Effects of superoxide dismutase (SOD) on relaxation of phenylephrine-induced tone in rat aortic rings produced by activated J774.7 cells. Cells were treated overnight with lipopolysaccharide (LPS, 100 ng ml^{-1}) and interferon-gamma (IFN-γ, 10 u ml^{-1}) and were added to the organ baths cumulatively. Ring were contracted with phenylephrine (10^{-8}-10^{-7} M) and cells added in the presence (●) or absence (■) of SOD (250 u ml^{-1}). Each point represents the mean ± s.e mean of 6 observations. * $P<0.05$ and *** $P<0.001$ indicate a significantly greater relaxation in the presence of SOD.

at 10^6 cells ml^{-1}.

In view of the potentiating effect of SOD on macrophage-induced relaxation, all further experiments were conducted in the presence of this agent at a concentration of 250 u ml^{-1}.

5.3 Effects of haemoglobin on relaxation of phenylephrine-induced tone by activated J774.7 macrophages

Haemoglobin binds NO and inhibits its actions (Martin *et al.*, 1985). If J774.7 cells produce their vasodilator action by releasing NO, then this effect would be expected to be blocked by haemoglobin. When haemoglobin (Hb, $3x10^{-6}$ M) was added to the tissue baths, the relaxing action of J774.7 cells (10^4-10^6 cells ml^{-1} final concentration), activated by LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}) for approx. 18 hours, was powerfully inhibited; maximum relaxation was 100 ± 10.2 % in the absence of Hb falling to 10.3 ± 3.2 % in the presence of Hb (Fig 5.3a & 5.3b).

5.4 Effects of LY 83583 on relaxation of phenylephrine-induced tone by activated J774.7 macrophages

LY 83583 is known to inhibit the actions of NO by generating superoxide anion both intracellularly and extracellularly (Mülsch *et al.*, 1989). Consequently, its effects were examined on the relaxation of rat aortic rings produced by J774.7 cells that had been activated for approx. 18 hours with LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}). Catalase was present in the baths to prevent the accumulation of hydrogen peroxide derived

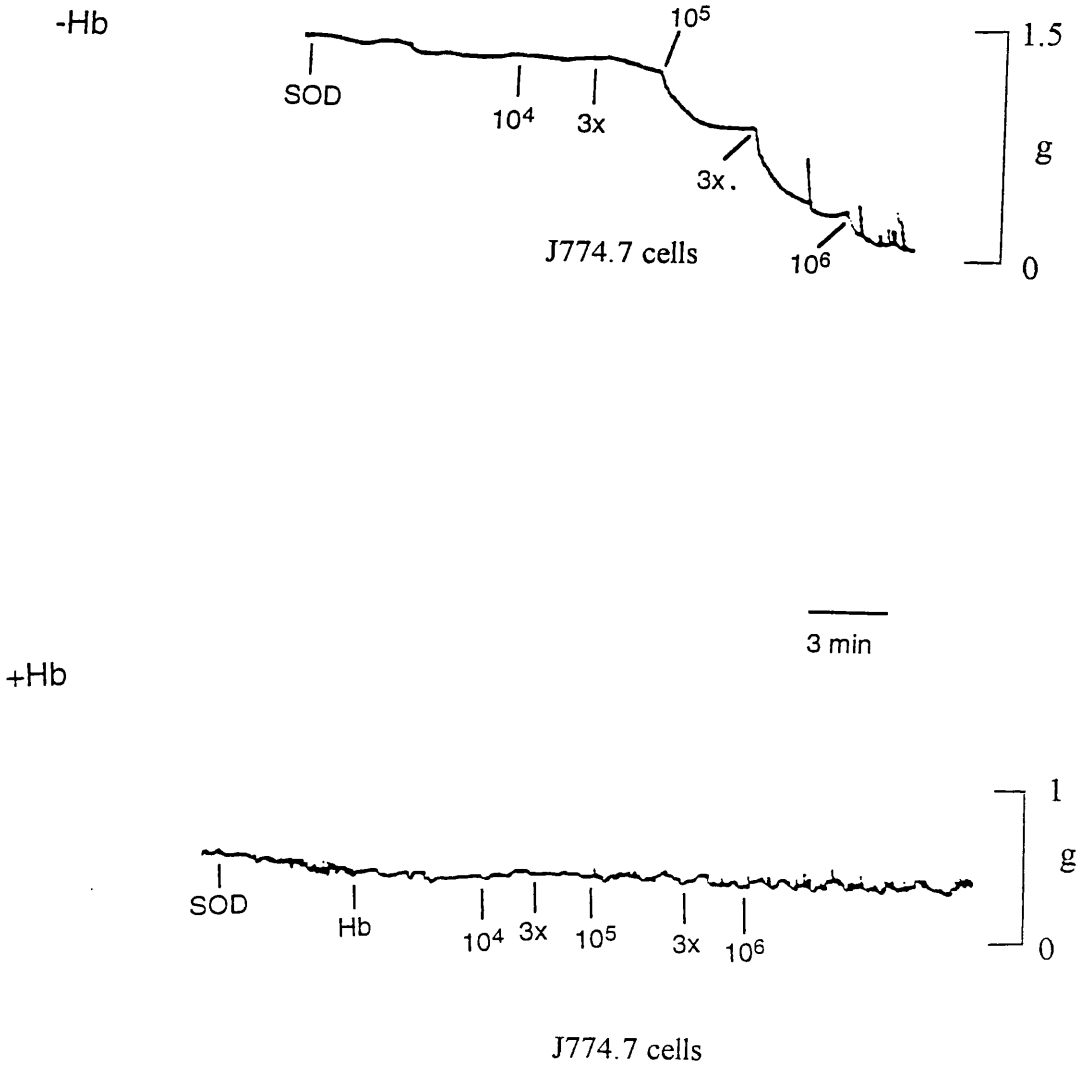


Figure 5.3a Traces demonstrating the ability of haemoglobin (Hb) to block relaxation induced by activated J774.7 cells in phenylephrine-contracted rings of rat aorta. Cells were treated overnight for approximately 18 hours with lipopolysaccharide (LPS, 100 ng ml^{-1}) and interferon-gamma (IFN-γ, 10 u ml^{-1}). They were then re-suspended in Krebs and added to the organ baths cumulatively. Concentrations of cells are given per ml of solution. Rings were contracted with phenylephrine (PE, $3x10^{-8}$ M), treated with superoxide dismutase (SOD, 250 u ml^{-1}) and cells were added in the absence or presence of Hb ($3x10^{-6}$ M).

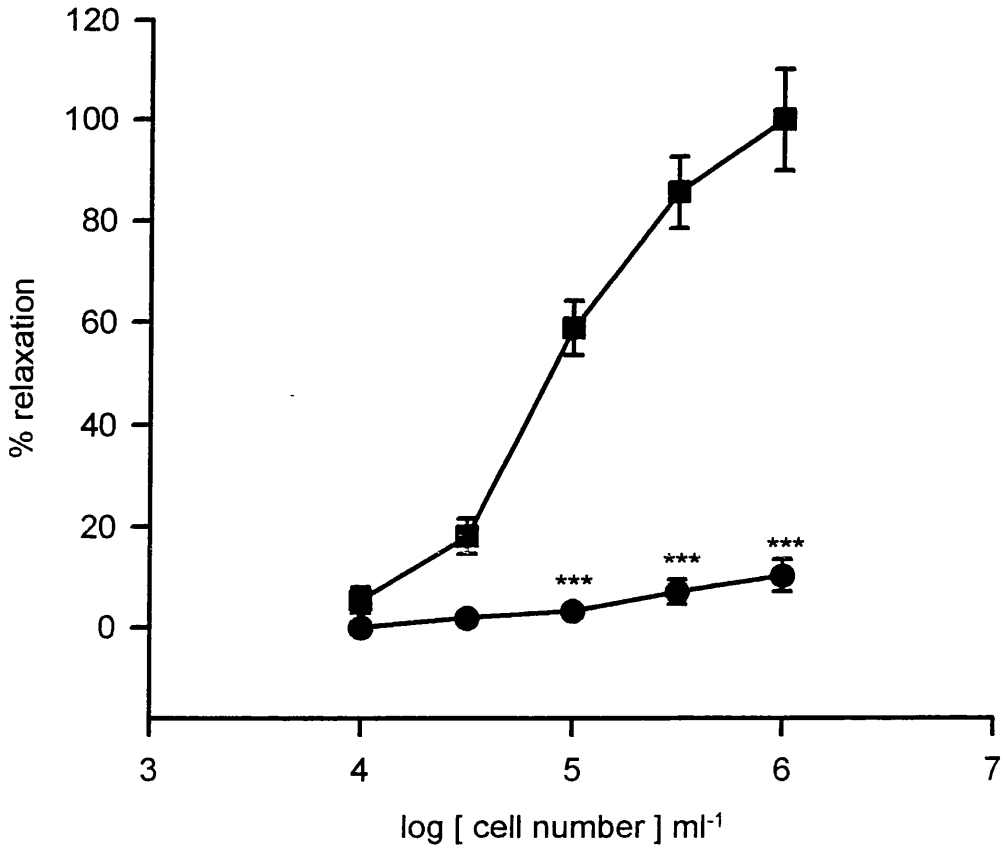


Figure 5.3b Effects of haemoglobin on relaxation of phenylephrine-induced tone in rat aortic rings produced by activated J774.7 cells. Cells were treated overnight with lipopolysaccharide (LPS, 100 ng ml^{-1}) and interferon-gamma (IFN-γ, 10 u ml^{-1}) and were added to the organ baths cumulatively. Ring were contracted with phenylephrine (10^{-8}-10^{-7} M), treated with SOD (250 u ml^{-1}) and cells were added in the presence (●) or absence (■) of Hb ($3x10^{-6}$ M). Each point represents the mean ± s.e mean of 6 observations. *** $P<0.001$ indicates a significant decrease in the presence of Hb.

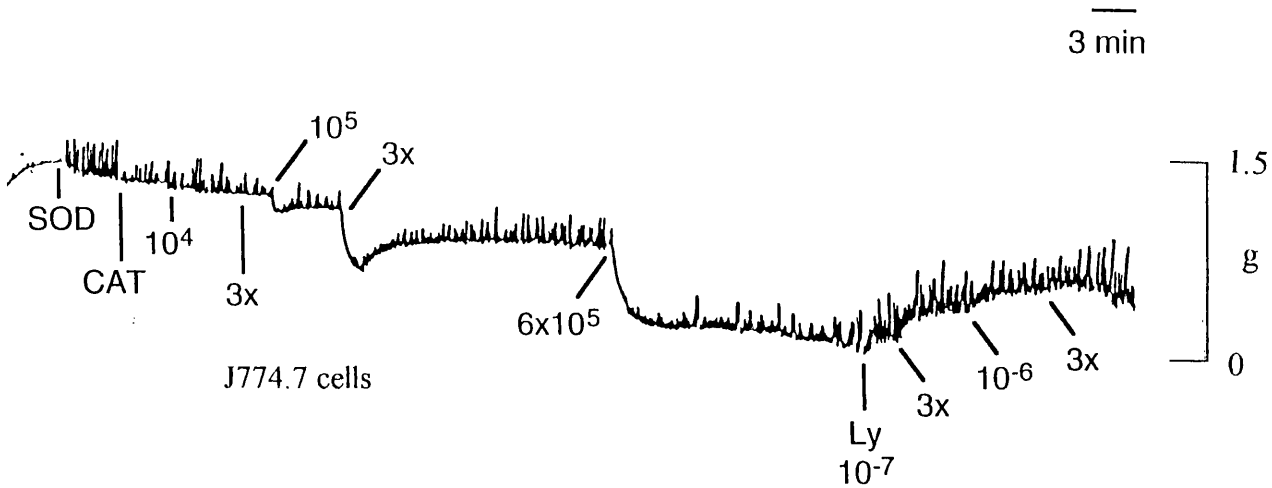


Figure 5.4a Trace demonstrating the typical ability of LY 83583 to reverse relaxation of phenylephrine-induced tone in rat aortic rings produced by activated J774.7 cells. Cells were treated overnight with lipopolysaccharide (LPS, 100 ng ml^{-1}) and interferon-gamma (IFN-γ, 10 u ml^{-1}). They were then re-suspended in Krebs and were added to the organ baths cumulatively. Concentrations of cells are given per ml of solution. Rings were contracted with phenylephrine (PE, $3x10^{-8}$ M) and treated with catalase (CAT, 1000 u ml^{-1}) to remove any hydrogen peroxide generated from superoxide anion, and superoxide dismutase (SOD, 250 u ml^{-1}) to potentiate the actions of NO, before the addition of activated cells. Once the relaxation had stabilised, LY 83583 was added cumulatively.

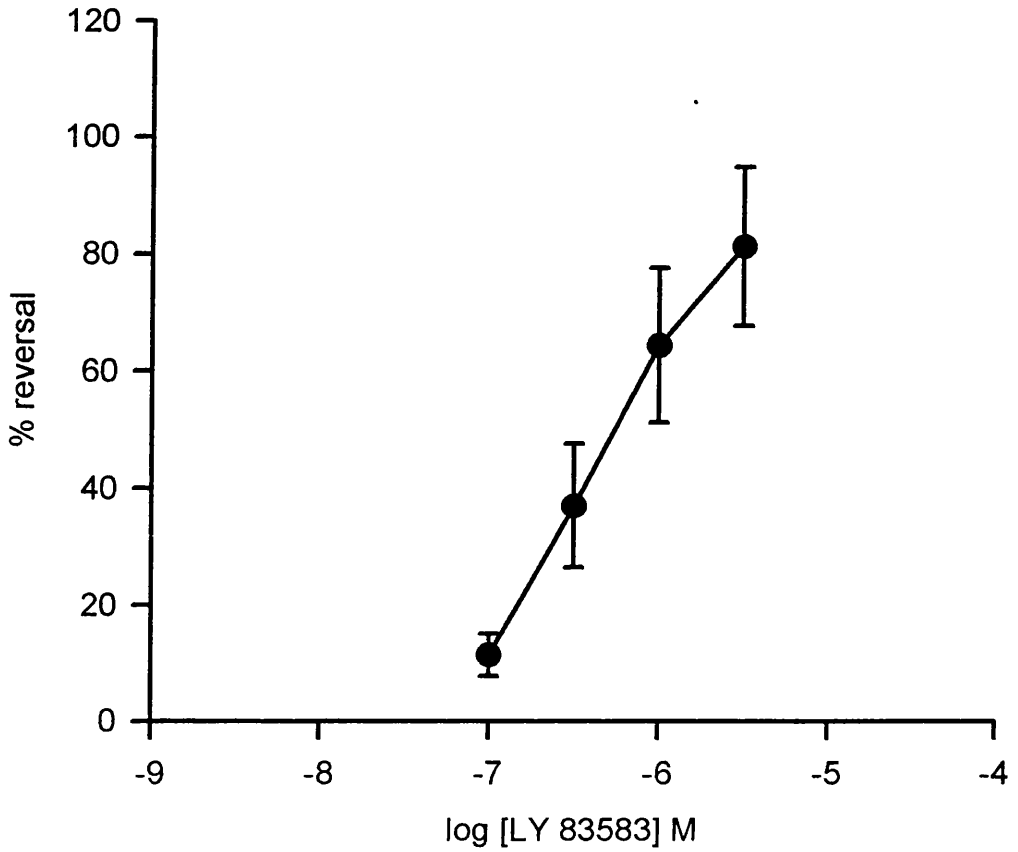


Figure 5.4b The ability of the superoxide anion generator, LY 83583, to reverse the relaxation of phenylephrine-induced tone in rat aortic rings produced by activated J774.7 cells. Cells were treated overnight with lipopolysaccharide (LPS, 100 ng ml^{-1}) and interferon-gamma (IFN-γ, 10 u ml^{-1}) and were added to the organ baths to achieve a final concentration of $6x10^5$ cells ml^{-1}. Rings were contracted with phenylephrine (10^{-8}-10^{-7} M) and treated with SOD (250 u ml^{-1}) to potentiate the effects of NO and with catalase (1000 u ml^{-1}) to remove any hydrogen peroxide generated from superoxide anion, before the addition of cells. Each point represents the mean ± s.e mean of 6 observations.

from superoxide anion. Addition of LY 83583 (10^{-7}-$3x10^{-6}$ M) during an established relaxation induced by activated J774.7 cells ($6x10^5$ cells ml^{-1}) led to a concentration-dependent reversal of relaxation (Figs 5.4a & 5.4b); maximum of 81.3 ± 13.6 %. SOD (250 u ml^{-1}) was also present to potentiate the actions of NO. Although the presence of SOD might appear unwarranted since it would be expected to scavenge superoxide anion, it would only be expected to act extracellularly and not inhibit the principal intracellular actions of LY 83583.

5.5 Effects of L-NMMA on relaxation of phenylephrine-induced tone by activated J774.7 macrophages

If the relaxation of aortic rings produced by activated J774.7 cells results from release of NO, then this action would be expected to be blocked by inhibitors of NO synthase. The NO synthase inhibitor, L-NMMA, has already been shown to be effective in the J774.7 macrophage cell line (see 3.2.4). The effects of L-NMMA were therefore investigated on relaxation of rat aortic rings induced by J774.7 cells activated by LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}). Phenylephrine (10^{-8} M)-contracted rings of rat aorta, treated with SOD (250 u ml^{-1}) to protect the actions of NO, were relaxed by the addition of 10^4 activated cells. During this relaxation, L-NMMA (10^{-4} M) was added and resulted in a partial reversal of the relaxation (Fig 5.5). This was often associated with the development of repeated transient relaxations. Addition of L-NMMA at a concentration of $3x10^{-4}$ M, however, resulted in a complete reversal of the relaxation.

