THE L-ARGININE-NO PATHWAY IN THE PATHOPHYSIOLOGY OF BLOOD VESSELS FROM SOLID TUMOURS AND RATS IN ENDOTOXIC SHOCK

Stuart Kenward Bisland

A Thesis Submitted for the Degree of PhD
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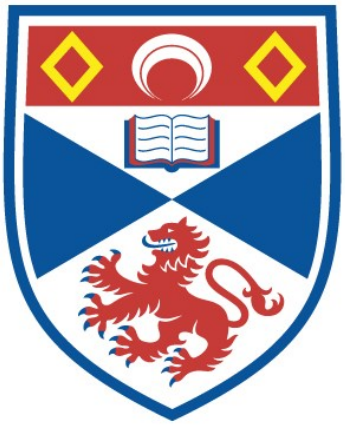

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The L-Arginine-NO Pathway In the Pathophysiology of Blood Vessels From Solid Tumours and Rats in Endotoxic Shock.

By **Stuart Kenward Bisland**

for the degree of
Doctor of Philosophy

Submitted
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Department of Biological and Medical Sciences.

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I hereby certify that Stuart Kenward Bisland has fulfilled the regulations to the Degree of Doctor of Philosophy.

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I dedicate this thesis to;

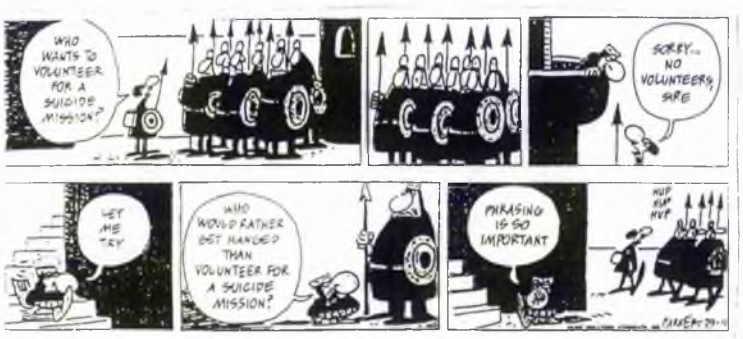
To certain individuals who have inspired me over the years especially -

My family, in particular my mum, without whom I would not have had the determination to succeed.

Marion, for being a major part of my life and providing me with continued support and an equally enduring friendship.

Debra, for her love.

And finally, to the many distractive friends I have made in St. Andrews, without whom I would probably have been finished an awful lot sooner.



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ABSTRACT

Tumour-associated arteries are a potential target for therapeutic strategies aimed at reducing or stopping tumour growth and metastases. However, the tumour vasculature exists in a state of 'near maximal vasodilation' and is markedly hyporesponsive to vasoactive agents generally. The role of nitric oxide (NO) in the growth and maintenance of solid tumours is unclear. Recent evidence suggests that it may be responsible for some of the characteristics of angiogenically-derived vessels found within the tumour and also for those of 'normal' vessels which become incorporated into the surrounding host tissue. A marked hyporesponsiveness to vasoactive agents also characterises vessels from animals in endotoxin-induced shock. One aim of the present study then was to compare the properties of arteries which formerly supplied an implanted solid tumour (tumour epigastric artery, or TEA) with arteries taken from experimental animals (rat tail artery, or RTA) injected with bacterial lipopolysaccaride (LPS).

The extent of endotoxaemia resulting from LPS treatment was assessed by (a) monitoring arterial blood pressure, using the tail cuff method; (b) measuring accumulated plasma nitrite and nitrate; (c) monitoring the development of hyporesponsiveness of RTAs to phenylephrine (PE); (d) detecting expression of inducible nitric oxide synthase (iNOS) by Western blot and immunocytochemical analysis; and (e) monitoring the loss of sensitivity of pre-contracted vessels from LPS-treated rats exposed to the vasodilator S-nitroso-N-acetylpenicillamine.

RTAs from LPS-treated rats and TEAs were less sensitive to PE than control arteries. This difference was abolished by non-selective NOS inhibitors (L-NAME and L-NMMA) and by the isoform-selective inhibitor aminoguanidine (AG). Furthermore, the protein synthesis inhibitor cycloheximide also reversed the hyporesponsiveness to PE of both types of vessel. The 'restoring' effect of cycloheximide was abolished when given after indomethacin, a specific cyclooxygenase inhibitor.