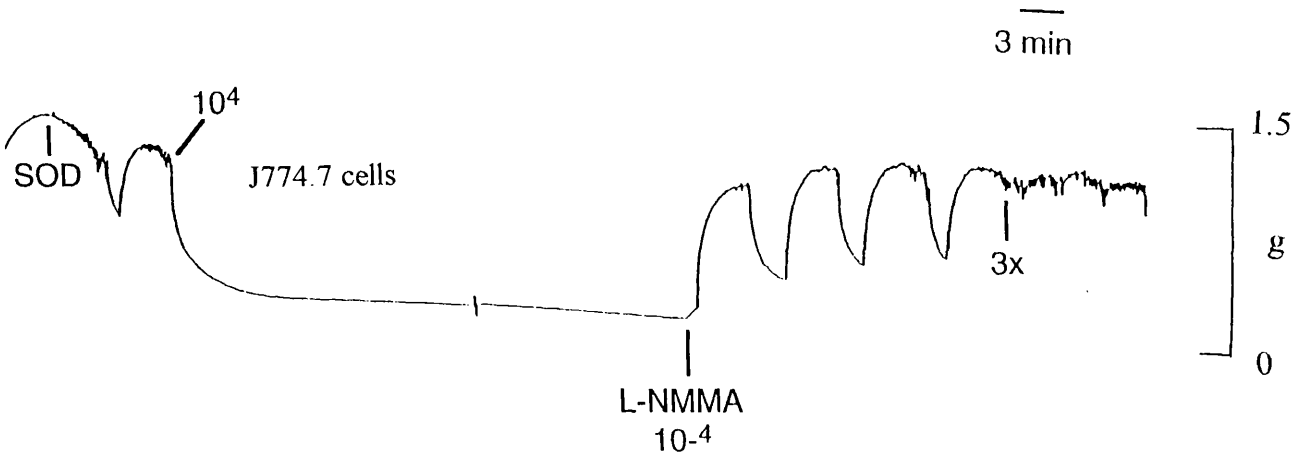


Figure 5.5 Trace demonstrating the ability of L-NMMA to reverse relaxation of phenylephrine-induced tone in rat aortic rings produced by activated J774.7 cells. Cells were treated overnight with lipopolysaccharide (LPS, 100 ng ml^{-1}) and interferon-gamma (IFN-γ, 10 u ml^{-1}). They were then re-suspended in Krebs and added to the organ baths cumulatively. Concentrations of cells are given per ml of solution. Rings were contracted with phenylephrine (PE, 10^{-8} M) and treated with superoxide dismutase (SOD, 250 u ml^{-1}) to potentiate the actions of NO before the addition of activated cells. Once the relaxation had stabilised, L-NMMA (10^{-4} M then $3x10^{-4}$ M) was added.

5.6 Effects of L-NAME on relaxation of phenylephrine-induced tone by activated J774.7 macrophages

Although L-NAME is an established inhibitor of NO synthase, it is not as effective as L-NMMA at inhibiting the inducible form of the enzyme in J774.7 cells (see Chapter 3, 3.2.5). The effects of L-NAME were therefore investigated on relaxation of rat aortic rings induced by J774.7 cells activated by LPS (100 ng ml^{-1}) and IFN-γ (10 u ml^{-1}). Addition of activated cells produced immediate cell number-dependent relaxation (Fig 5.6). L-NAME (10^{-4}-$3x10^{-3}$ M) produced only a very slight degree of reversal of the relaxation induced by activated cells; maximum reversal 26.4 ± 19 % (n=3) at $3x10^{-3}$ M.

Thus, all of the data obtained in this chapter are consistent with the macrophage-derived vasodilator being NO.

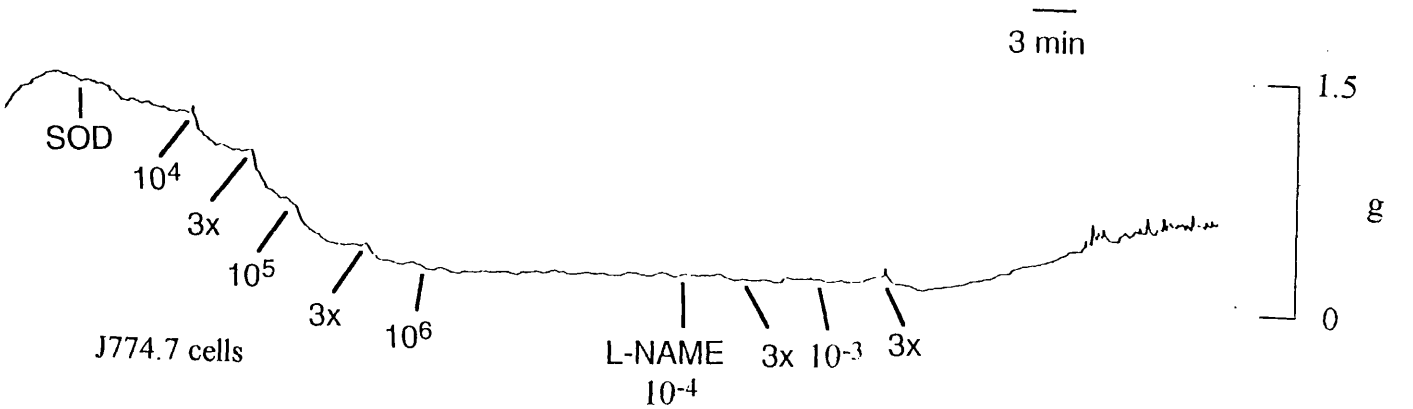


Figure 5.6 Trace demonstrating the ability of L-NAME to reverse relaxation of phenylephrine-induced tone in rat aortic rings produced by activated J774.7 cells. Cells were treated overnight with lipopolysaccharide (LPS, 100 ng ml^{-1}) and interferon-gamma (IFN-γ, 10 u ml^{-1}). They were then re-suspended in Krebs and added to the organ baths cumulatively. Concentrations of cells are given per ml of solution. Rings were contracted with phenylephrine (PE, 10^{-8} M) and treated with superoxide dismutase (SOD, 250 u ml^{-1}) to potentiate the actions of NO before the addition of activated cells. Once the relaxation had stabilised, L-NAME (10^{-4}-3x10^{-3} M) was added cumulatively.

CHAPTER 6

INVESTIGATION OF PRODUCTION OF REACTIVE OXYGEN SPECIES BY THE J774.7 MACROPHAGE CELL LINE

6.1 Production of reducing species

As described in the Methods section, production of reducing species can be detected by the use of cytochrome C, whereby its oxidised (Fe^{3+}) form is converted to the reduced (Fe^{2+}) form, resulting in an increase in absorbance at 550 nm. Cells involved in inflammatory processes can be stimulated to produce superoxide anion, a reducing agent with cytotoxic actions. An attempt was therefore made to examine the production of superoxide anion by the J774.7 macrophage cell line.

6.1.1 Effects of PMA and LPS on superoxide production

PMA and LPS are well documented for their ability to induce bactericidal and tumouricidal activity in macrophages (Amano *et al.*, 1985; Mayer *et al.*, 1993). The ability to induce superoxide production by J774.7 cells was therefore investigated. Cells were treated with PMA (10^{-7} M) and the effects

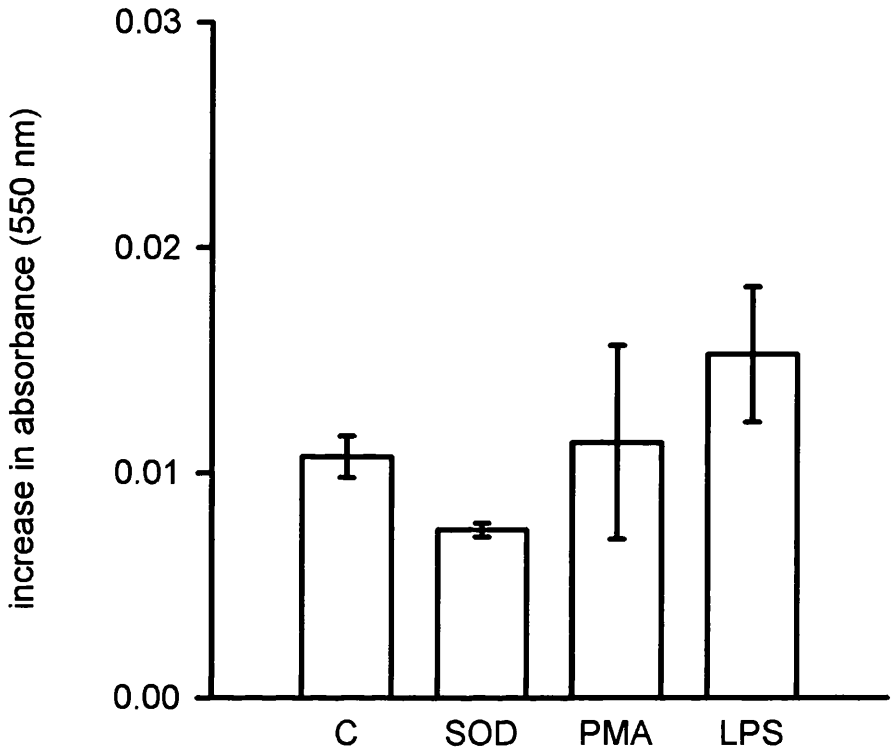


Figure 6.1 Effects of unstimulated and stimulated J774.7 cells (10^6 cells ml^{-1}) on the absorbance of oxidised cytochrome C (10^{-5} M) at 550 nm during a 1 hour incubation at 37°C. Cells were treated with phorbol 12-myristate 13-acetate (PMA, 10^{-7} M) during the 1 hour incubation, or were pretreated with lipopolysaccharide (LPS, 100 ng ml^{-1}) for 24 hours and then washed prior to the 1 hour incubation. Catalase (100 u ml^{-1}) was present in all cases to prevent the re-oxidation of cytochrome C. The effects of superoxide dismutase (SOD, 100 u ml^{-1}) on absorbance in the presence of unstimulated cells (C) is also shown. Each column represents the mean ± s.e mean of 6 observations. A standard reading from a 10^{-5} M solution of cytochrome C was subtracted from each reading.

on the absorbance of cytochrome C assessed during a 1 hour incubation. In a separate experiment, cells were pretreated with LPS (100 ng ml^{-1}) for 24 hours. They were then washed and their effects on the absorbance of oxidised cytochrome C assessed during a 1 hour incubation. Catalase (100 u ml^{-1}) was present in each case to prevent any hydrogen peroxide produced from re-oxidising cytochrome C. Unstimulated J774.7 cells and cells stimulated by PMA and LPS failed to induce reduction of cytochrome C (Fig 6.1), suggesting that superoxide anion was not being produced. Furthermore, J774.7 cells treated with SOD (100 u ml^{-1}), a scavenger of superoxide anions, had no effect on the absorbance of cytochrome C, confirming that there was no basal production of superoxide anion.

Due to the apparent lack of both LPS and PMA to stimulate production of superoxide anion, verification was sought that the assay used to detect production was in fact functioning.

6.1.2 Effects of the hypoxanthine/xanthine oxidase free radical generating system on oxidised cytochrome C in the absence and presence of cells

The hypoxanthine/xanthine oxidase (HX/XO) system is commonly used for the generation of superoxide anion (Berman & Martin, 1993). An experiment was therefore conducted to determine if the cytochrome C assay could detect superoxide anion generated by this system. Addition of HX ($2x10^{-4}$ M) and XO (20 mu ml^{-1}) to cytochrome C in the absence of cells produced a large increase in absorbance during a 1 hour incubation (Fig 6.2). This increase was reduced in the presence of SOD (100 u ml^{-1}) by

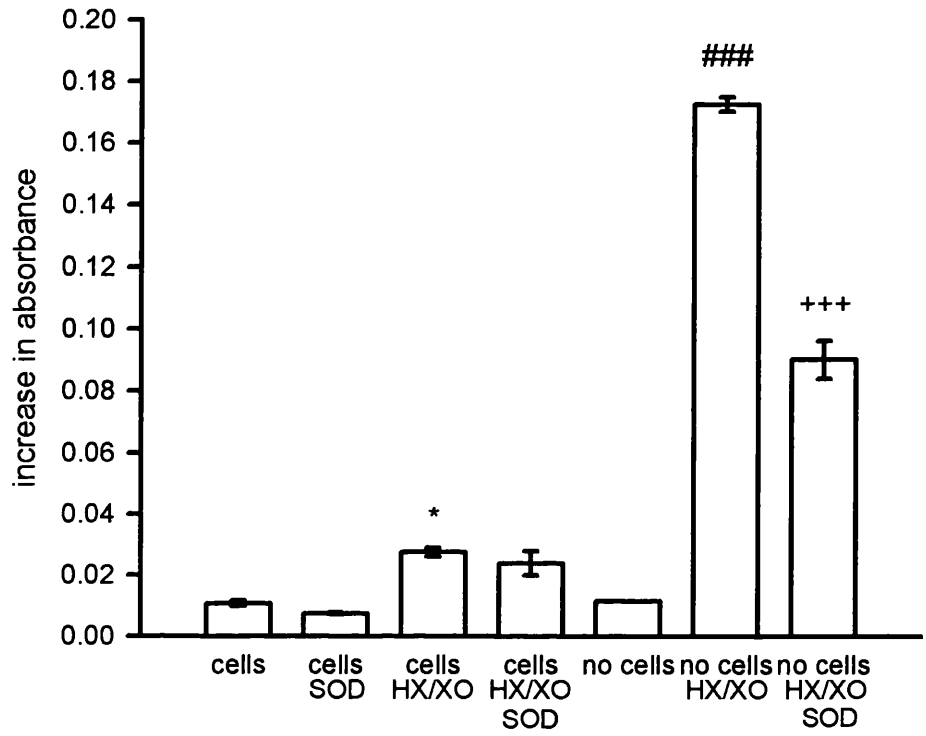


Figure 6.2 The effects of the hypoxanthine (HX)/xanthine oxidase (XO) superoxide anion generating system on the absorbance of oxidised cytochrome C (10^{-5} M) at 550 nm in the absence and presence of J774.7 cells during a 1 hour incubation at 37°C. HX ($2x10^{-4}$ M) /XO (20 mu ml^{-1}) produced a large increase in absorbance and this was reduced by superoxide dismutase (SOD, 100 u ml^{-1}). The presence of cells resulted in an almost complete abolition of the rise induced by HX/XO. Each column represents the mean ± s.e. mean of 6 observations. * $P<0.05$ indicates a significant increase in absorbance from cells alone. ### indicates a significant rise in the absorbance of cytochrome C in the presence of HX and XO. +++ indicates a significant decrease by SOD of the increase in absorbance produced by HX/XO. Catalase (100 u ml^{-1}) was present in each case. A standard reading from a 10^{-5} M solution of cytochrome C was subtracted from each reading.

47.8 ± 5.3 %. The assay therefore appeared to be functioning as expected. Surprisingly, when cells were added to a solution of cytochrome C the ability of HX/XO to reduced cytochrome C was almost completely abolished.

Clearly, some property of the cells was preventing the reduction expected by superoxide anion. The possibility that this action was due to production of a powerful oxidant by the J774.7 cells was investigated.

6.2 Production of an oxidising species

Upon activation by various stimuli, macrophages release a number of reactive oxygen species which play a role in the host defence mechanism. Although the superoxide anion released is a reducing agent, all the others including hydroxyl radical, hydrogen peroxide and peroxynitrite, are powerful oxidising species.

In Section 1, the reduction of oxidised cytochrome C was used as an index of production of the reducing agent, superoxide anion. In this section, the reduction in the absorbance of cytochrome C at 550 nm which accompanies oxidation of reduced cytochrome C was used as an index of production of an oxidising species by J774.7 cells.

6.2.1 Effects of J774.7 cells on the absorbance of reduced cytochrome C

Reduced cytochrome C was prepared by treatment with sodium dithionite (10^{-4} M). Addition of J774.7 cells (10^5 cells ml^{-1}) produced a time-

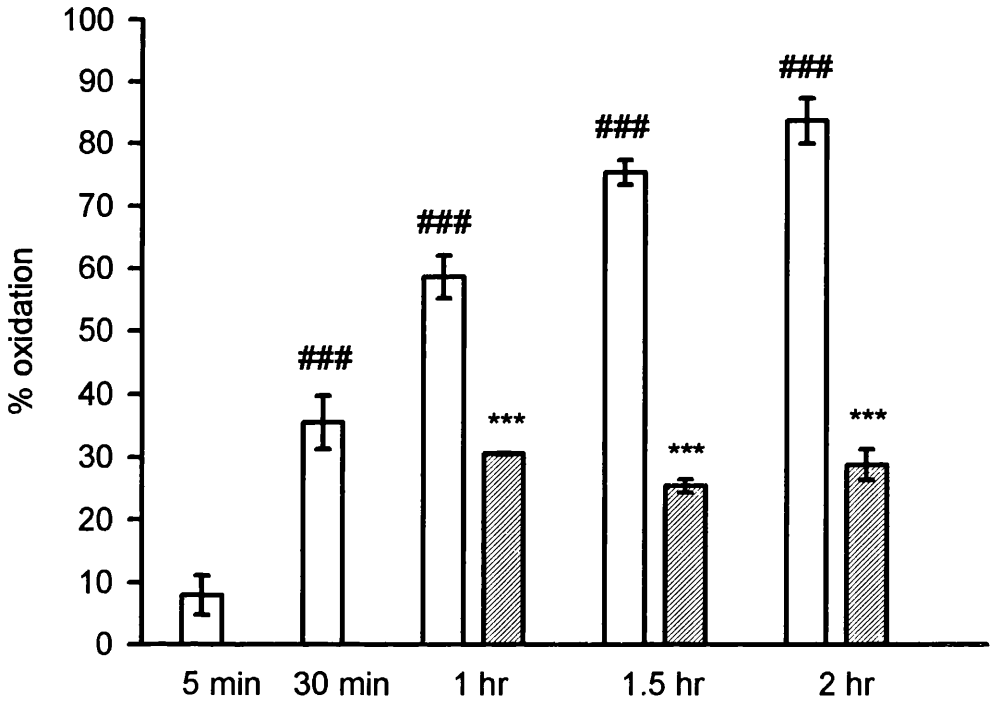


Figure 6.3 The oxidising effects of unstimulated J774.7 cells (10^5 cells ml^{-1}) on reduced cytochrome C (10^{-5} M) during an incubation at 37°C. Cells were added to reduced cytochrome C, and were left in contact for times of 5 min, 30 min, 1 hr, 1.5 hr and 2 hours, after which absorbance was measured (open columns). In a parallel experiment, J774.7 cells were left in contact with cytochrome C for 30 min after which the solution containing cytochrome C was removed, and its absorbance measured for the duration of the 2 hour incubation (hatched columns). Each column represents the mean ± s.e. mean of 6 observations. ### $P<0.001$ indicates a significant increase in the extent of oxidation compared with the 5 min time point.*** $P<0.001$ indicates a significant decrease in the extent of oxidation compared to cytochrome C in the presence of cells at the respective time points.

dependent oxidation of cytochrome C (10^{-5} M) during a 2 hour incubation at 37°C, as assessed by a fall in absorbance at 550 nm (Fig 6.3). In a parallel experiment, cells were left in contact with cytochrome C for 30 min, the solution containing the cytochrome C was then removed and its absorbance monitored for the duration of the 2 hour incubation. Fig 6.3 shows that when the cytochrome C was removed from the cells, the process of oxidation stopped.

Clearly, either the cells have to be in contact with the cytochrome C or the cells release a labile oxidant which decays rapidly in the supernatant. An attempt was made to determine if the oxidising action of the cells was due to production of known macrophage-derived oxidants.

6.2.2 Effects of catalase on oxidation of cytochrome C

The effects of catalase, a scavenger of hydrogen peroxide, were investigated on the oxidation of reduced cytochrome C by J774.7 cells. Cells were incubated at 37°C with reduced cytochrome C (10^{-5} M) and absorbance was monitored over the next 2 hours in the presence or absence of catalase (100-1,500 u ml^{-1}). Catalase (1,500 u ml^{-1}) did not block the oxidation of cytochrome C by J774.7 cells at any time during the 2 hour incubation (Fig 6.4). Lower concentrations of catalase also failed to affect the oxidation of cytochrome C (data not shown). Cell-mediated oxidation of cytochrome C is therefore unlikely to be due to production of hydrogen peroxide.

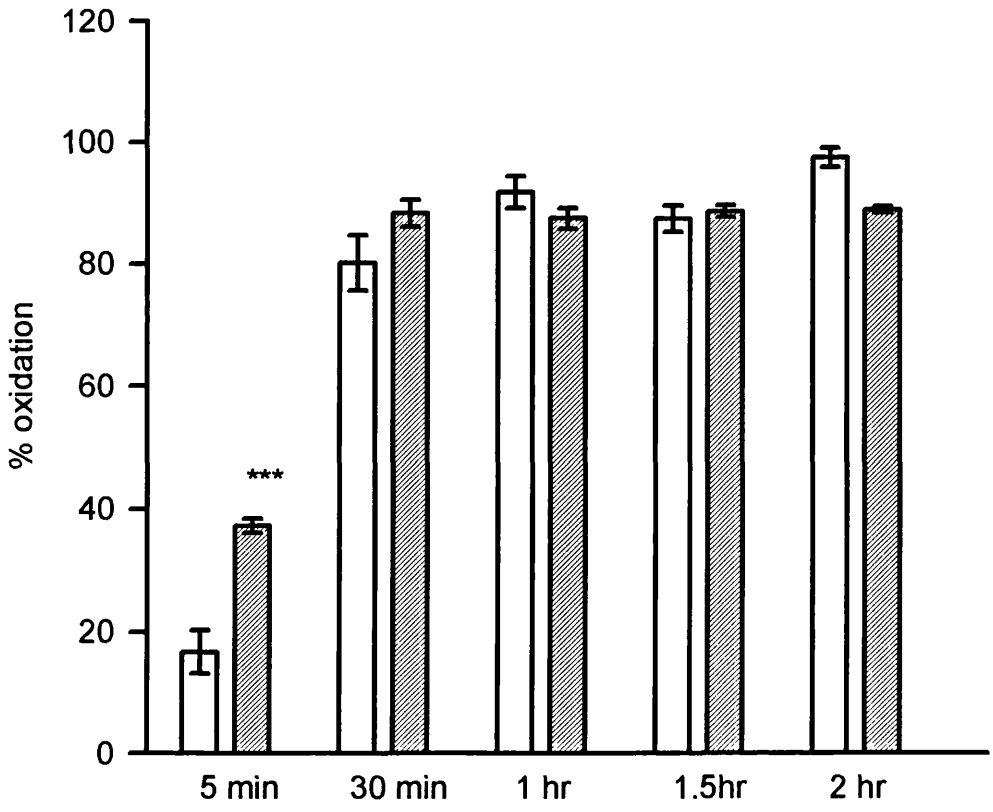


Figure 6.4 Effects of catalase (1,500 u ml^{-1}) on the oxidation of reduced cytochrome C (10^{-5} M) by J774.7 cells ($3x10^5$ cells ml^{-1}). Cells were left in contact with cytochrome C for 5 min, 30 min, 1 hr, 1.5 hr or 2 hours in the presence (hatched columns) or absence (open columns) of catalase, after which times the absorbance of cytochrome C was measured at 550 nm. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant increase in the absorbance of cytochrome C in the presence of catalase.

6.2.3 Effects of L-NAME and L-NMMA on oxidation of cytochrome C

Peroxynitrite is formed by the reaction of NO with superoxide anion (Freeman, 1994). Blocking NO synthase, thereby inhibiting the production of NO, should therefore prevent the formation of peroxynitrite. The effects of the NO synthase inhibitor, L-NAME, were therefore investigated on the oxidation of reduced cytochrome C by J774.7 cells. Cells were incubated at 37°C with reduced cytochrome C (10^{-5} M) in the presence or absence of L-NAME ($5x10^{-4}$ M) and the absorbance monitored over 2 hours. Although L-NAME reduced the oxidation of cytochrome C by J774.7 cells (10^6 cells ml^{-1}) at 5 min, it had no effect at any other time point (Fig 6.5).

In this experiment the oxidation of cytochrome C by J774.7 cells was complete by approx. 30 min and it was therefore possible that this high degree of oxidation obscured a possible slight inhibitory action of L-NAME. To test this, further experiments were conducted using cells at lower densities (10^5 and $3x10^5$ cells ml^{-1}) so that oxidation took place at a slower rate. Even at these lower cell densities, however, L-NAME was unable to block oxidation of cytochrome C by J774.7 cells (data not shown).

L-NMMA is known to be a more effective inhibitor the inducible form of NO synthase than L-NAME in the J774.7 macrophage cell line (see 3.2.4 and 3.2.5). Therefore, the effects of L-NMMA were investigated on the oxidation of reduced cytochrome C by J774.7 cells. However, like L-NAME, L-NMMA ($5x10^{-4}$ M) had no inhibitory effect on the oxidation of cytochrome C by J774.7 cells at any time during the 2 hour incubation (Fig 6.6).

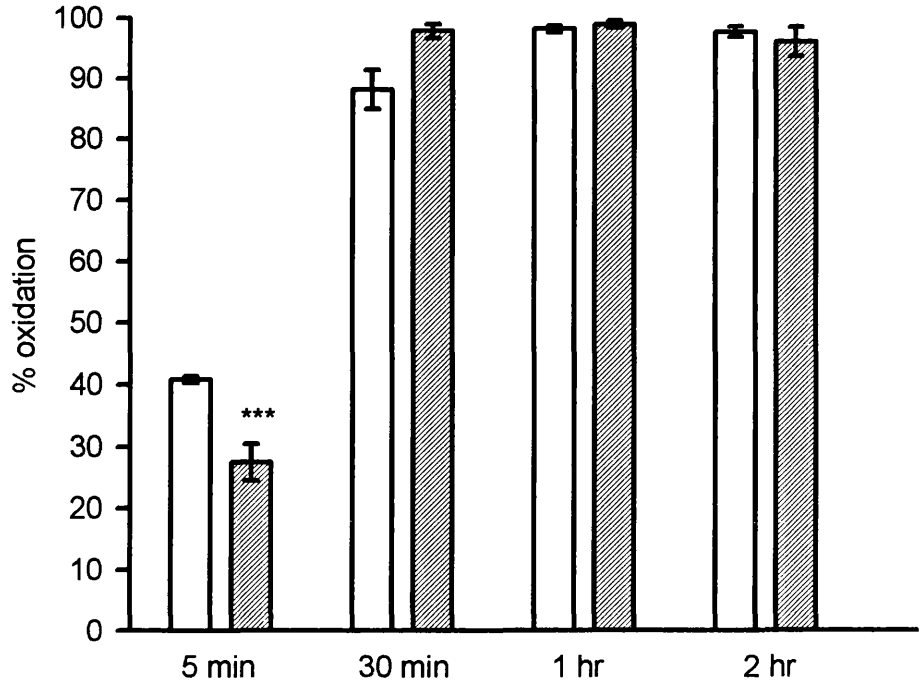


Figure 6.5 Effects of N^G-nitro L-arginine methyl ester (L-NAME, $5x10^{-4}$ M) on the oxidation of reduced cytochrome C (10^{-5} M) by J774.7 cells (10^6 cells ml^{-1}) during an incubation at 37°C. Cell were left in contact with cytochrome C for 5 min, 30 min, 1 or 2 hours in the presence (hatched columns) or absence (open columns) of L-NAME, after which times the absorbance of cytochrome C was measured at 550 nm. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant decrease in oxidation of cytochrome C by L-NAME.

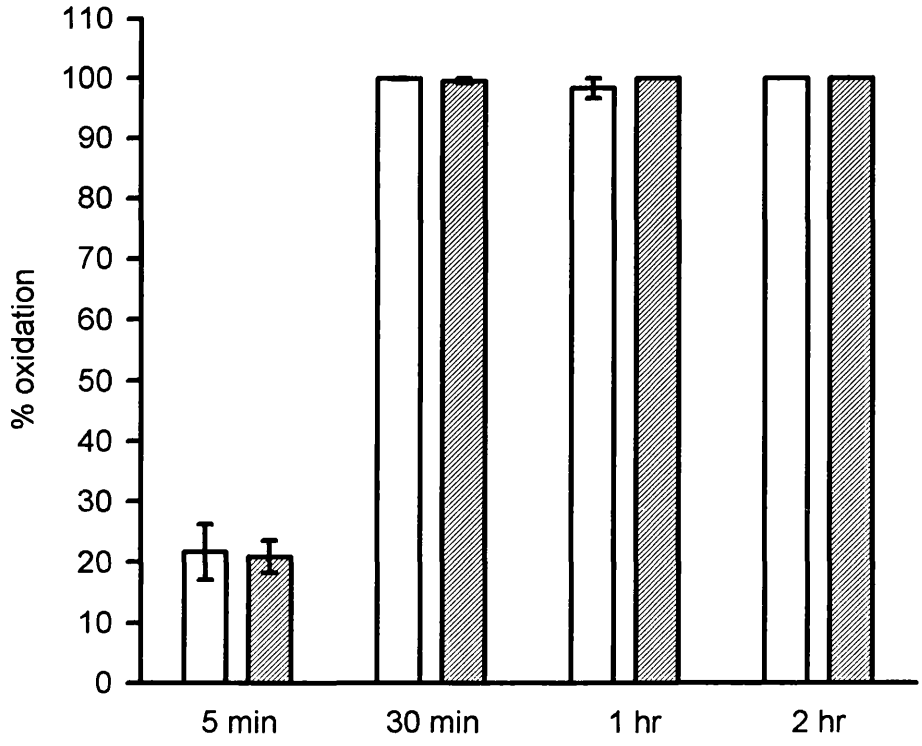


Figure 6.6 Effects of N^G-monomethyl-L-arginine (L-NMMA, $5x10^{-4}$ M) on the oxidation of reduced cytochrome C (10^{-5} M) by J774.7 cells ($3x10^5$ cells ml^{-1}) during an incubation at 37°C. Cells were left in contact with cytochrome C for 5 min, 30 min, 1 or 2 hours in the presence (hatched columns) or absence (open columns) of L-NMMA, after which times the absorbance of cytochrome C was measured at 550 nm. Each column represents the mean ± s.e. mean of 6 observations.

These data suggest that cell-mediated oxidation of cytochrome C does not involve production of peroxynitrite.

6.2.4 Effects of SOD on oxidation of cytochrome C

An alternative means of preventing the formation of peroxynitrite is to remove superoxide anion using SOD. The effects of SOD were therefore investigated on the oxidation of reduced cytochrome C by J774.7 cells. Cells were incubated at 37°C with reduced cytochrome C (10^{-5} M) in the presence or absence of SOD (100 u ml^{-1}) and the absorbance monitored over 2 hours. SOD had no inhibitory effect on oxidation of cytochrome C by J774.7 cells (Fig 6.7), again suggesting lack of involvement of peroxynitrite.

6.2.5 Effects of mannitol and DMTU on oxidation of cytochrome C

Mannitol and dimethythiourea (DMTU) are both scavengers of the oxidising species, hydroxyl radical (Fox, 1984; Wasil *et al.*, 1987). Mannitol cannot permeate cell membranes, whereas DMTU is cell permeable. The effects of each of these agents were investigated on the oxidation of reduced cytochrome C by J774.7 cells. Cells were incubated at 37°C with reduced cytochrome C (10^{-5} M) in the presence or absence of mannitol (10^{-3} M) or DMTU (10^{-3} M) and the absorbance monitored over 2 hours. Although mannitol (Fig 6.8) and DMTU (Fig 6.9) appeared to enhance oxidation at 30 min and 5 min, respectively, neither agent inhibited the oxidation of cytochrome C by J774.7 cells at any time point. These data suggest that the cell-mediated oxidation does not involve the production of hydroxyl radical.

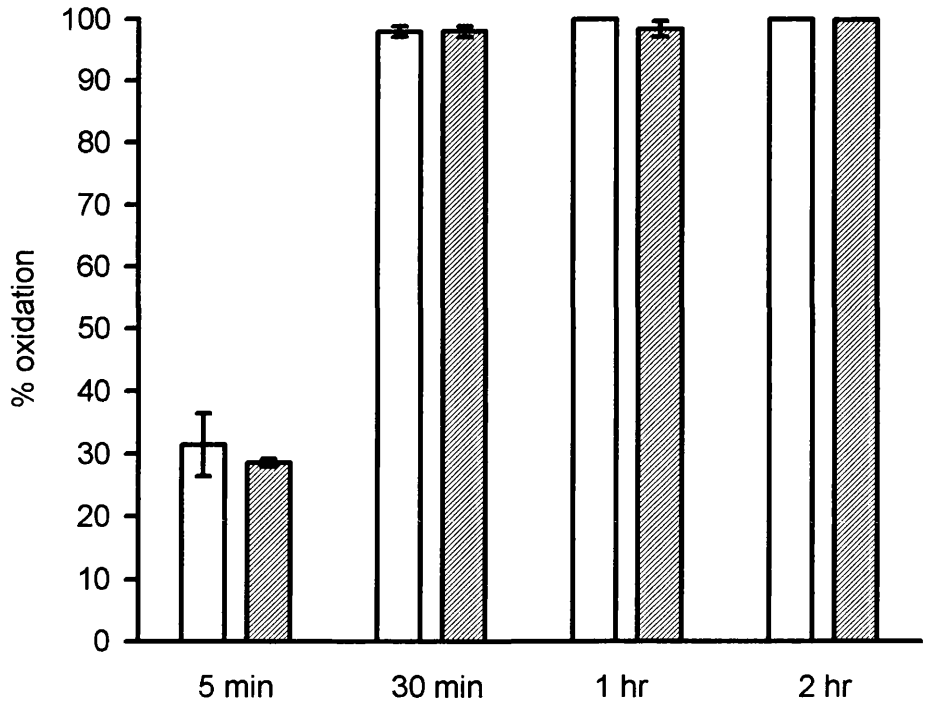


Figure 6.7 Effects of superoxide dismutase (SOD, 100 u ml^{-1}) on the oxidation of reduced cytochrome C (10^{-5} M) by J774.7 cells ($3x10^5$ cells ml^{-1}) during an incubation at 37°C. Cells were left in contact with cytochrome C for 5 min, 30 min, 1 or 2 hours in the presence (hatched columns) or absence (open columns) of SOD, after which times the absorbance of cytochrome C was measured at 550 nm. Each column represents the mean ± s.e. mean of 6 observations.

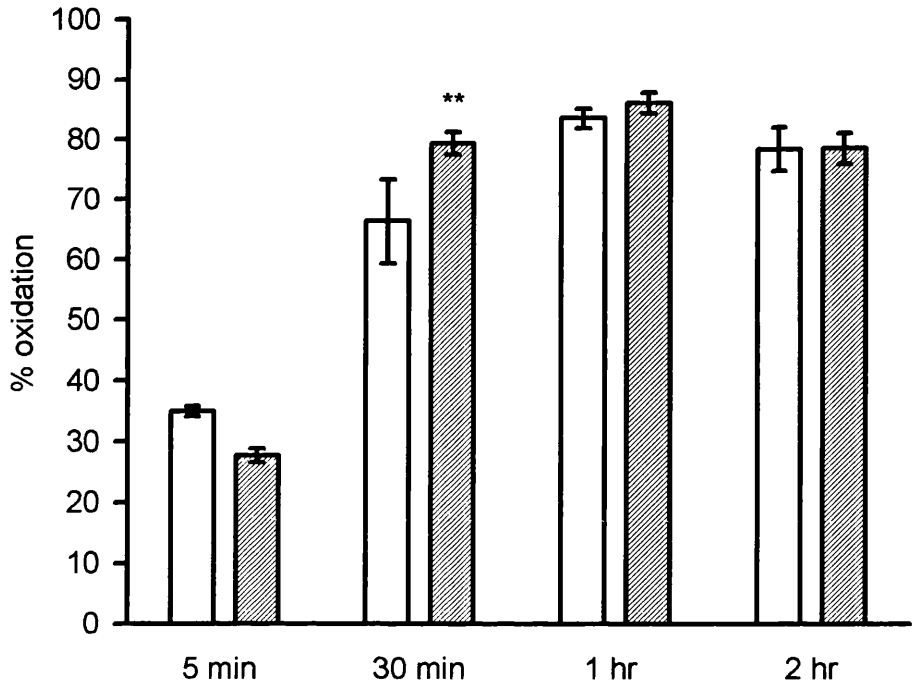


Figure 6.8 Effects of the hydroxyl radical scavenger, mannitol (10^{-3} M), on the oxidation of reduced cytochrome C (10^{-5} M) by J774.7 cells ($3x10^5$ cells ml^{-1}) during an incubation at 37°C. Cell were left in contact with cytochrome C for 5 min, 30 min, 1 or 2 hours in the presence (hatched columns) or absence (open columns) of mannitol, after which times the absorbance of cytochrome C was measured at 550 nm. Each column represents the mean ± s.e mean of 6 observations. ** $P<0.01$ indicates a significant increase in the extent of oxidation in the presence of mannitol.