Chronic, oral administration of L-NAME or AG to tumour-bearing rats (*via* the drinking water) significantly slowed tumour growth. When L-NAME treatment was halted, tumour growth resumed at pre-L-NAME (control) rates. A single i.p. injection of LPS also impaired tumour growth: this was dependent upon the time of injection, with no effect seen 24hrs prior to tumour implantation and the maximum delaying effect at 11 days post-implantation. LPS *potentiated* the growth retarding effects of L-NAME but *abolished* those of AG.

These results provide evidence for the involvement of iNOS-derived NO in regulating the tone of arteries supplying a solid tumour and also of those from animals in septic shock. They suggest that NO may assist tumour growth by helping to maintain an adequate flow of blood into the tumour and they highlight the L-arginine/NO pathway as a potential target for the design of improved therapeutic interventions.

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ABBREVIATIONS

Oxides of Nitrogen

NO/NO·	Nitric Oxide
NO^+	Nitrosonium ion
NO^-	Nitroxide ion
NO_2^-	Nitrite
NO_3^-	Nitrate
NO_2	Nitrogen dioxide
N_2O_3	Dinitrogen trioxide

Drugs used or referenced

SNAP	S-Nitroso-N-acetyl-DL-penicillamine
RBS	Roussins Black Salt
PE	Phenylephrine
oxyHb	Oxyhaemoglobin
metHb	Methaemoglobin
Hb	Ferro-haemoglobin
SOD	Superoxide Dismutase
NBT	Nitroblue Tetrazolium
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
N-dia	NADPH-diaphorase
cyclo	Cycloheximide
indo	Indomethacin
Ach	Acetylcholine
DAHP	2,4, diamino-6-hydroxypyrimidine
SNP	Sodium nitroprusside
NA	Nicotinamide

Biologically-derived Compounds

EDRF	Endothelium-derived relaxing factor
PGI_2	Prostacyclin
PG	Prostaglandin
GC	Gyanylate cyclase
sGC	Soluble gyanylate cyclase
GTP	Guanosine triphosphate
BH_4	(6R)-5,6,7,8-tetrahydrobiopterin
CaM	Calmodulin
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
CPR	Cytochrome-P450-reductase
O_2^-	Superoxide anion
H_2O_2	Hydrogen peroxide
OH·	Hydroxyl radical
NANC	Non-adrenergic, non-cholinergic
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ET-1	Endothelin-1
TXA_2	Thromboxane A_2
INP_3/IP_3	Inositol trisphosphate

PLC	Phospholipase C
PKC	Phosphokinase C
1,2 DAG/DG	1,2 diacylglycerol
PKA	cAMP-dependent kinase
PKG	cGMP-dependent kinase
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular calcium concentration
PDEs	Phosphodiesterases
G	G-protein receptor
IP4	1,3,4,5-tetrakiphosphate
PPLC	guanine nucleotide (G)-protein-phospholipase C
GAPDH	glyceraldehyde-3-phosphate dehyrogenase
NADH	Reduced nicotinamide adenine dinucleotide
NAD^+	Nicotinamide adenine dinucleotide
$ONOO^-$	Peroxynitrite
TNF-α	Tumour necrosis factor-alpha
IL-2	Interleukin-2
VPF	Vascular permeability factor
EDNO	Endothelium-derived nitric oxide
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
INF-γ	Interferon-gamma
LPS	Lipopolysaccharide
PDGF	Platelet-derived growth factor
IL-1β	Interleukin-1 beta
(i)AS	(inducible) Argininosuccinate synthetase
AL	Argininosuccinate lyase
(i)COX	(inducible) cyclooxygenase

L-arginine and NO-synthase Inhibitors

L-NMMA	N^{ω} monomethyl-L-arginine
L-NA/L-NARG	N^{ω} nitro-L-arginine
L-NAME	N^{ω} nitro-L-arginine methyl ester
AG	Aminoguanidine
DMA	N^{ω} N^{ω} dimethylarginine
BAEE	Nα-benzoyl-L-arginine
L-arg	L-arginine

NO-Synthases

NOS	Nitric oxide synthase
iNOS	Inducible NOS
cNOS	Constitutive NOS
eNOS	Endothelium-derived NOS
macNOS	macrophage-derived NOS
nNOS	neuronal-derived NOS