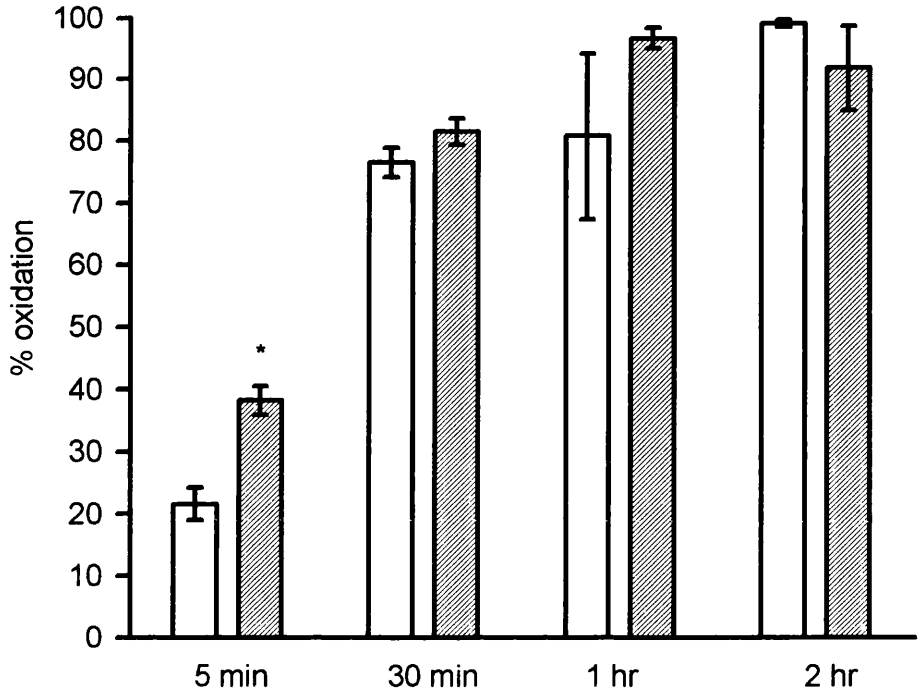


Figure 6.9 Effects of the hydroxyl radical scavenger, dimethylthiourea (DMTU, 10^{-3} M), on the oxidation of reduced cytochrome C (10^{-5} M) by J774.7 cells ($3x10^5$ cells ml^{-1}) during an incubation at 37°C. Cells were left in contact with cytochrome C for 5 min, 30 min 1 or 2 hours in the presence (hatched columns) or absence (open columns) of DMTU, after which times the absorbance of cytochrome C was measured at 550 nm. Each column represents the mean ± s.e. mean of 6 observations. * $P<0.05$ indicates a significant increase in the extent of oxidation in the presence of DMTU.

6.2.6 Effects of dithiothreitol on oxidation of cytochrome C

The reducing agent, dithiothreitol, is commonly employed to inhibit the oxidising effects of hypochlorous acid (Eley *et al.*, 1989). The effects of this agent were investigated on the oxidation of reduced cytochrome C by J774.7 cells. Cells were incubated at 37°C with reduced cytochrome C (10^{-5} M) in the presence or absence of dithiothreitol ($3x10^{-3}$ M) and absorbance monitored over 2 hours. Dithiothreitol appeared to almost completely inhibit the oxidation of cytochrome C by J774.7 cells, suggesting that the oxidising species is hypochlorous acid (Fig 6.10). However, when the actions of dithiothreitol were examined further, it was found that it could promote reduction of cytochrome C even in the absence of cells (data not shown). The apparent inhibition of cell-mediated oxidation by dithiothreitol is therefore explained by its ability to reduce cytochrome C directly and not by inhibition of the oxidant action of hypochlorous acid.

These experiments therefore failed to identify the mechanism or mediator of the oxidation of cytochrome C by J774.7 cells.

6.3 Regulation of production of an oxidising species

In the previous section it was established that unstimulated J774.7 cells promoted oxidation of reduced cytochrome C, although the nature of this oxidation process was not established. In this section, the possibility was examined that the oxidising action of J774.7 cells could be

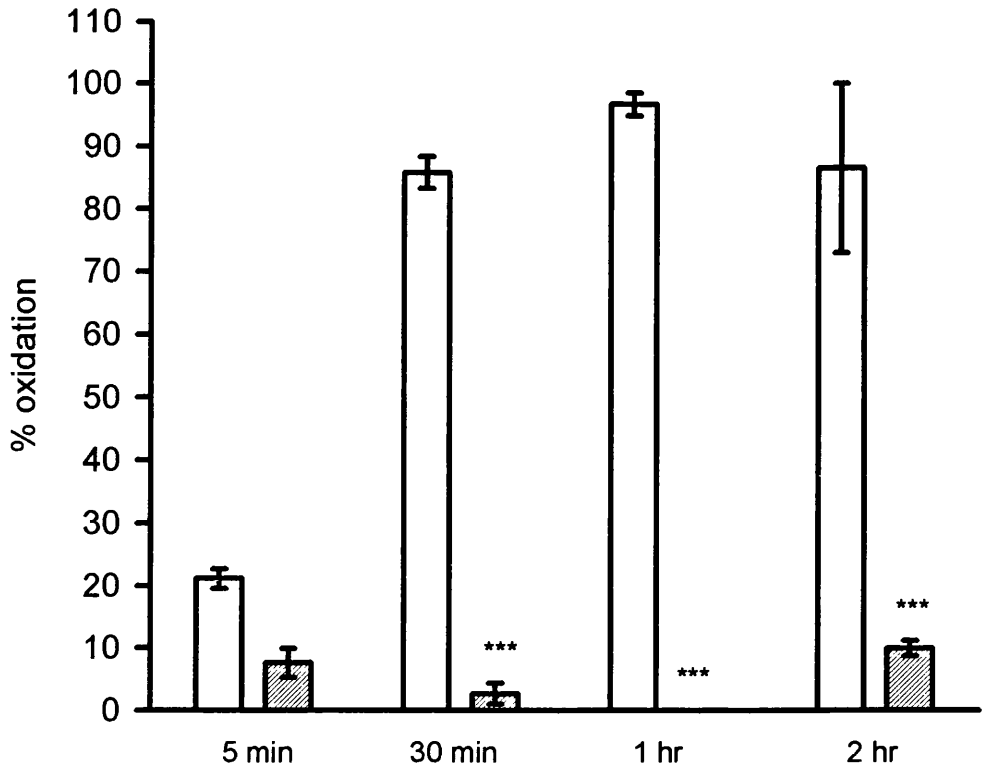


Figure 6.10 Effects of dithiothreitol ($3x10^{-3}$ M) on the oxidation of reduced cytochrome C (10^{-5} M) by J774.7 cells ($3x10^{-5}$ cells ml^{-1}) during an incubation at 37°C. Cells were left in contact with cytochrome C for 5 min, 30 min, 1 or 2 hours in the presence (hatched columns) or absence (open columns) of dithiothreitol, after which time the absorbance of cytochrome C was measured at 550 nm. Each column represents the mean ± s.e. mean of 6 observations. *** $P<0.001$ indicates a significant reduction in the extent of oxidation induced in the presence of dithiothreitol at that time point.

modulated by agents which are known either to activate or inhibit the activation of J774.7 cells.

6.3.1 Effects of LPS and IFN-γ on production of oxidising species

LPS and IFN-γ are well characterised activators of inflammatory cells and their ability to stimulate expression of inducible NO synthase is discussed elsewhere in this thesis (see Chapter 3, 3.2.2). The possibility that treatment with LPS and IFN-γ might affect the ability of J774.7 cells to promote oxidation of reduced cytochrome C was therefore investigated. Cells were incubated overnight (23 hours) with LPS (100 ng ml^{-1}) and IFN-γ (1 or 10 u ml^{-1}) alone and in combination. They were then washed and incubated in Krebs solution at 37°C for 30 min. When used alone, LPS and IFN-γ (both 1 and 10 u ml^{-1}) produced statistically significant but extremely small (<10%) increases in the extent of oxidation of cytochrome C by J774.7 cells (Figure 6.11). In other experiments, LPS and IFN-γ alone failed to affect the oxidation of cytochrome C by J774.7 cells (data not shown). The combination of LPS and IFN-γ (1 or 10 u ml^{-1}) also had no effect on the extent of oxidation.

6.3.2 Effects of dexamethasone on production of an oxidising species

Corticosteroids powerfully inhibit the inflammatory response, and their inability to inhibit the expression of the inducible form of NO synthase is discussed else where in this thesis (see Chapter 3, 3.2.7). The possibility that treatment with dexamethasone might affect the ability of J774.7 cells to promote oxidation of cytochrome C was therefore investigated. Cells were incubated overnight (23 hours) with dexamethasone (10^{-6} M). They were

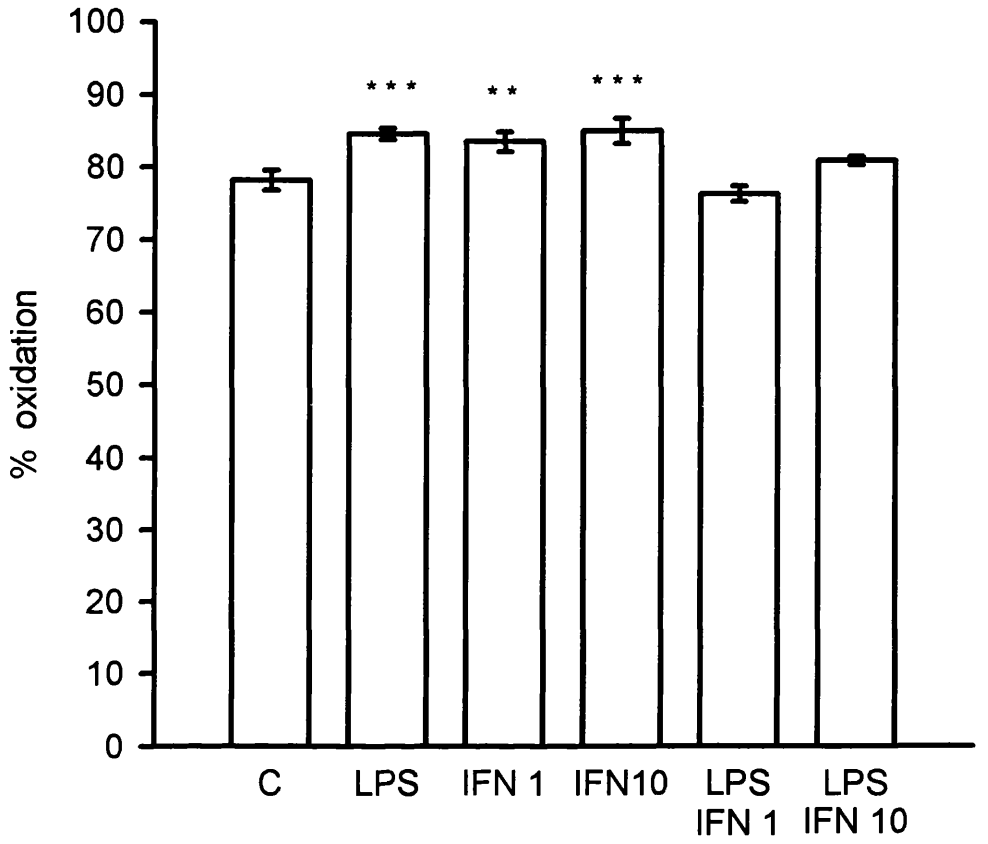


Figure 6.11 Effects of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) on the oxidation of reduced cytochrome C (10^{-5} M) by J774.7 cells (10^5 cells ml^{-1}). Cells were incubated overnight (23 hours) with LPS (100 ng ml^{-1}) and IFN-γ (1 or 10 u ml^{-1}) alone or in combination. They were then washed and incubated at 37°C in Krebs solution containing cytochrome C for 30 min, after which time the absorbance was measured at 550 nm. Each column represents the mean ± s.e. mean of 6 observations. ** $P<0.01$ and *** $P<0.001$ indicate a significant increase in oxidation from untreated (C) cells.

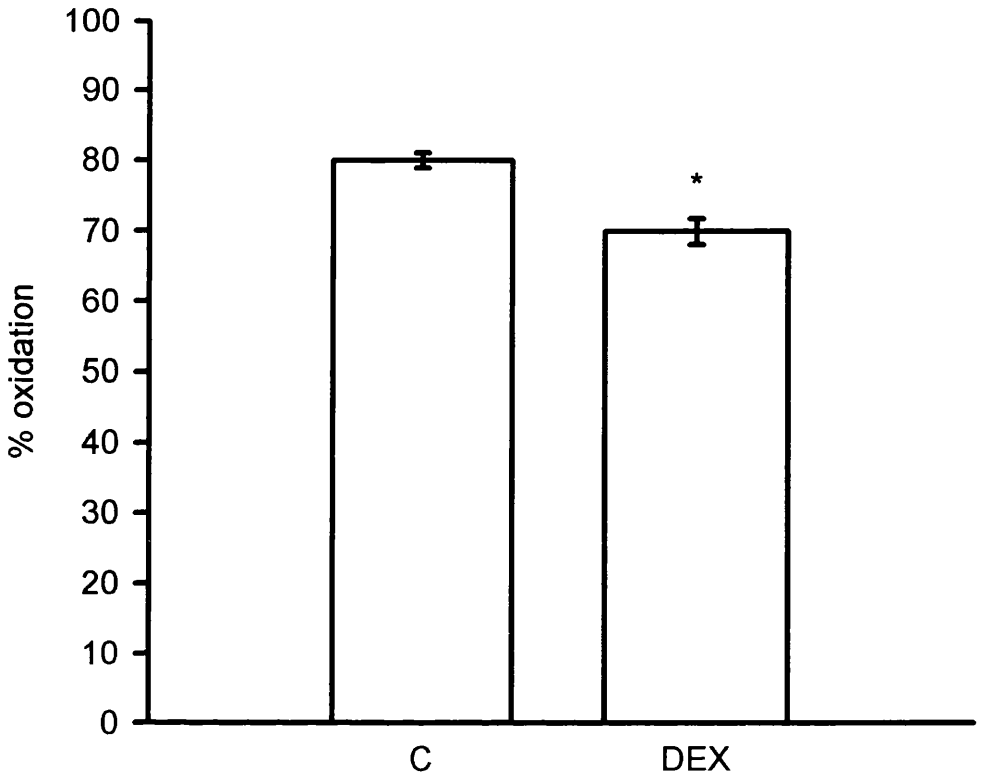


Figure 6.12 Effects of dexamethasone (DEX) on the oxidation of reduced cytochrome C (10^{-5} M) by J774.7 cells (10^5 cells ml^{-1}). Cells were incubated overnight with dexamethasone (10^{-6} M) and were then washed and incubated in Krebs solution at 37°C containing cytochrome C for 30 min, after which time the absorbance of cytochrome C was measured at 550 nm. Each column represents the mean ± s.e. mean of 6 observations. * $P<0.05$ indicates a significant decrease in oxidation from untreated (C) cells.

then washed and incubated in Krebs solution at 37°C for 30 min. Although dexamethasone produced a statistically significant reduction in the oxidation of cytochrome C produced by J774.7 cells, the magnitude of the inhibition was very small (12.6 ± 0.33 %) (Fig 6.12).

Thus, neither powerful activators of macrophages (LPS and IFN-γ) nor an inhibitor (dexamethasone) had any convincing effect on the ability of J774.7 cells to promote oxidation of cytochrome C.

DISCUSSION

CHAPTER 7

Macrophages play a major role in the host defence mechanism by their ability to release both NO and reactive oxygen species. The aim of this thesis was to investigate in mouse macrophages: (i) the induction of NO synthase and (ii) the production of reactive oxygen species.

Macrophage cell lines provide a good model to study activation by LPS and cytokines, since they are homogeneous and free from cellular contaminants frequently found in isolated primary cultures. They may also be grown in large numbers, thus providing a convenient source of cells for analysis. Furthermore, in culture, a wide variety of drugs can be used with which to manipulate different enzyme cascades, a situation difficult to obtain *in vivo* due to the complex nature of the whole animal. However, there is also the disadvantage that the cells may differ in some way from native macrophages, and may differ in the way they respond to drugs in comparison to freshly isolated cells. For example, it was found in this particular study that once the J774.7 cells were passaged many times, they no longer responded to the combined stimulus of LPS and IFN-γ, demonstrating that the characteristics of the cells changed over time. This had to be taken into consideration when comparing the results obtained from cells of a low passage number with those having a higher passage number. The practical way this was dealt with was that when a batch of J774.7 cells failed to respond well to LPS and IFN-γ, new, low-passage batches of cells were thawed from storage in liquid nitrogen.

A great deal of the work outlined in this thesis involves examination of the role of a variety of effector pathways that may result in the induction of NO synthase in murine macrophages. A limitation of the study is that it was carried out from a purely pharmacological perspective, using drugs known to block specific biochemical pathways. The additional use of biochemical and molecular biological techniques would certainly have provided a more comprehensive understanding of the mechanisms by which the drugs used produced their effects.

Direct measurement of NO is difficult because of its short half-life. A simple indirect way to assess NO production is by measurement of its stable end products, nitrite and nitrate. It is known that the majority of NO in aqueous solution decays to nitrite (Ignarro *et al.*, 1993), and thus measurement of accumulation of this into the medium bathing the macrophages was used as an index of NO production by the cells. Nitrite is relatively stable and the Griess reaction adopted is a simple and convenient assay to undertake. A more sensitive way in which to measure the effects of drugs on NO production would be to use chemiluminescence (Palmer *et al.*, 1987) or measure output of L-citrulline (Paul *et al.*, 1995). In fact, a Dasibi chemiluminescence detector was in use in our laboratory (threshold sensitivity of around 0.1 μM) but it could process only 20 samples per day as opposed to two hundred or so that could be dealt with using the Griess reaction. Moreover, the amounts of nitrite formed by macrophages were so high that the additional sensitivity afforded by chemiluminescence was not required.

7.1 Effects of LPS on nitrite accumulation

LPS is well established as an agent capable of inducing NO synthase not only in macrophages but in a variety of other cell types including Kupffer cells (Gaillard *et al.*, 1992), endothelial cells (Akarasereenont *et al.*, 1994), astrocytes (Feinstein *et al.*, 1993) and hepatocytes (Hortelano *et al.*, 1992). When used alone to stimulate J774.7 cells in this study, LPS proved to be rather ineffective, and produced inconsistent results. On some occasions, the cells produced a small rise (2.5-fold) in nitrite production following stimulation overnight, but on others, little response to LPS was seen.

7.2 Effects of drugs on LPS-stimulated nitrite accumulation

7.2.1 NO synthase inhibitors

The effects of two inhibitors of NO synthase, L-NMMA and L-NAME, were examined to determine if nitrite production by the cells in response to LPS alone arose from the L-arginine/NO system. Both L-NMMA and L-NAME when present during the overnight incubation slightly inhibited basal nitrite accumulation, suggesting a low basal activity of inducible NO synthase even in unstimulated cells. This may have been due to a slight endotoxin contamination of the medium bathing the cells. Alternatively, there could have been some constitutive NO synthase activity in this cell line that had so far gone unreported. Nevertheless, both L-NMMA and L-NAME reduced the increase in nitrite accumulation induced by LPS, indicating that it arose from induction of the L-arginine/NO pathway. L-NMMA appeared slightly more effective at inhibiting nitrite accumulation than L-NAME. In order to determine if this was generally the case, the effects of the drugs were investigated on an alternative macrophage cell line, i.e. RAW 264 cells.

LPS was found to produce a 2-fold rise in nitrite accumulation during an overnight incubation. In contrast to their effects on J774.7 cells, L-NMMA and L-NAME were equipotent and equi-effective at reducing nitrite production by RAW 264 cells stimulated by LPS alone. In fact, using RAW 264 cells, nitrite accumulation was almost completely abolished at the highest concentration of each drug used ($5x10^{-5}$ M for each), whereas, using J774.7 cells, the same concentration for each drug induced a far lesser degree of inhibition (67.7 ± 1.9% and 49.9 ± 1.9% for L-NMMA and L-NAME, respectively). Thus inhibition by these drugs demonstrates that nitrite produced in response to LPS alone arises from induction of the L-arginine/NO pathway in RAW 264 cells. These findings indicate a difference in the susceptibility of inducible NO synthase to inhibition in J774.7 cells and RAW 264 cells. Whether this results from differences in the nature of the NO synthase or to differential handling of the inhibitors, e.g. differential metabolism or uptake, by the two cell lines remains to be determined.

7.2.2 Polymyxin B

The question of whether contamination by endotoxin was responsible for the low but measurable basal production of nitrite by J774.7 cells was addressed using polymyxin B, an antibiotic which binds LPS with high affinity and blocks its actions (Lasfaragues *et al.*, 1989). When present during the overnight incubation, polymyxin B reduced basal nitrite accumulation (by 40.8 ± 2.8 %). This finding, taken together with the ability of L-NMMA and L-NAME to reduce basal nitrite accumulation, suggested that despite our best efforts, the culture medium was sometimes contaminated with sufficient LPS to produce a low-level induction of NO synthase. Polymyxin B also significantly reduced the production of nitrite by the cells stimulated by LPS, confirming its effectiveness as an inhibitor of endotoxin.

Thus, LPS alone appeared to stimulate expression of inducible NO synthase in J774.7 cells and RAW 264 cells. It was not, however, a powerful or reliable agent for induction.

7.3 Effects of IFN-γ on nitrite accumulation

As LPS was not a reliable stimulus for inducing nitrite accumulation, it was decided that an alternative stimulus should be sought. IFN-γ induces tumoricidal activity in macrophages (Celada & Schreiber, 1987) and it was hoped it would prove more effective than LPS in inducing NO synthase in J774.7 cells. However, in most cases, when used alone to activate J774.7 cells, IFN-γ proved even less effective than LPS, with very poor stimulation of nitrite production. In contrast, on isolated occasions, IFN-γ induced a massive rise (to approx. 50 μM) in nitrite accumulation. It is likely, however, in those experiments where IFN-γ appeared to be effective on its own that the rise in nitrite accumulation was in fact due to synergy between IFN-γ and endotoxin contaminating the medium (see Section 7.4 below). Evidence for this came from experiments where the LPS-selective binding agent, polymyxin B, reduced the ability of IFN-γ to stimulate nitrite production.

7.4 Effects of the combination of LPS and IFN-γ on nitrite accumulation

The combination of LPS and IFN-γ is well documented to produce a synergistic expression of NO synthase in murine macrophages (Stuehr & Marletta, 1987b) and this also proved true for J774.7 cells. As indicated above, both LPS and IFN-γ alone produced very little stimulation of nitrite accumulation, but when they were used in combination, a massive increase was seen. Numerous possible mechanisms have been proposed to explain the

synergy between LPS and IFN-γ. For example, LPS can apparently stabilise the increase in inducible NO synthase mRNA expressed by exposure to IFN-γ (Weisz *et al.*, 1994). In addition, IFN-γ may positively regulate the transcription activation of the gene for NO synthase in macrophages by LPS (Lorsbach *et al.*, 1993). LPS and IFN-γ are known to act at two distinct locations of the promoter region of the mouse gene for inducible NO synthase. LPS binds to region I and IFN-γ to region II, and simultaneous activation of the two regions synergistically increases expression of the gene (Lowenstein *et al.*, 1993).

The timing of stimulation with LPS and IFN-γ can profoundly influence the extent to which NO synthase is induced. It has been shown that either simultaneous exposure to the two agents or pre-treatment for between 2 and 24 hours with IFN-γ followed by activation with LPS results in full NO synthase induction (Lorsbach & Russell, 1992; Walter *et al.*, 1994; Hanano & Kaufmann, 1995). In all experiments performed in this study, LPS and IFN-γ were added simultaneously, thus ensuring that optimum induction of NO synthase was produced.

7.5 Time course of nitrite accumulation

It has been reported that, following stimulation, NO synthase activity in J774 cells peaks after 12 hours, decays to a low level by 48 hours, and is undetectable by 72 hours (Assreuy *et al.*, 1993). This did not appear to be the case with J774.7 cells in this study, as nitrite production continued to rise in an almost linear fashion for at least 48 hours. Thus, enzyme activity in our J774.7 cells is maintained for longer periods than for the unspecified clone of J774 cells used by Assreuy *et al.* (1993). Whether this is due to a longer

period of synthesis or slower rate of inactivation of NO synthase in this sub-clone is not known.

It has been proposed that L-arginine, the substrate for NO synthase, can become rate limiting due to the high levels of NO that are produced by the inducible enzyme. The concentration of L-arginine can thus be critical for macrophage function at sites of tissue injury and infection, as levels can rapidly diminish (Albina *et al.*, 1989). Transport of amino acids across plasma membranes occurs by diffusion and via specific membrane bound carrier proteins (White, 1985). It is likely that uptake of L-arginine into J774 cells is mediated by the system y^+ amino acid transporter (Bogle *et al.*, 1992a; Baydoun *et al.*, 1994). Reports indicate that LPS, TNF and IL-1 up-regulate transmembrane L-arginine transport in endothelial cells (Cendan *et al.*, 1995) and in J774 cells (Bogle *et al.*, 1992b). The finding in this study that J774.7 cells produced nitrite in an almost linear fashion for up to 48 hours suggests that L-arginine was not rate limiting. Thus, it is possible that increased L-arginine uptake by the y+ carrier system had allowed for adequate supply of substrate during the 48 hour incubation. Also, L-citrulline, the by-product of NO synthesis, is recycled to L-arginine by activated J774 cells (Baydoun *et al.*, 1994), thus providing a further mechanism by which adequate L-arginine is provided for continuous NO synthesis. Thus, these two systems, in addition to the levels of L-arginine present in the medium (400 μM) bathing the cells, appear to maintain a sufficient supply of L-arginine to maintain a steady production of NO by J774.7 cells over 48 hours.

7.6 Effects of drugs on the combined stimulus of LPS and IFN-γ

7.6.1 NO synthase inhibitors

The NO synthase inhibitor, L-NMMA, when present during the overnight incubation, produced a concentration-dependent inhibition of nitrite accumulation into the medium bathing J774.7 cells stimulated by LPS and IFN-γ, with a maximum inhibition of approx. 73 % at $3x10^{-4}$ M. Nitrite accumulation is therefore likely to have arisen from induction of the L-arginine/NO pathway. On occasion, L-NMMA (10^{-4} M) also produced an inhibition (approx. 52%) of basal nitrite production by the cells, suggestive of a slight endotoxin contamination of the bathing medium. However, at a lower concentration ($3x10^{-6}$ M), L-NMMA produced a paradoxical effect, i.e. it produced a 20% increase rather than a decrease in nitrite production. It has been shown that L-NMMA can, in addition to its ability to block NO synthase, function as an alternative substrate (Archer & Hampl, 1992; Olken & Marletta, 1993). Thus, where it produced an increase in nitrite accumulation, L-NMMA may have been acting as an alternative substrate to enhance nitrite accumulation, rather than producing its expected inhibitory effect. However, at higher concentrations, the overall action is a profound inhibition of NO production. In contrast to the effects of L-NMMA, L-NAME was much less effective at inhibiting nitrite accumulation stimulated by the combination of LPS and IFN-γ, with a maximum inhibition of around 25 %, compared to almost 75 % by L-NMMA. Similar observations have been made by McCall *et al.* (1991) using J774 cells. Why L-NAME is so ineffective at inhibiting nitrite accumulation stimulated by LPS and IFN-γ is not known. It is possible that J774.7 cells destroy or inactivate L-NAME in some way. In addition, LPS mediates an increased expression of the y^+ transporter in cells, including J774 cells, allowing for increased L-arginine transport. L-NMMA has been shown to inhibit the uptake of L-arginine into

endothelial cells by the y^+ transport system (Bogle *et al.*, 1992a), whereas L-NAME has little effect. This is because L-NMMA is taken up into J774 cells via the y^+ transporter system, whereas L-NAME enters J774 cells by a neutral transporter (Baydoun & Mann, 1994). These findings therefore provide a possible explanation of why L-NMMA is a more effective inhibitor of nitrite accumulation as it is more effectively taken up into J774 cells via the up-regulated y^+ transporter. Also, L-NMMA, acting via inhibition of L-arginine uptake, may produce a situation where L-arginine becomes rate limiting, thus reducing NO production by the cells due to lack of substrate. With regards to inhibition of LPS-stimulated nitrite production by RAW 264 cells, both L-NAME and L-NMMA were equally effective and therefore perhaps there is no selectivity in the uptake of the two inhibitors into this cell line.

7.6.2 Polymyxin B

Polymyxin B significantly reduced nitrite production by J774.7 cells stimulated by LPS and by the combination of LPS and IFN-γ, thus demonstrating its utility as an inhibitor of LPS. In addition, polymyxin B reduced the stimulatory effects of IFN-γ alone on nitrite production. Although the possibility has been discussed (see 7.3) that this action may have been due to loss of the synergistic action of IFN-γ with a slight contamination of the culture medium by endotoxin, another action may contribute. Specifically, it has been suggested that the effector pathway for IFN-γ to stimulate expression of NO synthase is through activation of PKC (Severn *et al.*, 1992). Polymyxin B at concentrations such as those used in our experiments (10 $\mu g\ ml^{-1}$), is known to have an additional action as a PKC inhibitor (Nel *et al.*, 1985). Inhibition of PKC might therefore explain a

component of the depression of IFN-γ-induced nitrite accumulation seen upon treatment of J774.7 cells with polymyxin B.

7.6.3 Dexamethasone

Expression of the inducible form of NO synthase is time-dependent, involves *de novo* synthesis of protein and can be inhibited by glucocorticoids such as dexamethasone (Di Rosa *et al.*, 1990; Knowles *et al.*, 1990; Assreuy & Moncada, 1992). The NO production by J774.7 cells, detected as nitrite in this study, is almost certain to have come from the inducible form of NO synthase, as demonstrated by the inhibitory effects of dexamethasone. Dexamethasone inhibits induction of NO synthase probably at the level of transcription of mRNA. Two receptors exist for glucocorticoids; the type I receptor where dexamethasone binds at low concentrations, and the type II receptor, which is subdivided into type IIα and IIβ, where dexamethasone will bind after saturation of the type I receptor (Wilckens, 1995). Type I and type II receptors interfere with transcription factors at a low affinity glucocorticoid response element with a binding site for a transcription factor, and can lead to blockade of transcription (Wilckens, 1995). Type I and type II receptors have been found to be functionally antagonistic (Wilckens, 1995) and thus may explain why a biphasic effect of dexamethasone was seen in this study, where inhibition was observed at concentrations of 10^{-7} and $3x10^{-7}$ M, whereas at higher concentrations, the inhibitory effect was lost. It is not, however, known at this time if J774.7 cells express both of these receptors for dexamethasone, or whether an alternative explanation is required to explain the biphasic effect of the drug. Glucocorticoids mediate some of their biological effects via formation of the 37 kD protein, lipocortin 1 (Flower & Rothwell, 1994). The findings of Wu *et al.* (1995) indicate that the suppressive effects of dexamethasone on induction of NO synthase by LPS

may be due to modification of transcription regulators, thus producing a modulation of gene expression. Furthermore, it has been demonstrated that the promoter region for mouse macrophage inducible NO synthase does not contain a glucocorticoid response element (Lowenstein *et al.*, 1993). Thus, it is likely that the inhibitory effects of dexamethasone in this system are mediated indirectly, perhaps by the induction of lipocortin 1 which may reduce expression of the NO synthase gene by reducing the effectiveness of transcription regulators. In this study, dexamethasone produced only a small inhibitory effect on nitrite production stimulated by LPS and IFN-γ, with maximum inhibition of around 15-20 %. Dexamethasone was, however, more successful at inhibiting nitrite accumulation induced by LPS alone, where the level of inhibition was around 45 %. This is in contrast to the findings of Di Rosa *et al.* (1990) and Schoedon *et al.* (1993), who reported the ability of dexamethasone to inhibit by up to 80 % NO and nitrite production stimulated by LPS and IFN-γ in J774 cells and murine peritoneal macrophages, respectively. However, our findings are similar to those of Baydoun *et al.* (1993), who found that dexamethasone completely inhibited nitrite production stimulated by LPS, but only slightly reduced that stimulated by the combination of LPS and IFN-γ in J774 cells. Bryant *et al.* (1995) demonstrated that the inhibitory effects of dexamethasone on inducible NO synthase stimulated by LPS in J774.2 cells are partially mediated by lipocortin, but this was not the case for IFN-γ. As lipocortin is the most likely mediator of dexamethasone's effects, this might explain why in this study, dexamethasone was more effective in inhibiting the effects of LPS alone than of LPS and IFN-γ in combination. Furthermore, since LPS and IFN-γ act synergistically at the level of transcription of NO synthase mRNA (Lorsbach *et al.*, 1993), and LPS can stabilise the increase in inducible NO synthase mRNA induced by exposure to IFN-γ (Weisz *et al.*, 1994), perhaps it is not surprising that the combined stimulus of LPS and

IFN-γ is not as susceptible to inhibition by dexamethasone as LPS used alone. In contrast to the majority of findings, Fantuzzi *et al.* (1995) reported that dexamethasone stimulated an increase in NO release from murine liver by inducing cytochrome P_{450}. Although J774.7 macrophages may not contain cytochrome P_{450}, the possibility cannot be ruled out that dexamethasone may induce some other enzyme system that could result in an increased production of NO. This could therefore act as an alternative explanation for the loss of the inhibitory effects of dexamethasone seen at higher concentrations.

A further possible mechanism to explain the ability of dexamethasone to reduce nitrite production by J774.7 cells is via a post-transcriptional effect. Kunz *et al.* (1994) reported that dexamethasone inhibited NO synthase protein expression without inhibiting mRNA levels. Thus, it is possible that dexamethasone may have reduced nitrite accumulation by interfering with mRNA translation, or by inducing degradation of NO synthase protein. However, this is as yet an unsupported finding, and the favoured view of inhibition is via the interference of transcription by dexamethasone. A further factor which should be considered is that LPS has been shown to decrease dexamethasone binding sites on peritoneal macrophages (Jiayi & Chen 1992). If this also occurs with the J774.7 cell line, it would certainly be expected to impair the suppressive effect of dexamethasone on NO synthase expression. In contrast, a study on RAW 264 cells reported that LPS led to an increase in receptors for glucocorticoids (Salkowski & Vogel, 1992), thus making difficult construction of a unified hypothesis on the effects of LPS on steroid binding sites.

Another glucocorticoid, hydrocortisone, had no effect on nitrite accumulation by the combination of LPS and IFN-γ in this study, suggesting that this

glucocorticoid was unable to affect transcription of inducible NO synthase. Inhibition expression of NO synthase by cycloheximide (Oguchi *et al.*, 1994) has also been reported, thus demonstrating the need for *de novo* protein synthesis. In this study, cycloheximide was effective at inhibiting nitrite production in response to LPS and IFN-γ in J774.7 cells. However, at all concentrations of the drug which reduced nitrite production, detachment of cells was observed, indicative of generalised toxicity.

7.7 Effects of cyclic nucleotides

7.7.1 Cyclic AMP

The second messenger, cyclic AMP, was first discovered in 1958. It is synthesised from ATP by adenylate cyclase, and is hydrolysed to 5'-AMP by a family of phosphodiesterase enzymes. Cyclic AMP has a variety of effects on cellular function including regulation of the contractile state of smooth muscle, energy metabolism and cell differentiation. Many of the effects of cyclic AMP are mediated through the activation of protein kinase A. The adenylate cyclase/cyclic AMP system can be regulated by a variety of mechanisms. The binding of messengers to certain plasma-membrane receptors causes the receptors to activate, via a G-protein, the enzyme adenylate cyclase, which is located on the inner surface of the membrane. For example, prostaglandins E_2 and I_2 stimulate adenylate cyclase thus raising cyclic AMP levels (Marotta *et al.*, 1992). Forskolin can activate the catalytic subunit of adenylate cyclase (Seamon & Daly, 1981), thus elevating intracellular cyclic AMP levels. A variety of analogues exist which mimic the effects of cyclic AMP, including the membrane permeant agent, dibutyryl cyclic AMP (Sowa & Przewlocki, 1994). There are five known families of phosphodiesterase enzyme, three of which hydrolyse cyclic AMP and the other two, cyclic GMP (Beavo & Reifsnyder, 1990). Drugs are available

which selectively inhibit specific phosphodiesterase enzymes. For example, of those that hydrolyse cyclic AMP, the type III cyclic GMP-inhibited family and the type IV cyclic AMP-specific family, are inhibited by the drugs SKF 94120 and rolipram, respectively (Beavo & Reifsnyder, 1990). Thus, inhibition of these enzymes would be expected to lead to elevated cyclic AMP levels within cells.

7.7.1.1 Effects of dibutyryl cyclic AMP

Elevation of cyclic AMP levels is known to suppress the induction of NO synthase at the level of transcription and/or translation in J774 cells (Marotta *et al.*, 1992; Bulut *et al.*, 1993). However, in our study, overnight pre-treatment with dibutyryl cyclic AMP was only able to produce a small (12%) degree of inhibition of nitrite production by J774.7 cells. A possible explanation for this poor reduction in nitrite accumulation is that dibutyryl cyclic AMP was only partially able to penetrate the membrane of J774.7 cells, or perhaps it was inactivated in some way within the cells.

It is known that inducible NO synthase has a consensus sequence for phosphorylation by protein kinase A (Lowenstein *et al.*, 1992). Therefore, agents which elevate cyclic AMP levels may have the ability to regulate the activity of NO synthase by activating PKA leading to possible phosphorylation of the enzyme. Thus, it is possible that elevation of cyclic AMP in J774.7 cells mediates two effects; firstly, an effect on the expression of NO synthase and secondly, an effect on NO synthase activity mediated by PKA. Measuring nitrite accumulation over 24 hours would not separate these events, since only the overall effect would be seen. Thus, it was necessary to examine the effects of the various cyclic AMP elevating agents when they were added after NO synthase had been induced. By examining the effects of

the drugs after the induction process was complete, it was hoped to determine if any of the agents affected enzyme activity.

An attempt was made to determine if dibutyryl cyclic AMP could affect activity of the already induced NO synthase. When added after NO synthase had been induced by LPS and IFN-γ, dibutyryl cyclic AMP (10^{-3} M) produced a 10 % inhibition of nitrite accumulation, which did not differ significantly from that obtained (approx. 12 %) when it was added before LPS and IFN-γ. These effects are therefore consistent with dibutyryl cyclic AMP mediating its inhibitory effect on nitrite accumulation via activation of PKA, inducing phosphorylation of NO synthase, thereby decreasing the activity of the enzyme.