Analytical Techniques

HPLC	High Performance Liquid Chromatography
ESR/EPR	Electron spin/paramagnetic resonance
FITC	Fluorescein isothiocyanate
ABC	avidin-biotin complex

ECL	Enhanced Chemiluminescence
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Miscellaneous

EA	Epigastric artery
TEA	Tumour supplying EA
CEA	Non-tumour associated (control) EA
RTA	Rat tail artery
VSM	Vascular smooth muscle
CNS	Central nervous system
PNS	Peripheral nervous system
ED_{150}	Equivalent dose to produce contractile perfusion pressure =150 mmHg
P_{max}/P	Contractile perfusion pressure produced by $5x10^{-3}$M PE
pp	Perfusion pressure
D.R.	Dose response
i.p.	Intraperitoneal
i.v.	Intravenous

Part A

Biology of Nitric Oxide

1.1 Introduction

Nitric oxide (NO) was quietly 'going about its business' long before humans evolved. Yet, only within the last 10 years or so have we belatedly recognised that it appears to be everywhere in the body and relevant to many, if not all, physiological functions. One of the smallest biological molecules known, NO has finally energed from obscurity to become one of the most talked about topics in modern biology. It is ironic that having long been studied by industrial chemists as an environmental pollutant, contributing to smog and acid rain, it should turn out to be one of the most important mediators of physiological function in mammals.

In order to readily appreciate the key findings which have helped determine the course of NO research, and provide us with discernible evidence for its role, a chronological diary of publications is listed below.

1 The first indication that mammals (including humans) could generate nitrate (NO_3^-) was in 1916 (Mitchell *et al.*, 1916). This study measured NO_3^- levels in urine and concluded that more NO_3^- was being excreted than ingested in the diet.

2 More than sixty years elapsed before Mitchell's observation was confirmed, at which time Tannenbaum *et al.*, (1978) established that humans could synthesise NO_3^- and nitrite (NO_2^-).

3 In 1980, Furchgott and Zawadzki coined the term 'endothelium-derived relaxing factor' (EDRF) to describe the substance, released by endothelial cells in response to acetylcholine (Ach), which could relax vascular smooth muscle.

4 Injected bacteria were found to stimulate mammalian macrophages to release high levels of NO_2^- and NO_3^- (Steuhr & Marletta, 1985).

5 It was established that neither NO_2^- nor NO_3^- is responsible for the cytotoxic effect of activated macrophages on tumour cells (Hibbs Jr. *et al.*, 1987).

6 The production of NO_2^- and NO_3^- requires oxidation of a guanidino nitrogen of L-arginine which produces an intermediate oxide of nitrogen more reactive than either NO_2^- or NO_3^- (Miwa *et al.*, 1987).

7 EDRF released by Ach-stimulated endothelial cells is neither NO_3^- nor NO_2^- but more likely NO· (Palmer *et al.*, 1987; Ignarro *et al.*, 1987).

8 The ability of activated macrophages to kill tumour cells resides in the conversion of L-arginine to L-citrulline and NO. The production of NO and the cytotoxic effect were both inhibited by N^G-methyl-L-arginine (Hibbs Jr. *et al.*, 1988).

9 L-arginine is identified as the precursor for NO synthesis in vascular endothelial cells (Palmer *et al.*, 1988).

10 Neuronal cells of the brain can be stimulated to release a factor similar to that released from endothelial cells and macrophages (Garthwaite *et al.*, 1988).

So in the ten years since the initial discovery that humans produce NO_2^- and NO_3^- it emerged that three major cell types, (macrophages, endothelium and neurons), spanning diverse areas of physiological function (cytotoxicity, vasorelaxation and neurotransmission), are stimulated to synthesise the same labile mediator from L-arginine. The identity of this mediator is most likely NO although this has not been proven as yet and so remains an area of intense research and debate.

The studies listed above paved the way for an avalanche of research which was to follow and ultimately resulted in NO being named 'Molecule of the year' in 1992 by the American Association for the Advancement of Science. Now that the presence of NO has been established considerable research now focuses on trying to decipher the biosynthetic mechanism(s) responsible for its production. In what must be considered a relatively short period of time since the discovery of NO in mammals, a tremendous amount has already been learned about the enzymes involved in its synthesis. The details concerning the synthesis of NO are dissussed in the 'NOS' section of this introduction.