7.7.1.2 Effects of forskolin

Although dibutyryl cyclic AMP produced little inhibitory effect on nitrite production by J774.7 cells, pre-treatment with forskolin, a drug which activates the catalytic subunit of adenylate cyclase (Seamon & Daly, 1981), inhibited nitrite accumulation stimulated by LPS and IFN-γ much more effectively (by 43 %) when used at high concentrations ($3x10^{-5}$-10^{-4} M). Thus, these findings are consistent with reports that elevated cyclic AMP levels suppress the induction of NO synthase in macrophages.

However, when used at relatively low concentrations, forskolin (10^{-7} & $3x10^{-7}$ M) induced a slight (maximum of 15 % at 10^{-7} M) increase in nitrite accumulation. In some cell types, cyclic AMP has been shown to potentiate NO production. This appears to be a fairly common phenomenon in vascular smooth muscle cells. For example, following elevation of cyclic AMP levels using forskolin and prostaglandins, IFN-γ-induced production of nitrite and

expression of NO synthase mRNA levels were enhanced (Koide *et al.*, 1993). Furthermore, isoprenaline, dibutyryl cyclic AMP, forskolin and the PDE inhibitor, Ro 20-1724, all enhanced IL-1β-induced nitrite production (Hirokawa *et al.*, 1994; Schini-Kerth *et al.*, 1994; Scott-Burden *et al.*, 1994). Potentiation of the inflammatory response has also been seen in rat peritoneal macrophages where cholera toxin and dibutyryl cyclic AMP each enhanced LPS-stimulated nitrite production, and each also led to production of nitrite when used in the absence of LPS (Sowa & Przewlocki, 1994). In fact, in vascular smooth muscle cells, forskolin induced an increase in nitrite accumulation in the absence of any other stimulus (Koide *et al.*, 1993). The exact mechanism by which elevated cyclic AMP levels lead to increased expression of NO synthase is unclear, although it appears that direct activation of gene transcription and *de novo* synthesis of NO synthase protein occur. It is possible that the gene for inducible NO synthase belongs to a cyclic AMP-inducible family (Koide *et al.* 1993) since many other genes have been shown to contain a cyclic AMP response element. However, there is no evidence for a cyclic AMP response element on the mouse inducible NO synthase gene (Xie *et al.*, 1993). Although forskolin appeared able to induce a slight rise in nitrite accumulation at low concentrations, these increases were small compared to the inhibition (43%) seen at higher concentrations, and thus it appears that forskolin produced an overall inhibitory action on nitrite production by J774.7 cells.

An attempt was made to determine if the inhibitory effect seen with forskolin (at 10^{-5} & $3x10^{-5}$ M) was due to suppression of the induction process or a decrease in enzyme activity. When added after NO synthase had been induced by LPS and IFN-γ, forskolin ($3x10^{-5}$ M) produced only a 4 % increase in nitrite accumulation, compared to a 24% inhibition when added before LPS and IFN-γ. The slight rise in nitrite accumulation that resulted

when forskolin was added after LPS and IFN-γ suggests that forskolin may, like dibutyryl cyclic AMP, have induced phosphorylation of NO synthase resulting in an increase in enzyme activity. However, the rise in nitrite accumulation was so small it was probably due to random biological variation. Thus, it appears that forskolin mediates mainly inhibitory effects through suppression of the induction process rather than of enzyme activity.

7.7.1.3 Effects of phosphodiesterase inhibitors

The presence of the phosphodiesterase (PDE) type IV isoenzyme, inhibited by rolipram, has been demonstrated in both human (Dent *et al.*, 1994) and guinea-pig (Dent *et al.*, 1991) eosinophils, guinea-pig peritoneal macrophages (Turner *et al.*, 1993; Turner & Wood, 1994) and human monocytes (Prabhakar *et al.*, 1994; Verghese *et al.*, 1995). There is, however, no evidence in the literature concerning the presence of the type III isoenzyme in any of these cell types.

Pretreatment with the type IV PDE inhibitor, rolipram, produced a slight increase in nitrite production by J774.7 cells stimulated with LPS and IFN-γ, with a maximum increase of 30 % at $3x10^{-7}$ M. This drug did not inhibit nitrite accumulation at any concentration used. The type III PDE inhibitor, SKF 94120 also produced a slight increase (37 %) in nitrite accumulation at $3x10^{-7}$ M, but produced a decrease (26 %) at 10^{-4} M.

An attempt was made to determine if the effects produced by rolipram and SKF 94120 were due to an effect on induction of NO synthase, or an effect on enzyme activity. When added after NO synthase had been induced by LPS and IFN-γ, rolipram ($3x10^{-6}$ M) had no effect on nitrite accumulation, whereas when added before LPS and IFN-γ, it produced a 12% increase in

nitrite accumulation. Thus, it appears that rolipram produced an increase in nitrite accumulation by enhancing the expression of NO synthase rather than by increasing the activity of the enzyme. When added after NO synthase had been induced by LPS and IFN-γ, SKF 94120 (10^{-6} M) had no effect on nitrite accumulation whereas when added before LPS and IFN-γ, it produced a 24% decrease in nitrite accumulation. Thus, it appears that SKF 94120 produced a decrease in nitrite accumulation by suppressing the induction process rather than by decreasing the activity of the enzyme.

Thus, the two PDE inhibitors appeared to have differing actions on nitrite production by J774.7 cells since common effects would have been expected if they each acted by elevating cyclic AMP levels in the cells. It is likely therefore that the effects of these drugs may have resulted from non-selective actions.

7.7.1.4 Combination of forskolin and PDE inhibitors

In combining forskolin with the two PDE inhibitors, it was expected that a greater elevation of intracellular cyclic AMP levels would occur than with either drug used on its own. Thus, it was anticipated that an additive effect would be seen on nitrite production by the cells. Combining a relatively high concentration of forskolin ($3x10^{-5}$ M) with relatively high concentrations of rolipram ($3x10^{-6}$ M) or SKF 94120 (10^{-6} M), led to no further inhibition. This may have been because the drugs were already exerting a maximum inhibitory effect when used alone. Combining submaximal concentrations of rolipram ($3x10^{-7}$ M) and forskolin ($3x10^{-6}$ M) did induce a slight degree of additivity, but the overall inhibition never exceeded around 30 %. Surprisingly, the combination of a submaximal concentration of SKF 94120 (10^{-7} M) with forskolin ($3x10^{-6}$ M) led to an abolition of all inhibitory effects.

A possible explanation for this antagonistic effect is that a submaximal concentration of SKF 94120 may stimulate transcription of the inducible NO synthase gene rather than suppress it. This would mask any suppressive effects of forskolin, and therefore result in no overall change in nitrite production. However, the reason why none of the agents, when used in combination, produced an increase in nitrite accumulation as when used alone, is unknown. A more complete explanation of the actions of PDE inhibitors on nitrite production in J774.7 cells must await more detailed analysis of levels of cyclic AMP, protein kinase A, inducible NO synthase mRNA and inducible NO synthase activity.

7.7.2 Cyclic GMP

Cyclic GMP is involved as a second messenger in a wide spectrum of events including renal ion transport, smooth muscle contractility and the retinal rod response to light (Schmidt *et al.*, 1993). The two systems known to generate cyclic GMP are particulate (membrane bound) guanylate cyclase and soluble (cytoplasmic) guanylate cyclase. Both enzymes convert GTP to cyclic GMP. Particulate guanylate cyclase is activated by ligands such as atrial natriuretic peptide and brain natriuretic peptide. These bind to cell membrane receptors which then activate intracellular guanylate cyclase via transmembrane domains (Schmidt *et al.*, 1993). Soluble guanylate cyclase is a cytoplasmic enzyme which has a ferrous haem moiety receptor for the binding of NO (Gerzer *et al.*, 1981). Furthermore, cyclic GMP can regulate cyclic AMP levels by influencing the activity of the type II cyclic GMP-stimulated PDE or the type III cyclic GMP-inhibited PDE. Cyclic GMP itself is hydrolysed by the type I calcium-calmodulin-dependent family and the type V cyclic GMP-specific family of PDEs, both of which are inhibited by zaprinast.

Although NO leads to elevation of cyclic GMP levels via stimulation of soluble guanylate cyclase, little evidence has been presented for a role of cyclic GMP in modulating the activity of NO synthase. This is despite a large number of examples where NO appears to exert a negative feedback action on NO production. For example in J774 cells, NO synthase activity induced by LPS and IFN-γ was inhibited by exposure to the NO donor, S-nitroso-acetyl-penicillamine (SNAP) (Assreuy *et al.*, 1993). Furthermore, in endothelial cells the activity of constitutive NO synthase was reduced by the addition of NO or the NO donor, SNAP (Buga *et al.*, 1993). In addition, in rat cortex the activity of neuronal NO synthase was inhibited by the NO donors, sodium nitroprusside, hydroxylamine and glyceryl trinitrate (GTN) (Vickroy & Malphurs, 1995). Moreover, in alveolar macrophages the activity of muramyldipeptide-induced NO synthase was reduced by the addition of the NO donors, SIN-1 and nitrosoglutathione (Morin *et al.*, 1994). Inhibition of NO synthase could potentially have been due to elevated cyclic GMP levels suppressing the induction of NO synthase. However, there is no evidence that a response element exists for cyclic GMP on the gene for inducible NO synthase. A further possible mechanism is that elevated cyclic GMP levels may have led to activation of a cyclic GMP-dependent protein kinase, found in human mononuclear phagocytes (Pryzwansky *et al.*, 1995). If a consensus sequence for PKG exists on NO synthase, then this protein kinase will phosphorylate the enzyme, potentially modulating its activity. The feedback effect of NO in each of the above cases, however, appeared to be independent of cyclic GMP (Assreuy *et al.*, 1993; Durieu-Trautmann *et al.*, 1993; Koide *et al.*, 1993; Hirokawa *et al.*, 1994), and is therefore unlikely to have occurred as a consequence of phosphorylation by protein kinases. Instead, a likely mechanism for the inhibitory feedback effect of NO on NO synthase is an irreversible toxic

effect on the enzyme resulting from the formation of haem-nitrosyl complexes (Hurshman & Marletta, 1995).

In our study, the membrane permeant analogue of cyclic GMP, 8-bromo-cyclic GMP, had little effect on nitrite production by J774.7 cells stimulated by LPS and IFN-γ over 24 hours, with only a small rise (8.8 ± 0.2 %), probably due to biological variation. Glyceryl trinitrate (GTN), a drug converted intracellularly to NO via reductive enzymes (Armstrong *et al.*, 1980; Feelisch, 1991; Schröder, 1992), produced a biphasic effect on nitrite accumulation stimulated by LPS and IFN-γ. Initially, at $3x10^{-7}$-10^{-6} M, a decrease in nitrite accumulation occurred (max. 19% at 10^{-6} M), followed by an increase (max. 20% at 10^{-5} M). The most likely mechanism by which GTN led to a reduction in nitrite accumulation is via inhibition of NO synthase by NO, as discussed above (Hurshmann & Marletta, 1995). Although no evidence exists for a suppressive role for cyclic GMP on NO production, it is possible that GTN can mediate some of its effects through elevation of cyclic GMP and via nitration of NO synthase. As the concentration of GTN increased, we would have expected to see a more pronounced inhibition of nitrite accumulation, but in fact an increase was seen. It is likely that, as the concentration of GTN increased, the NO generated from it would have decayed to nitrite, thus adding to that produced by J774.7 cells. This large generation of nitrite would thus have masked any inhibitory effects of NO on NO synthase. Overall, it is likely in J774.7 cells that GTN exerts an inhibitory effect on nitrite accumulation via suppression of NO synthase activity through the generation of a nitrosyl-haem complex with the enzyme.

Zaprinast, which inhibits type I and type V PDE enzymes (Beavo & Reifsnyder, 1990), was used to potentially elevate cyclic GMP levels in

J774.7 cells by inhibiting hydrolysis of this cyclic nucleotide. Zaprinast produced a maximum reduction of nitrite accumulation of 33% at $3x10^{-5}$ M. These results were thus consistent with an inhibitory effect of cyclic GMP . However, no evidence exists to support the presence either of a cyclic GMP-dependent response element on the NO synthase gene or of a consensus sequence on NO synthase for PKG. Moreover, it is not known if J774.7 cells contain either the type I or type V families of PDEs. There is thus the possibility that zaprinast exerted its inhibitory effect via a non-selective action. However, this seems unlikely, as the degree of inhibition was so reproducible. No measurements of levels of cyclic GMP, inducible NO synthase mRNA or inducible NO synthase activity were made, so a firm conclusion cannot be made as to the mechanism by which zaprinast suppresses nitrite accumulation by J774.7 cells.

Combining zaprinast and GTN, it was hoped to raise maximally cyclic GMP levels and so enhance any action occurring via this pathway. Surprisingly, however, no greater degree of inhibition was produced when the drugs were used in combination, suggesting that GTN did not produce its inhibitory action via this cyclic nucleotide. As explained above, it is possible that the true extent of inhibition by the drugs may not be apparent, as the NO generated from GTN would decay to nitrite, and thus mask any inhibitory action taking place on NO synthase.

Thus, although the cyclic nucleotide, cyclic AMP, has the ability to affect both the induction and activity of NO synthase, there is less evidence to support a role for cyclic GMP in the regulation of nitrite production by J774.7 cells stimulated with LPS and IFN-γ.

7.8 Involvement of protein kinase C

The protein kinase C (PKC) family plays a key role in many cellular functions including cell proliferation, gene expression and signal transduction (Nishizuka, 1988). The gene structure of several isozymes have been established so far, namely α, βI, βII, γ, δ, ε, η, θ, μ, ι/λ and ζ isoforms (Fujihara *et al.*, 1994). Four of the isozymes, α, βI, βII and γ, require calcium in the presence of phosphatidylserine and DAG for activation and are classified as conventional protein kinase Cs. The novel isotypes, δ, ε, ζ and θ are calcium-independent and DAG-activated and the atypical isoforms ι/λ are not DAG-activated (Parker *et al.*, 1995). The newly discovered PKC μ has a long N-terminal region which has a potential transmembrane domain, a feature not found in other PKC isoforms (Gómez *et al.*, 1995). Tumour promoting phorbol esters are often used to mimic the activation of PKC normally produced by DAG. Elevated calcium levels which contribute to the activation of certain isoforms of PKC, also lead to activation of another protein kinase, calcium/calmodulin-dependent protein kinase.

7.8.1 Effects of PMA and A23187 on induction of NO synthase

Activation of protein kinase C is one of the proposed pathways involved in the induction of NO synthase in macrophages (Severn *et al.*, 1992; Paul *et al.*, 1995). In an attempt to mimic the stimulation of PKC in the induction of NO synthase, the calcium ionophore A23187 and the phorbol ester, phorbol 12-myristate 13-acetate (PMA), a widely used activator of protein kinase C (Rodriguez-Pena & Rozengurt, 1984; Hortelano *et al.*, 1993), were used. However, neither treatment with PMA nor elevation of intracellular calcium with A23187, or even the combination of the two led to an increase in nitrite production by J774.7 cells over a 24 hour period. Thus, activation of PKC or

of calcium/calmodulin dependent kinase either alone or in combination did not seem sufficient for induction of NO synthase in these cells. The inability of A23187 to induce any rise in nitrite accumulation also suggests the absence of any constitutive NO synthase activity in J774.7 cells, as this would have been expected to be stimulated by the calcium/calmodulin complex (Nathan & Xie, 1994a). In the development of tumoricidal activity, IFN-γ primes macrophages and subsequent exposure to LPS leads to full expression (Celada & Schreiber, 1986; Somers *et al.*, 1986). In this situation, IFN-γ can be substituted by the combination of a phorbol ester and ionophore A23187, demonstrating the need for activation of protein kinase C and possibly also calcium/calmodulin-dependent protein kinase. By analogy, experiments were conducted in this study to examine if the stimulation of PKC using PMA, and raising calcium levels using A23187, could substitute for IFN-γ in the induction of NO synthase in the J774.7 macrophage cell line. However, when each of these two stimuli was combined with LPS, the rise in nitrite accumulation obtained was similar to the small increase induced by LPS alone. Furthermore, the combination of PMA, A23187 and LPS also produced an increase in nitrite accumulation that was no greater than with LPS alone and did not mimic the large rise in nitrite accumulation induced by the combination of LPS and IFN-γ. Thus, it seems that the factors regulating induction of NO synthase differ from those for tumoricidal activity (Celada & Schreiber, 1986; Somers *et al.*, 1986) in that activation of PKC and elevation of intracellular calcium cannot substitute for IFN-γ in induction of the former. In addition, a wide range of concentrations of PMA were unable to induce an increase in nitrite accumulation when used alone, findings in agreement with Jun *et al.* (1994a), confirming that activation of PKC alone is not sufficient to induce NO synthase.

7.8.2 Effects of PKC on induction of NO synthase

7.8.2.1 Effects of phorbol ester

Phorbol esters have complex effects on the activity of PKC; they have the ability to activate and then down-regulate the enzyme (Rodriguez-Pena & Rozengurt, 1984; Dieter & Fitzke, 1991), although the different subspecies of PKC are not equally affected (Parker *et al.*, 1995). Although phorbol esters can both stimulate and down-regulate PKC, more than only a very short period of exposure results in down-regulation (Dieter & Fitzke, 1991). The ability of phorbol esters to inhibit induction of NO synthase has previously been reported in J774 cells, where 4 hours' (Fujihara *et al.*, 1994) or 48 hours' (Severn *et al.*, 1992) exposure to PMA reduced nitrite production by the cells. In our work, since J774.7 cells were exposed to PMA for 24 hours, it is likely that this length of time would have resulted in down-regulation of PKC. In these experiments, pre-treatment with PMA inhibited by about 30 % nitrite accumulation stimulated by the combination of LPS and IFN-γ . It is possible therefore that this inhibition of nitrite accumulation arose as a consequence of down-regulation of PKC by PMA. Thus, this finding provides evidence for the involvement of PKC in the induction process of NO synthase in J774.7 cells stimulated by LPS and IFN-γ.

PKC has been shown to decrease neuronal NO synthase by phosphorylation (Nakane *et al.*, 1991). It is possible therefore that a consensus sequence may exist on inducible NO synthase for phosphorylation by PKC. However, there is as yet no evidence to suggest that such a consensus sequence exists. Nevertheless, the effects of PMA on nitrite accumulation were investigated after NO synthase had been induced by LPS and IFN-γ to determine if post-translation modification of the enzyme was possible.

When added 12 hours after NO synthase had been induced by LPS and IFN-γ, PMA (10^{-7} M) produced a 59 % increase in nitrite accumulation, compared to a maximal 27 % inhibition when PMA was added before the activating stimuli. This enhancement is likely to have resulted from a post-translational action of PKC on NO synthase activity. The nature of this post-translational action, whether due to stimulation of PKC activity or to its down-regulation, is unknown. This increase in nitrite accumulation would confirm that the inhibition produced upon pre-treatment with PMA was due to suppression of induction rather than to inhibition of NO synthase activity.

7.8.2.2 Effects of PKC inhibitors

7.8.2.2.1 Effects of PKC inhibitors on expression of NO synthase

PKC can be inhibited by a number of agents including staurosporine (Tamaoki *et al.*, 1986; Dieter & Fitzke, 1991), Ro 31-8220 (Keller & Niggli, 1993; Walker & Watson, 1993) and also by the highly selective inhibitor, chelerythrine chloride (Herbert *et al.*, 1990; Barg *et al.*, 1992). Further investigation of the involvement of PKC was therefore carried out using a variety of inhibitors of this protein kinase. Pre-treatment with either staurosporine or Ro 31-8220 produced a concentration-dependent inhibition of nitrite accumulation stimulated by LPS and IFN-γ, in agreement with the findings of Paul *et al.* (1995), Severn *et al.* (1992) and Jun *et al.* (1994a). The maximum inhibitory effect produced by staurosporine (at non-toxic concentrations) was 58% at 10^{-7} M, and by Ro 31-8220 was 73 % at 10^{-5} M. It must be pointed out, however, that staurosporine is not entirely selective for PKC since it has been shown to inhibit other protein kinases including protein kinase A, tyrosine kinase, protein kinase G and myosin light chain kinase (Tamaoki *et al.*, 1986; Persaud & Jones, 1994). Ro 31-8220,

however, is regarded as being more selective than staurosporine (Keller & Niggli, 1993).

Surprisingly, pre-treatment with chelerythrine chloride, a highly selective inhibitor of PKC, produced a maximum inhibition of only 20 % at the highest concentration used ($3x10^{-5}$ M), where the drug was in fact toxic to the cells and induced detachment. This poor inhibitory effect thus casts doubt on the role of PKC in the induction of NO synthase. It has been shown that J774.7 cells possess a variety of isoforms of PKC (Fujihara *et al.*, 1994). Although the βII and ε isoforms are present and are suspected to play a role in the induction process (Fujihara et al., 1994), it is possible that chelerythrine is unable to inhibit the appropriate isoform of PKC. Staurosporine, on the other hand, is fairly effective at inhibiting all the isoforms of PKC with the exception of the ζ subtype (Hoehn *et al.*, 1995). However, at some concentrations, chelerythrine chloride produced an increase in nitrite accumulation. In addition to the proposal that stimulation of PKC is involved in the induction of NO synthase, there is evidence to suggest that activation of PKC may suppress NO synthase expression. This hypothesis has previously been presented by Mühl & Pfeilschifter (1994) who demonstrated that short-term activation of PKC by a phorbol ester for less than one hour in rat mesangial cells reduced cytokine-induced nitrite production, and down-regulation of PKC resulted in super-induction of inducible NO synthase mRNA. A similar effect was also observed in rat aortic smooth muscle cells (Geng *et al.*, 1994), where PMA antagonised cytokine-induced nitrite production, and this antagonism was blocked by the PKC inhibitor, calphostin C, demonstrating that the inhibitory effect of PMA was mediated via PKC activation. Additionally, Hortelano *et al.* (1992; 1993) demonstrated a clear antagonism between NO production induced by LPS or by PMA in rat hepatocytes and peritoneal macrophages. The findings of Hortelano *et al.*

could be explained by the phorbol ester activating PKC, and thus inhibiting the ability of LPS to induce NO synthase. In our study, both chelerythrine chloride ($3x10^{-8}$, 10^{-6} & 10^{-5} M) and Ro 31-8220 (10^{-8} & $3x10^{-7}$ M) produced a slight enhancement of nitrite accumulation by J774.7 cells, with a maximum increase of 23% at $3x10^{-5}$ M with Ro 31-8220, and 23 % at 10^{-6} M with chelerythrine chloride. With regards to the reduction in nitrite accumulation produced by PMA, it is possible, but unlikely, that this resulted from stimulation of PKC, rather than down-regulation. If PKC activation did have an inhibitory effect on NO synthase induction, we would have expected that use of PKC inhibitors would universally potentiate NO production. In fact, the majority of findings (Fujihara *et al.*, 1994; Paul *et al.*, 1995) show that inhibition of PKC in fact blocks the expression of NO synthase.

7.8.2.2.2 Effect of PKC inhibitors on activity of NO synthase

An attempt was made to determine if staurosporine had any effect on the activity of the already induced NO synthase. When added 12 hours after NO synthase had been induced by LPS and IFN-γ, staurosporine (10^{-7} M) produced only a slight degree of inhibition (13 %) of nitrite accumulation compared to that (58%) when it was added before LPS and IFN-γ. Thus, it appears that staurosporine exerts its inhibitory action by suppressing induction of NO synthase rather than directly inhibiting NO synthase activity. An attempt was also made to determine if Ro 31-8220 had any effect on the activity of the already induced NO synthase. When added 12 hours after NO synthase had been induced by LPS and IFN-γ, Ro 31-8220 (10^{-5} M) produced only a slight degree of inhibition (12 %) of nitrite accumulation compared to that (71%) when it was added before LPS and IFN-γ. Thus, it appears that Ro 31-8220 also exerts its inhibitory effect on nitrite accumulation by suppressing induction of NO synthase rather than directly

inhibiting NO synthase activity. Thus, these effects are consistent with staurosporine and Ro 31-8220 exerting inhibitory effects via induction of NO synthase in J774.7 cells. When chelerythrine chloride (10^{-5} M) was added to the cells 12 hours after LPS and IFN-γ, it produced a slight increase in nitrite accumulation (17.2 %) which was no different from that which occurred upon addition of the drug prior to LPS and IFN-γ. Thus, it appears that chelerythrine chloride may produce a slight increase in nitrite accumulation via a direct stimulatory effect on the activity of NO synthase.

7.8.2.3 Combination of PMA and PKC inhibitors

Although, as was discussed above, it was felt that the inhibitory effect of PMA on nitrite accumulation resulted from down-regulation of PKC, additional experiments were conducted in which the effects of this agent were examined in combination with the inhibitors of PKC, staurosporine and Ro 31-8220. The rationale for these experiments was that if stimulation of PKC was the mechanism by which nitrite accumulation was reduced, the effects of PMA would be blocked by PKC inhibitors which act via inhibition of the catalytic site (Budworth & Gescher, 1995). Alternatively, if down-regulation accounted for the actions of PMA, then PKC inhibitors could possibly mimic or even potentiate the action of PMA.

The combination of staurosporine (10^{-8} M) with PMA (10^{-8} M) produced an additive inhibitory effect (62%) on nitrite accumulation compared to no inhibition by staurosporine and 35 % inhibition by PMA when used alone. Thus, this would suggest that the mechanism by which PMA induced inhibition of nitrite accumulation is via down-regulation of PKC activity. The combination of PMA ($3x10^{-9}$ M) with Ro 31-8220 (10^{-6} M) produced no additive inhibitory effects. In fact, it was found that relatively high

concentrations of Ro 31-8220 (10^{-6} & $3x10^{-6}$ M) blocked the inhibitory effects of PMA. If the PMA concentration was increased (10^{-8} M and above), however, the suppressive effect of Ro 31-8220 was lost. This antagonistic effect was not seen when PMA was combined with staurosporine. As the degree of inhibition induced by PMA was less than that in the presence of Ro 31-8220, modification of PKC at the catalytic subunit by Ro-31-8220 may render PKC less susceptible to down regulation by PMA. PKC is cleaved by a neutral protease, calpain, a process thought to be responsible for down-regulation. However, phorbol esters are thought to increase the susceptibility to proteolysis through exposure of a linker region between the regulatory and catalytic domains (Kishimoto *et al.*, 1989). Therefore modification of the catalytic site by Ro 31-8220 may not directly interfere with the proteolytic process. Alternatively, this decrease by Ro 31-8220 in the ability of PMA to inhibit nitrite accumulation may indicate that PMA exerts a component of its inhibitory effects via stimulation of PKC. As levels of PKC activity were not measured it is thus difficult to explain precisely the mechanisms by which each of these agents produced their effects on nitrite accumulation. Overall, it appears that PMA produced a reduction in nitrite accumulation by inducing down-regulation of PKC. The majority of findings in this work suggest that PKC is involved in the induction of NO synthase in J774.7 cells by LPS and IFN-γ.

7.9 Involvement of tyrosine kinase

Tyrosine kinases play a central role in signal transduction pathways involving regulation of cell proliferation and differentiation. The kinases can be divided into two main groups based on their structures. One group, which possesses extracellular domains, is the receptor protein tyrosine kinases such as the receptors for platelet-derived growth factor (PDGF), epidermal growth

factor (EGF), nerve growth factor (NGF) and insulin (Ullrich & Schlessinger, 1990). The second major group is the non-receptor protein kinases which lack extracellular domains (Bolen *et al.*, 1992). There are a variety of agents available which selectively inhibit tyrosine kinase, including herbimycin A (Fukazawa *et al.*, 1991; Satoh *et al.*, 1992), erbstatin (Akarasereenont *et al.*, 1994) and genistein (Akiyama *et al.*, 1987). Alternatively, levels of tyrosine kinase activity can be increased by inhibitors of phosphotyrosyl-protein phosphatase, such as the drug sodium orthovanadate (Swarup *et al.*, 1992).

Tyrosine kinase is another second messenger candidate proposed to be involved in induction of NO synthase in J774 cells (Akarasereenont *et al.*, 1994), where stimulation of the cells with LPS led to generation of nitrite, a process blocked by two inhibitors of tyrosine kinase, genistein and erbstatin . This protein kinase has also been suggested to be involved in the induction of NO synthase stimulated by the combination of LPS and IFN-γ in a variety of other cell types including C3h/HeN cells (Dong *et al.*, 1993c), astrocytes (Feinstein *et al.*, 1994), and RAW 264 macrophages (Paul *et al.*, 1995), In these studies, the activity of NO synthase was reduced by various inhibitors of tyrosine kinase, including genistein, herbimycin A and tyrphostin. Also, in rat aortic smooth muscle cells, induction of NO synthase by LPS or IL-1β was blocked by the tyrosine kinase inhibitors, genistein and geldanamycin (Marczin *et al.*, 1993).

7.9.1 Effects of herbimycin A on nitrite production

In our work, it was found that pre-treatment with herbimycin A, a selective and irreversible inhibitor of tyrosine kinase (Fukazawa *et al.*, 1991; Satoh *et al.*, 1992; Dong *et al.*, 1993b), produced an almost complete inhibition of nitrite accumulation stimulated by LPS and IFN-γ in J774.7 macrophages.

This finding suggested an involvement of tyrosine kinase in the induction of NO synthase in J774.7 cells. In contrast to every other concentration used, 0.01 μg ml^{-1} herbimycin A induced an increase (17%) rather than a decrease in nitrite accumulation (max. 96% at 1 μg ml^{-1}) suggesting the possibility of a dual action. There have, however, been no other reports of tyrosine kinase inhibitors increasing NO production, and it can only be assumed that at this concentration, herbimycin A was affecting other processes in the cell which culminate in either an increase in expression of NO synthase or an increase in its activity.

An attempt was made to determine if herbimycin A could affect activity of the already induced NO synthase. When added 12 hours after NO synthase had been induced, herbimycin A (0.3 μg ml^{-1}) produced only a 24 % inhibition of nitrite accumulation, which was significantly smaller than that (63%) obtained when it was added before LPS and IFN-γ. Thus, it would appear that herbimycin A mediated most of its inhibitory effect on nitrite accumulation via inhibition of the induction of NO synthase by inhibiting tyrosine kinase. A lesser effect by directly suppressing NO synthase activity also appears to be taking place.

7.9.2 Effects of genistein on nitrite production

Pre-treatment with genistein, which competitively inhibits ATP binding to tyrosine kinase (Akiyama *et al.*, 1987), prior to stimulation with LPS and IFN-γ, also produced powerful concentration-dependent inhibition (max. 51% at $3x10^{-5}$ M). It was, however, around 100-fold less potent than herbimycin A. Thus, the inhibitory effect of genistein supports the view that tyrosine kinase is involved in the induction of NO synthase in J774.7 cells stimulated by LPS and IFN-γ.

As with herbimycin A, an attempt was also made to determine if genistein could affect activity of the already induced NO synthase. When added 12 hours after NO synthase had been induced, genistein ($3x10^{-5}$ M) had no effect on nitrite accumulation, compared to the 51% inhibition obtained when it was added before LPS and IFN-γ. Thus, it would appear that genistein mediated all of its inhibitory effect on nitrite accumulation via inhibition of induction of NO synthase, rather than via a direct suppressive effect of NO synthase activity.

7.9.3 Effects of sodium orthovanadate on nitrite production

Pretreatment of J774.7 cells with sodium orthovanadate, an inhibitor of tyrosine phosphatase activity (Swarup *et al.*, 1982), had little effect on nitrite accumulation stimulated by LPS and IFN-γ. This might suggest that inhibition of tyrosine phosphatase is not important in the induction process. However, it is more likely that sodium orthovanadate either is unable to permeate the cell membrane, or perhaps an isoform of tyrosine phosphatase exists in these cells which is resistant to inhibition by this drug.

Thus, from the findings of this section, it appears that tyrosine kinase has an important role in the induction of NO synthase in J774.7 cells stimulated with LPS and IFN-γ.

7.10 Involvement of both tyrosine kinase and protein kinase C

Due to the synergistic stimulation of the cells by LPS and IFN-γ, we suspected that at least two pathways, perhaps tyrosine kinase and PKC, must be working in concert to induce NO synthase. If so, combining inhibitors of

tyrosine kinase with inhibitors of PKC might be expected to result in enhanced inhibition. In this study, when concentrations of herbimycin A and staurosporine that were effective on their own were combined, additive inhibition of nitrite production by J774.7 cells was obtained, consistent with this hypothesis. However, this may have resulted from a non-selective action of staurosporine (Tamaoki *et al.*, 1986), since the more selective inhibitors of PKC, Ro 31-8220 and chelerythrine chloride failed to augment the inhibitory action of herbimycin A. The lack of additivity when inhibiting both tyrosine kinase and PKC makes it unlikely that the two pathways are acting in parallel. Activation of tyrosine kinase may sequentially produce activation of PKC somewhere down the line in the pathway leading to activation of the inducible NO synthase gene. For example, activation of tyrosine kinase can lead to activation of phospholipase C and generation of DAG and thus activation of PKC (Ullrich & Schlessinger, 1990). This may explain why no further inhibition was produced when blocking PKC, as inhibition of tyrosine kinase would automatically render PKC inactive, if tyrosine kinase was required for activation of PKC. Thus, more in-depth analysis involving examination of kinase activities would be necessary to explain fully the complex interplay between these two second messenger pathways in the regulation of NO synthase expression.

7.11 Effects of J774.7 macrophages on rat aortic rings

7.11.1 Production of NO or a NO-containing complex

Although it is generally excepted that EDRF is NO, it has been suggested that a dinitrosyl-iron-cysteine complex (Vanin, 1991), S-nitrosocysteine (Myers *et al.*, 1990; Rubanyi *et al.*, 1991), nitroxyl ion or hydroxylamine (Feelisch *et al.*, 1994) may also account for its action. For example, in cultured bovine aortic endothelial cells, it was suggested that EDRF was

more likely to be the nitrosothiol, S-nitrosocysteine than NO (Myers *et al.*, 1990) as the amount of NO released was insufficient to account for the vasorelaxant activities of EDRF. Also, it was proposed that the EDRF released from canine femoral arteries was more likely to be S-nitrosocysteine than NO (Rubanyi *et al.*, 1991), as free NO could not account for the bioactivity of the relaxant agent, whereas S-nitrosocysteine apparently could. The identity of the bovine retractor penis inhibitory factor has also been questioned, with the proposal that it is in fact an S-nitrosothiol rather than NO (Kerr *et al.*, 1992), and it has been shown that S-nitrosothiols are potent relaxants of the mouse anococcygeus (Gibson *et al.*, 1992). Furthermore, it has been proposed that S-nitrosothiols mediate the relaxation of smooth muscle by certain nitrovasodilators (Ignarro *et al.*, 1981).

Although macrophages are known to release large amount of NO to induce cytostatic or cytotoxic effects on invading organisms, there appear to be very few reports of damage to the macrophage itself. It is possible therefore that macrophages have evolved a means of protecting themselves against the high concentrations of NO they produce. One potential means of protection could be by binding NO in the form of a complex, thus inhibiting its toxic actions to the host cell. In endothelial cells, it has been shown that NO generated by constitutive NO synthase is incorporated into dinitrosyl iron complexes (Mülsch *et al.*, 1993). Also, in macrophages, it has been shown that NO produced following stimulation with LPS reacts with proteins carrying iron-sulphur clusters to form dinitrosyl ferrous iron complexes, which are released from the cells and can yield NO (Vanin *et al.*, 1993). Thus, it is possible that NO released from our J774.7 macrophages may not be in a free form, but may be released as a complex. In order to investigate this possibility, the effects of J774.7 cells were investigated on the tone of rat aortic rings. Also, these experiments provided additional information to those in which the

Griess reaction was used as a means of assessing NO production by J774.7 cells.

7.11.2 Relaxation of aortic rings by activated and unactivated J774.7 cells

J774.7 macrophages were stimulated with LPS and IFN-γ for around 18 hours to induce NO synthase within the cells. The cells were then added to rings of rat aorta which had been denuded of endothelium, thus preventing constitutive NO from exerting any effect. Addition of activated J774.7 cells produced a powerful, rapidly-developing, cell number-dependent relaxation of the pre-contracted aortic rings. This powerful relaxation is consistent with the production of large amounts of NO by the cells. Unactivated cells were much less effective in inducing relaxation, as we would expect, since such cells release only small amounts of nitrite over 18 hours. The relaxation produced by these unstimulated cells was also very slow in developing. Perhaps the small amount of nitrite accumulating was enough to induce a slight relaxation of the rings, as nitrite itself is a weak relaxant.