1.2 NO is an Endothelium-Derived Relaxing Factor

It was first shown in 1980 by Furchgott and Zawadzki that acetylcholine could stimulate the release of a factor from the endothelium which relaxed precontracted rabbit aortic rings. This was termed an endothelium-derived relaxing factor (EDRF) and has been identified as NO by two research groups working independently of each other, one led by Moncada at the Wellcome laboratories in England and the other in Los Angeles by Louis Ignarro (Ignarro *et al.*, 1987).

Moncada's group, Palmer *et al.*, (1987), showed that authentic NO and endogenous EDRF release from cultured porcine aortic endothelial cells both had virtually identical effects on precontracted strips of denuded rabbit aorta. Furthermore, the levels of NO released by bradykinin, determined using a chemiluminescence method, could account for vasorelaxation of the bioassay strip produced by EDRF.

Similar perfusion bioassay systems, in which the transit time of the effluent from endothelial cells to the denuded preparations can be varied, have been used to study the rate of decay of EDRF. Decay is responsible for the diminished relaxation caused by EDRF and NO as they pass down the cascade (Palmer *et*

al., 1987; Khan & Furchgott, 1987). EDRF and NO display similar diminished relaxation suggesting that they have similar chemical instability.

A considerable range of half-life values (3 to 50 sec.) for EDRF has been reported (Rubanyi *et al.*, 1985; Gryglewski *et al.*, 1986; Griffith *et al.*, 1984; Cocks *et al.*, 1985; Feelisch *et al.*, 1994), raising some doubt as to its chemical identity. Discrepancies concerning instability are largely due to the presence of superoxide free radicals (O_2^-) within the experimental solutions (Gryglewski *et al.*, 1986). Different laboratories with different experimental design for perfusion, superfusion and oxygenation will ultimately have different levels of O_2^- in their medium. NO reacts with O_2^- to produce peroxynitrite ($ONOO^-$), a powerful oxidant which may be responsible for many cytotoxic effects attributed to NO (Beckman *et al.*, 1990). The endogenous enzyme, superoxide dismutase (SOD), can be added to the superfusate to prolong the half-life of EDRF by catalysing the conversion of O_2^- to hydrogen peroxide.

The vascular smooth muscle (VSM) relaxation associated with EDRF (unstimulated or Ach-stimulated) is mediated by cyclic 3', 5',-monophosphate (cGMP; Rapoport & Murad, 1983) and is inhibited by haemoglobin or methylene blue (Griffith *et al.*, 1984; Martin *et al.*, 1985) in much the same way as NO (Murad *et al.*, 1978), although it has been suggested more recently that these inhibitors of EDRF/NO· also generate O_2^- (Marczin *et al.*, 1992).

In 1990 it was proposed that while spontaneous, *de novo* NO synthesis in endothelial cells accounts for basal EDRF activity, stimulated EDRF release occurs from *S*-nitrosothiol stores which decompose releasing NO· by exocytosis (Ignarro, 1990). Indeed, light sensitive S-nitrosothiol stores are thought to exist in VSM cells (Venturini *et al.*, 1993) which can be depleted and later replenished by endothelium-derived NO in an N^G-monomethyl-L-arginine-sensitive manner (Megson *et al.*, 1995). The physiological relevance of a photosensitive store of NO in VSM is still unclear. Iron-sulphur cluster nitrosyls eg. Roussins black salt

($[Fe_4S_3(NO_7)]$) can enter endothelial cells and decompose to release NO and may represent another potential store in vascular tissues (Flitney *et al.*, 1992).

A comparison of NO, *S*-nitrosothiol and EDRF effects using rabbit aortic rings (Furchgott *et al.*, 1992) revealed that although relaxations induced by *s*-nitrosothiol and Ach-stimulated EDRF were mediated by NO, they were only slightly inhibited by xanthine + xanthine oxidase (O_2^- generators), while the relaxation induced by authentic NO was completely inhibited. This seemed to suggest that the behaviour of abluminal EDRF more closely resembles that of an *s*-nitrosothiol which can enter VSM cells before releasing NO, than NO itself.

In contrast, perfusion bioassay experiments suggest that EDRF release into the lumen of vessels is most probably NO, since both display a similar diminished relaxation with increasing transit time to the bioassay ring (Furchgott *et al.*, 1991). This was supported by Feelisch *et al.*, (1994), who concluded that *s*-nitrosylated compounds (including *s*-nitrosoglutathione and *s*-nitrosocysteine) are up to 10-fold more potent than EDRF and, together with a number of other potential candidates for EDRF, (ie sodium nitroxyl, hydroxylamine and dinitrosyl-iron-cysteine complex), are more stable than EDRF and less susceptible to inhibition by oxyhaemoglobin.

Further indications that EDRF may not be NO are evident using a perfusion bioassay system in which infused NO relaxes certain nonvascular smooth muscle preparations while EDRF released from cultured bovine endothelial cells does not (Dustin *et al.*, 1987). EDRF also binds to anionic exchange resins while NO does not (Shikano *et al.*, 1988). EDRF and NO in solution at room temperature (R.T.) are undetectable using conventional electron paramagnetic resonance (EPR) spectroscopy (Henry *et al.*, 1993). However, because NO is paramagnetic EPR can detect NO adducts. The complexity surrounding spin-adducts compromises the efficiency of EPR, and with the potential for so many NO

interactions *in vivo*, there is a growing concensus that EDRF is not NO but more likely a precursor (Greenberg *et al.*, 1990).

1.3 Biochemical Properties and Interactions of NO

NO is a colourless gas at R.T. and due to its solubility in water is physiologically present as an aqueous solution. Its uncharged, hydrophobic properties promote its ability to diffuse readily across different cell membranes. NO has an unpaired electron in its antibonding π orbital and as such is paramagnetic with a reduced bond order of ~ 2.5 and an N-O bond length of ~1.5 angstroms (i.e. between triple and a typical double bond length) (Butler & Williams, 1993).

The nitrogen oxide species normally present in physiological systems are likely to be tightly regulated by cellular redox reactions influencing their oxidative state, the formation of reactive oxygen species and ultimately their interaction with thiols, metals and metalloproteins.

1.3.1 Interaction with Thiols

The highly reactive nature of NO stems from the redox couples it forms with the nitrosonium cation (NO^+) and the nitroxyl anion (NO^-) or its acid, HNO. NO is a poor nitrosating agent, but when oxidised to NO^+, *s*-nitrosothiols can be readily formed(equ. 1) which may transfer NO^+ to a second thiol (R^2S)(equ. 2) in a process called transnitrosation (refer to Butler *et al.*, 1995).

$$(1)\ RSH + NO^+ \rightarrow R^1SNO + H^+$$

$$(2)\ R^1SNO + R^2S^- \rightarrow R^1S^- + R^2SNO$$

S-nitrosothiols may therefore provide a mechanism for transporting and supplying NO^+, which would otherwise immediately form nitrous acid in aqueous solution.

It has been shown that NO^- can be reconverted to NO by SOD (Fukuto *et al.*, 1993). The physiological relevance of this has yet to be resolved although theoretically NO^- could react directly with molecular oxygen to produce peroxynitrite. The abundance of proteins with which NO may interact *in vivo* lessens the likelyhood of this happening.

1.3.2 Peroxynitrite Formation

The activated immune response results in overproduction of NO which can react with O_2^- and increase the formation of other potentially more toxic products, including peroxynitrite ($ONOO^-$)(equ. 3). $ONOO^-$ is almost instantly (half-life ~1sec.) protonated to peroxynitrous acid (ONOOH) which subsequently decomposes to nitrogen dioxide and hydroxyl radical(equ. 4) (Beckman *et al.*, 1990).

$$(3)\ NO\cdot + O_2^- \rightarrow ONOO^-$$

$$(4)\ ONOO^- + H^+ \rightleftharpoons ONOOH \rightarrow NO_2 + \cdot OH$$

The hydroxyl radical is an extremely reactive species and may be responsible for much of the damage caused by peroxynitrite, such as lipid peroxidation (Radi *et al.*, 1991) and nitration of several functional groups of amino acids including tyrosine (Ischiropoulos *et al.*, 1992). The nitrated tyrosine in proteins appears to be a specific biological marker of peroxynitrite and may offer significant insight into the role of peroxynitrite in the pathology associated with a number of inflammatory conditions, including sepsis, adult respiratory distress syndrome and atherosclerosis (Crow & Beckman, 1995).