7.11.2.1 Effects of superoxide dismutase

NO in an unprotected state is sensitive to attack by superoxide anions (Rubanyi & Vanhoutte, 1986; Rosen *et al.*, 1995), leading to loss of its vasodilator actions and the simultaneous production of the powerful oxidant, peroxynitrite. (Beckman *et al.*, 1990; Denicola *et al.*, 1993; Carreras *et al.*, 1994; Szabó & Salzman, 1995). Thus, in the case of NO regulating blood flow and blood pressure, production of superoxide anion would disrupt its normal homeostatic role. It has been shown that superoxide dismutase (SOD) can prolong the actions of NO produced in both isolated blood vessels

(Rubanyi & Vanhoutte, 1986) and cultured vascular endothelial cells (Gryglewski *et al.*, 1986b). Furthermore, it has been proposed that superoxide dismutase may protect vascular tissue by preventing the formation of peroxynitrite (Beckman *et al.*, 1990). However, although SOD has been shown to potentiate the effects of endothelial-derived NO, its effects on the large amounts of NO generated by activated macrophages may be of less significance. Fricker *et al.* (1995) demonstrated that addition of SOD to RAW 264 macrophages stimulated with LPS and IFN-γ had no effect on the extent of tumour cell killing by the cells, or on the level of nitrite produced. Thus, it appears that superoxide anion is not an important factor in the immunological effects of NO, whereas it is a major factor to consider when only small amounts of NO are released by the endothelium.

On the basis of the above, SOD was used as a tool to determine if the smooth muscle relaxant released from activated J774.7 cells had properties similar to NO. The presence of SOD, which dismutates superoxide to hydrogen peroxide, resulted in a significantly greater relaxation of the aortic rings upon addition of activated J774.7 cells. This suggests that the relaxant released from J774.7 cells, like NO, is sensitive to attack by superoxide anion. These superoxide anions may have been generated by the tissue or by the high oxygen tension in the tissue bath.

7.11.2.2 Effects of LY 83583

An alternative way to examine the susceptibility of the macrophage-derived relaxant to superoxide anion is to examine the inhibitory effects of drugs known to generate the free radical. The compound, LY 83583, is a superoxide anion generator, and it has been shown to inhibit the effects of NO released from rabbit aortic strips (Mülsch et al., 1988) and rabbit aortic

rings (Diamond, 1987). We therefore investigated the effects of LY 83583 on macrophage-mediated relaxation. When the precontracted aortic rings had been fully relaxed by addition of the activated J774.7 cells, LY 83583 induced an almost complete reversal of relaxation. This reversal confirmed that the relaxation induced by the activated cells was due to an NO-like vasodilator.

7.11.2.3 Effects of haemoglobin

Haemoglobin has the ability to bind NO, and will therefore inhibit its actions (Martin *et al.*, 1985). In the presence of haemoglobin in the tissue bath, the relaxation produced by the addition of activated macrophages to aortic rings was almost completely blocked. However, this inhibitory effect of haemoglobin does not tell us if the NO released form macrophages is in an unprotected form since haemoglobin can also inhibit the actions of NO donors such as nitrovasodilators and S-nitrosothiols (Martin *et al.*, 1985; Liu *et al.*, 1994b). Haemoglobin may have had an additional action which was not studied in our experiments. Specifically, NO has the ability to feed back and inhibit NO synthase (Morin *et al.*, 1994; Vickroy & Malphurs, 1995). One way of preventing this inhibition would be by the addition of haemoglobin, which will bind NO, and therefore prevent it from exerting its negative feedback effects. Such an action explains why addition of haemoglobin increases the rate of formation of NO from L-arginine by NO synthase (Hurshman & Marletta, 1995). Thus, addition of haemoglobin may exert two effects in this system: firstly, the rate of production of NO might increase due to suppression of any negative feedback effect, and secondly, the scavenging of NO by haemoglobin would prevent it from relaxing the smooth muscle cells. Thus, although the enzyme may be more efficient at generating NO in the presence of haemoglobin, any enhanced relaxation of

smooth muscle would not be observed due to the scavenging abilities of this protein.

7.11.2.4 Effects of inhibitors of NO synthase

Many analogues of L-arginine act as competitive inhibitors of NO synthase. One of the most widely used inhibitors is N^G-monomethyl-L-arginine (L-NMMA)(Hibbs *et al.*, 1987a), which inhibits both constitutive and inducible isoforms of NO synthase. L-arginine analogues exhibit some degree of isoform selectivity. For example, N^G-amino-L-arginine and N^G-nitro-L-arginine are about 100 times more potent that L-NMMA at inhibiting constitutive NO synthase in endothelial cells (Gross *et al.*, 1990, 1991). Furthermore, McCall *et al.* (1991) found that in J774 macrophages, N-iminoethyl-L-ornithine was a more potent inhibitor of NO synthase than L-NMMA, and that L-NAME was much less effective at inhibiting inhibition of platelet aggregation induced by the cells. In our work on measuring nitrite production, we had already demonstrated that L-NMMA is a much more effective inhibitor of inducible NO synthase in J774.7 cells than L-NAME. Thus, the effects of the two NO synthase inhibitors, L-NAME and L-NMMA were investigated on J774.7 cell-mediated relaxation of rat aortic rings. We found that L-NMMA almost completely reversed the macrophage-mediated relaxation whereas L-NAME had very little effect. Thus, due to the inhibitory effect of L-NMMA, it appears that NO is the agent responsible for mediating relaxation of rat aortic rings upon the addition of J774.7 cells. Furthermore, the greater effectiveness of L-NMMA over L-NAME found in these experiments is consistent with the findings obtained when measuring nitrite production by J774.7 cells.

Thus, J774.7 macrophages release a smooth muscle relaxing factor, formed by the L-arginine/NO pathway which is sensitive to attack by superoxide anion and to inhibition by haemoglobin. These properties are consistent with the relaxant being free NO, and not an NO releasing compound such as an S-nitrosothiol or a dinitrosyl ferrous iron complex. Whether J774.7 macrophages have an intracellular system to protect themselves against attack by the high concentrations of NO they produce remains to be determined.

7.12 Release of reactive oxygen species

Different cells of the immune system, including macrophages, Kupffer cells, and neutrophils, produce and release a number of reactive oxygen species, in addition to NO, as part of their anti-microbial effector system. These include superoxide anion ($\bullet O_2^-$), hydrogen peroxide (H_2O_2) and hypohalous acids (HOX), for example hypochlorous acid (HOCl). Production of superoxide and hydrogen peroxide can lead to the additional formation of singlet oxygen ($^1O_2$), hydroxyl radical ($\bullet OH^-$) and peroxynitrite ($ONOO^-$). Superoxide is generated by NADPH oxidase which transfers electrons from NADPH in the cytosol to oxygen to form superoxide and subsequently hydrogen peroxide and other oxidants in the phagocytic vacuole. This NADPH oxidase is found for example in monocytes (Landmann *et al.*, 1995) and macrophages (Assreuy *et al.*, 1994) and can be stimulated by phorbol esters and calcium ionophores (Robinson *et al.*, 1984). Most of the toxic effects of superoxide are mediated via generation of hydroxyl radicals and hydrogen peroxide. Hydrogen peroxide is formed by the dismutation of superoxide anion and can oxidise cellular enzymes and mediate DNA damage and mutagenesis. Peroxynitrite, formed by the combination of NO and superoxide anion can catalyse membrane lipid peroxidation in an iron-independent manner (Radi *et*

al., 1991b), oxidise DNA bases (Beckman *et al.*, 1990) and react with metals or metalloproteins to form the toxic nitronium (NO_2^+) ion (Ischiropoulos *et al.*, 1992a), making it one of the more damaging species. Hydroxyl radical is also a damaging macrophage-derived oxidant (Miller & Britigan, 1995a), as it is highly reactive, and can induce the initiation of lipid peroxidation (Farber, 1994). Hypochlorous acid, formed by the enzyme myeloperoxidase in mononuclear cells, has been shown to disrupt cell membrane integrity via lipid peroxidation and decarboxylation of membrane proteins (Albrich *et al.*, 1986) and may also inhibit DNA synthesis (Rosen *et al.*, 1990).

Measurement of production of oxygen-derived reactive species would therefore give an insight into the activity of J774.7 cells with respect to their ability to kill invading organisms.

7.12.1 Release of superoxide anions

Superoxide anions are produced by a variety of leucocytes via the NADPH oxidase system during the respiratory burst where large quantities of glucose are metabolised by way of the hexose monophosphate shunt (Segal, 1995). Assays using cytochrome C allow for the presence of superoxide anion, a reducing agent, to be assessed due to the increase in absorbance at 550 nm that takes place upon reduction. Phorbol myristate acetate (PMA) is a phorbol ester which activates protein kinase C, and is a well characterised stimulus for inducing the production of superoxide anions by cells (Wolfson *et al.*, 1993; Conde *et al.*, 1995). Both LPS and PMA are very effective at inducing superoxide production by cell types known to exhibit the respiratory burst, e.g. neutrophils (Maridonneau-Parini *et al.*, 1986), rat liver macrophages (Dieter *et al.*, 1991) and mouse peritoneal macrophages (Conde *et al.*, 1995). It appeared that PMA was not effective as a stimulus

for production of superoxide anions by J774.7 macrophages in this study as it failed to induce reduction of cytochrome C over 1 hour. No effect was also seen when cells were stimulated overnight with LPS, an agent well known for its ability to induce superoxide production in monocytes and macrophages (Amano *et al.*, 1985; Landmann *et al.*, 1995). It is possible that the subclone of cells used in this study (J774.7) is unable to synthesise superoxide anion upon stimulation by LPS or PMA. An alternative possibility is that the cells were producing superoxide, but for an unknown reason, the system was unable to detect its production.

The hypoxanthine (HX)/xanthine oxidase (XO) system, commonly used for the generation of superoxide anions (Berman & Martin, 1993), was added to J774.7 cells to determine the efficiency of our technique for the measurement of the radical. In the absence of cells, a significant increase in absorbance was observed, which was reduced by 100 u ml^{-1} SOD. This confirmed that the HX/XO system was effective in producing superoxide anions in a cell free environment. Surprisingly, addition of HX/XO in the presence of unstimulated J774.7 cells failed to result in the reduction of cytochrome C. One possible suggestion to explain this observation is that J774.7 cells were releasing an agent which scavenged superoxide anions produced by the cells. An alternative possibility is that the macrophages produced an agent that prevented the reduction of cytochrome C by superoxide anions. This could be an oxidising substance, which opposed the reduction of cytochrome C by superoxide anions. To investigate this, the reduced form of cytochrome C was prepared and used as a tool to investigate the presence of an oxidising agent. This indeed provided the answer since reduced cytochrome C was actually oxidised in the presence of unstimulated J774.7 cells. Further experiments were therefore conducted to determine the nature of the oxidising species derived from J774.7 cells.

7.12.2 Production of an oxidising species

It was apparent that unactivated J774.7 cells produced complete oxidation of reduced cytochrome C within 1-2 hours. Cells left in contact with cytochrome C for 30 minutes induced a slight degree of oxidation and on removal of cells from the cytochrome C, the degree of oxidation did not increase for the next 90 min. When comparing the degree of oxidation obtained upon the removal of cells to the degree of oxidation when cells were present throughout the 90 min incubation, it was apparent that the presence of the cells was vital for oxidation of cytochrome C to take place. One possible explanation for this observation is that the cells produce a short-lived oxidant. Alternatively, some aspect of the macrophage cell surface may have to remain in contact with the cytochrome C in order to oxidise it. For example, the plasma membrane of eukaryotic cells contains an NADH oxidase, responsible for the transfer of electrons across the membrane (Crane *et al.*, 1994). It is therefore possible that any cytochrome C which comes into contact with the surface of the J774.7 cells would be oxidised as a result of redox reactions occurring at the cell surface, but this would only be the case if the enzyme system was sufficiently externalised. No experiments were conducted to test this hypothesis. A series of experiments was, however, undertaken to determine if the oxidation of cytochrome C occurred as a consequence of the actions of known macrophage-derived oxidants.

7.12.2.1 Hydrogen peroxide as the oxidising species

One such oxidising species released from phagocytic cells is hydrogen peroxide, which is formed from the dismutation of superoxide anions. Potential toxic effects of hydrogen peroxide include lipid peroxidation, and

DNA damage (Miller & Britigan, 1995a). In order to test for the presence of hydrogen peroxide, the anti-oxidant enzyme, catalase, was used. Catalase removes hydrogen peroxide by converting it to oxygen and water. Catalase was unable to significantly affect the ability of J774.7 cells to oxidise cytochrome C over 2 hours, suggesting that H_2O_2 is not the oxidising species released by the cells.

7.12.2.2 Peroxynitrite as the oxidising species

Peroxynitrite, a strong oxidising agent, is formed from the simultaneous production of NO and superoxide anion (Beckman & Crow, 1993). Peroxynitrite is one of the more toxic reactive oxygen species released from macrophages with actions including lipid peroxidation (Radi *et al.*, 1991b), protein modification (Ischiropoulos & Al-Mehdi, 1995) and oxidation of DNA bases (Beckman *et al.*, 1990). Thus, the possibility that peroxynitrite was the macrophage-derived oxidant was tested using the NO synthase inhibitor, L-NAME. The basis for these experiments is that blocking NO production by the cells should prevent peroxynitrite formation. This was unsuccessful, however, as the addition of L-NAME was not effective in blocking the oxidation of cytochrome C by J774.7 cells to any extent over 2 hours. A slight degree of inhibition of oxidation of cytochrome C by J774.7 cells occurred after 5 min, but this inhibition was lost after 30 min. In this experiment, it was thought possible that the large number of cells used (10^6 cells ml^{-1}) produced so much peroxynitrite that inhibition by L-NAME may have been difficult to detect. Thus, lower numbers of cells (10^5 cells and $3x10^5$ cells ml^{-1}) were used to produce a smaller degree of oxidation, and thus make any inhibitory effect of L-NAME easier to detect. However, the use of lower cell numbers failed to reveal any ability of L-NAME to inhibit oxidation. Thus, it was likely either that the oxidising agent was not

peroxynitrite, or L-NAME was not able to inhibit NO synthase sufficiently to prevent the formation of peroxynitrite. This latter possibility was worthy of consideration since L-NAME appeared to be much less effective in blocking NO synthase in J774.7 cells than L-NMMA (see 3.2.4 & 3.2.5). Therefore, L-NMMA would be expected to be a more reliable inhibitor of production of peroxynitrite. However, L-NMMA was also unable to prevent the oxidation of reduced cytochrome C by J774.7 cells at any point over 2 hours. An alternative mechanism to prevent peroxynitrite production would be to remove superoxide anion by the use of SOD. However, SOD was also completely ineffective in reducing oxidation of reduced cytochrome C by J774.7 cells at any point over 2 hours. Criticism can be levelled at this experiment, however, since SOD is unable to permeate the cell membrane. It may therefore have been unable to prevent intracellular formation of peroxynitrite by J774.7 cells. However, due to the fact that L-NMMA (and L-NAME) did not inhibit the oxidation of cytochrome C by J774.7 cells, it seems unlikely that peroxynitrite is the oxidising species produced by the J774.7 cells.

7.12.2.3 Hydroxyl radical as the oxidising species

Peroxynitrite decomposes to form nitrogen dioxide radical and hydroxyl radical. Hydroxyl radical is one of the most potent oxidants released from cells and, due to its high reactivity, can react with many different molecules, leading to injury (Freeman & Crapo, 1982; Varani *et al.*, 1985; Guyton & Kensler, 1993). It can also be generated from hydrogen peroxide, but requires the presence of an iron catalyst (Halliwell & Gutteridge, 1984). It was therefore important to investigate if hydroxyl radical was the oxidising species released from J774.7 cells. Both dimethylthiourea (DMTU) and mannitol are scavengers of hydroxyl radical (Fox, 1984; Wasil *et al.*, 1987).

The former is able to permeate the membranes of cells and act intracellularly, but the latter is unable to enter cells and can therefore act only in the extracellular space. In our experiments, DMTU was unable to significantly reduce the oxidation of reduced cytochrome C by J774.7 cells. This was also the case for mannitol. Thus, it appears unlikely that hydroxyl radical is the oxidising species produced by J774.7.

7.12.2.4 Hypochlorous acid as the oxidising species

It is believed that monocytes but not macrophages contain the enzyme myeloperoxidase (Miller & Britigan, 1995a), and therefore perhaps hypochlorous acid should not be regarded as a possible candidate to explain the ability of J774.7 cells to oxidise cytochrome C. Nevertheless, experiments were conducted to determine if hypochlorous acid was responsible. These experiments made use of dithiothreitol, a general anti-oxidant which has been used previously to inhibit the actions of hypochlorous acid (Eley *et al.*, 1989). Dithiothreitol appeared to almost completely inhibit the oxidation of cytochrome C by J774.7 cells, initially suggesting that hypochlorous acid was the oxidising species. Upon closer inspection, however, dithiothreitol was found to promote the reduction of oxidised cytochrome C in the absence of cells. Its actions as a reducing agent probably accounted for this effect and consequently, it was not possible to determine if the oxidising agent released by J774.7 cells was hypochlorous acid. The lack of myeloperoxidase in macrophages (Miller & Britigan, 1995a), however, makes hypochlorous acid an unlikely candidate.

Due to the inability of any of the above agents, i.e. catalase, L-NMMA, L-NAME, SOD, mannitol or DMTU to inhibit oxidation, the oxidising species produced by cells has not been identified. None of the reactive oxygen

species known to be released from cells involved in the inflammatory process, namely hydrogen peroxide, peroxynitrite, hydroxyl radical or hypochlorous acid, seem to be responsible. Perhaps a novel oxidising agent is released from J774.7 cells that has yet to be characterised.

7.12.3 Attempted modulation of the oxidising activity of J774.7 cells

Although identification of the oxidising species proved difficult, further experiments were conducted to determine if, in addition to being released spontaneously by cells in the resting state, production of the oxidising species could be regulated. LPS and IFN-γ are known to stimulate the induction of NO synthase in J774.7 cells. Therefore, experiments were conducted to determine if these stimuli could regulate the production of the oxidising species by J774.7 cells. Treatment of cells for 23 hours with the combination of LPS and IFN-γ, which has been shown to be effective at inducing nitrite production by J774.7 cells, had no effect on their ability to oxidise cytochrome C. When LPS and IFN-γ were used alone, i.e. conditions that produce very little effect on nitrite accumulation, slight (<10%) but significant increases in oxidation of cytochrome C by J774.7 cells were produced. These small effects, however, were likely to have arisen from biological variation. In separate experiments, the peptide polymyxin B which has high affinity for LPS, and the corticosteroid, dexamethasone, produced slight decrease in oxidation. The magnitude of the reductions of oxidation produced by these agents were, however, so small that they too were likely to have arisen from biological variation

Thus, J774.7 cells appear to release spontaneously a very powerful oxidant which induces almost complete oxidation of cytochrome C over 30 min. We

are unable to identify the species, and assume therefore that it is not one of those already characterised as being released from inflammatory cells.

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CHAPTER 8

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