New evidence suggests that peroxynitrite may also assist in detoxification by reacting with certain sugars (eg. glucose) commonly found in physiological buffers to form an NO-donor with characteristics of an organic nitrite/nitrate

(Moro *et al.*, 1995). This will undoubtedly be relevant to *in vitro* and *ex vivo* studies and it remains to be clarified whether similar reactions also occur *in vivo*.

1.3.3 NO in Aqueous Solution

The reaction between NO and O_2 is not favourable at physiologically relevant levels of NO (ie. 1-100nM) and will more readily occur at supraphysiological [NO]. Lipopolysaccharide-induced high levels of NO *in vivo* may favour the reaction between NO and O_2.

In this respect it has been observed that in aqueous solution NO reacts much more slowly with O_2 (half-life of several hours) than predicted using *in vitro* bioassay systems (half-life of 3-50sec.; Kharitonov *et al.*, 1994). Interactions with O_2^- and redox metals, which typically contaminate bioassay buffers, will inactivate NO *in vitro*. The inactivation by O_2^- is less evident *in vivo* with no significant alteration in NO-mediated vasorelaxation in the presence of added SOD (Wolin *et al.*, 1990).

NO is oxidised by O_2 to nitrogen dioxide(equ. 5) which, under physiological conditions of temperature, oxygen tension and pH, usually reacts with more NO to form the anhydride of nitrous acid(equ. 6) and finally nitrous acid or nitrite ion, depending on pH(equ. 7). Alternatively nitrogen dioxide may react with water to produce a mixture of nitrate and nitrite(equ. 8), although this reaction is thought to be much slower (ca. 10x) than its reaction with NO (Feelisch *et al.*, 1991).

(5) $2NO + O_2 \rightarrow 2NO_2$

(6) $NO_2 + NO\cdot \rightarrow N_2O_3$

(7) $N_2O_3 + H_2O \rightarrow 2HNO_2 \rightarrow 2H^+ + 2NO_2^-$

(8) $2NO_2 + H_2O \rightarrow HNO_2 + HNO_3$

1.3.4 Interaction with Haemoglobin

The high oxygen tensions in arterial blood favour nitrate as the main breakdown product of NO's reaction with oxygen, rather than nitrite (NO_2^-) which is the main product in oxygenated aqueous solution. The interactions of NO and its metabolites (ie. NO_2^-) with haemoglobin (Hb) (and myoglobin) appear to be responsible for nitrate formation *in vivo* (Wennmalm *et al.*, 1993).

The relative amounts of methaemoglobin (metHb, Hb (Fe^{III})), nitrosylated Hb (HbNO) and nitrate formed is dependent on the oxygenation state of Hb within the erythrocytes. In arterial blood (O_2 saturation 94-99%) nearly all of the oxygenated Hb (oxyHb, Hb(Fe^{II})O_2) which binds NO is converted to metHb + nitrate (equ. 9) with little HbNO. In venous blood (O_2 saturation 36-85%) deoxygenated Hb (deoxyHb, Hb(Fe^{II})) reacts with NO to form mainly (Hb(Fe^{II})NO), with less nitrate(equ. 10) .

(9) $NO + (Hb(Fe^{II})O_2) \rightarrow (Hb(Fe^{III})) + NO_3^-$

(10) $NO + (Hb(Fe^{II})) \rightarrow (Hb(Fe^{II})NO)$

NO, like carbon monoxide, binds to the iron centre of oxyHb impeding the ability of erythrocytes to transport oxygen. MetHb does not accumulate in these cells as it is efficiently converted back to Hb(Fe^{II}) by the NADPH-dependent enzyme, methaemoglobin reductase (Wennmalm *et al.*, 1993). The nitrate produced is transferred into the blood serum and excreted via the kidneys into the urine, with a small amount being taken up into the saliva.

The interaction between NO and deoxyHb is similar to that of oxyHb however, NO can remain complexed with deoxyHb for up to 4hrs with a 10^6-fold greater affinity than O_2 (Henry. *et al.*, 1993). The mechanism responsible for releasing NO· from deoxyHb is not clear but may involve the oxidation of NO to nitrite.

Jia *et al.*, (1996) recently suggested that certain nitrosothiols can enter erythrocytes and complex with oxyHb in the lungs to form *s*-nitrosylated oxyHb (SNO-Hb(Fe^{II})O_2) without compromising O_2 transport. *S*-nitrosothiols are not subject to diffusional constraints imposed by Hb so can transport NO out of the lungs to the tissues. The idea that NO can mediate vasoactive effects away from its release site is controversial and will undoubtedly generate considerable interest.

1.4 Detecting NO

Accurate methods of detection have to be devised in order that the distribution and reactivity of NO *in vivo* be more fully appreciated. There is a growing number of such methods, although several do not measure NO directly.

1.4.1 Griess Reaction

The assay based on the Griess reaction does not measure NO directly, but relies instead on the propensity for NO to be oxidised to nitrite and nitrate under physiological conditions. The reagents detect nitrite so all nitrate is first reduced, using either a copper-coated cadmium column or a bacterial enzyme, nitrate reductase. The purple azo derivative which is formed in the reaction is monitored spectrophotometrically at 540nm (Green *et al.*, 1982).

This assay only infers NO production and offers little qualitative information as to whether the nitrite or nitrate is derived via NO metabolism or the diet. The technique is however quick and easy to use, with considerable sensitivity. The stability of nitrate in mammalian systems means that lipopolysaccharide (LPS)-

induced increases in NO production can be measured from blood serum samples (Schmidt & Kelm, 1996).

1.4.2 The Oxyhaemoglobin Assay

Another assay relying on spectrophotometry involves a shift in the visible spectrum peak absorbance from 415nm to 406nm as NO rapidly converts oxyHb to metHb and nitrate ion(equ. 12).

$$(12)\ (Hb(Fe^{II})O_2) + NO\cdot \rightarrow Hb(Fe^{III}) + NO_3^-$$

The oxyHb assay is a useful method for obtaining kinetic information concerning NO production under physiological conditions although with so many redox-active species within the biological milieu (ie. thiols, hydrogen peroxide, O_2^-, $ONOO^-$) inaccuracies in measurement must be anticipated. (Feelisch *et al.*, 1996).

1.4.3 Electron Paramagnetic Resonance (EPR)

Although NO is paramagnetic, in its unexcited state it is undetectable using EPR in solutions at room temperature. The intervention of EPR as a means of monitoring NO production relies on the formation of NO adducts. NO-specific adducts include organic spin-traps like Fe^{II}-diethyldithiocarbamate $(DETC)_2$ which complexes with NO to form $FeNO(DETC)_2$. This adduct has been used successively to detect NO in organs from animals pre-exposed to LPS. EPR can detect minute amounts of NO in specific cells, tissues and living animals and as such represents a powerful tool for measuring NO in various pathological conditions such as septic shock or diabetes mellitus (Henry *et al.*, 1993; Singel & Lancaster Jr., 1996).

1.4.4 Chemiluminescence Reaction with Ozone

NO reacts with ozone (O_3) in the gaseous phase to produce nitrogen dioxide in an excited state (NO_2^*)(equ. 13).

$$(13)\ NO + O_3 \rightarrow NO_2^* + O_2$$

As NO_2^* relaxes to its normal, unexcited state it emits light of a characteristic energy(equ. 14) which is detectable using a photomultiplier tube.

$$(14)\ NO_2^* \rightarrow NO_2 + h\nu$$

Chemiluminescence is one of the most accurate assays available and, in addition to measuring NO itself, it can also be used to measure the intermediates and end-products of NO oxidation. However, due to the diversity and variability of NO interactions *in vivo*, this procedure can only be used outwith the biological milieu. (Hampl *et al.*, 1996).

1.4.5 Electrochemical NO Probes

Electrochemical probes for NO rely on the propensity of NO to oxidise at the anode. The current generated is proportional to the amount of NO oxidised. Porphyrinic sensors have been used to detect the release of NO from a single cell *in situ* (Malinski & Taha, 1992) and more recently, the NO release associated with infused Ach and bradykinin in the human vein (Vallance *et al.*, 1995). Therefore NO probes provide further evidence that EDRF in humans is NO and will undoubtedly prove invaluable in assessing the roles of NO in physiological systems on a moment to moment basis. More recently other types of NO sensor have been reported to detect endogenous NO release *in vivo* (Leung *et al.*, 1996).

1.5 Physiological Roles of NO

1.5.1 Modulation of Arterial Tone

NO is an important mediator of vascular tone, maintaining local and systemic vascular resistance, blood flow and oxygen delivery. An imbalance in NO synthesis is implicated in a number of life threatening conditions eg. septic shock, resulting from excess NO production, or diabetes, ischaemia and systemic hypertension, all of which are associated with impaired NO production.

The monolayer of endothelial cells lining the inner surface of blood vessels is the source of numerous factors which modify VSM activity, either spontaneously (ie. basal release) or in response to stimulation (eg. shear stress or endothelium-dependent agonists). NO is just one of a number of endothelium-derived relaxing factors. Other EDRFs include prostaglandin I_2 (PGI_2) and endothelium-derived hyperpolarising factor (EDHF; Tare *et al.*, 1990). Constricting factors (EDCF) are also released from endothelium and include endothelin-1 (ET-1) and thromboxane A_2 (TXA_2).

Most of these factors generate second messengers within VSM which ultimately regulate vascular tone (ie. PGI_2 increases cAMP; NO increases cGMP; ET-1 and TXA_2 increase INP_3 and 1,2 DAG). Basal NO can be considered a tonic suppressor of vascular contractility whereby the true state of contraction is only unmasked when NO is inhibited eg. by haemoglobin. Agonist-stimulated (eg. phenylephrine; α_1 adrenoceptor agonist) contractions are also antagonised by basal NO release (Martin *et al.*, 1986).

Therefore, although the mechanisms employed to regulate vascular tone are varied, they principally deal with two opposing responses, vasorelaxation and vasoconstriction.

1.5.1a Blood Flow - Basal Tone

In the absence of any mechanism regulating vascular tone (ie. innervation, circulating and locally synthesised substances) small arteries reside in a partially constricted state *in vivo* due to the flow of blood through them. Blood flow exerts physical forces on vessel walls resulting in a persistant contraction, often referred to as basal tone. Although frequently overlooked, basal tone is essential to ensure adequate circulation and so prevent peripheral vascular collapse (Segal & Duling, 1987). Specific inhibitors of NOS eg. N^G-monomethyl-L-arginine (L-NMMA) increase arterial tone, both *in vitro* and *in vivo* (Moncada *et al.*, 1991) indicating that basal release of EDRF in unstimulated vessels regulates basal tone.

The synthesis of EDRF can also be enhanced in endothelial cells by a variety of mechanisms.

1.5.1b - Stretch and Shear Stress

Vessel walls are also subject to shear stress (a tangential force generated by friction between cells in the blood and endothelial lining) and stretch (pressure perpendicular to the endothelial layer associated with blood flow, volume, viscosity and velocity). As well as contributing to basal tone these physical forces also establish active tone (myogenic activity) within the vessels by elevating intracellular calcium concentration $[Ca^{2+}]_i$ (Bevan, 1991; Falcone *et al.*, 1993). Endothelial basal activity is principally determined by shear stress while stretch regulates arterial diameter in a smooth muscle-dependent manner (Johnson, 1991). This was demonstrated by altering fluid viscosity, which is proportional to shear stress, in isolated segments of artery and maintaining a constant pressure (Koller *et al.*, 1993).

Shear stress on endothelial cells may be detected by sialic acid residues built into its surface (Heckler *et al.*, 1993). This may provide a Ca^{2+}-independent activation of membrane bound eNOS. (See fig. 1.1).

Mechanosensitive ion channels (Ca^{2+}, K^+ and Cl^-) also exist on endethelial cells and are activated in response to shear stress (Oleson *et al.*, 1988). Tone is altered by changes in intracellular ionic concentrations, for instance K^+ selective channels can hyperpolarise endothelial cell membranes to cause an EDRF-independent relaxation (Oleson *et al.*, 1988), while stretch-induced Ca^{2+} influx (via stretch-activated Ca^{2+} channels) (Kirber *et al.*, 1988) and activated voltage-dependent channels (Harder *et al.*, 1987) can cause constriction (Laher & Bavan, 1987).

Shear stress is also thought to influence the release of ET-1, a potent endothelial-derived vasoconstrictor. Whether ET-1 release is suppressed (Malek & Izumo, 1992) or stimulated (Yoshizumi *et al.*, 1989) is thought to be determined by differences in the level of shear stress imposed on the endothelial cells (Morita *et al.*, 1993). It is also possible that NO inhibits ET-1 release (Boulanger & Luscher, 1990).

NO (Hutcheson & Griffith, 1991, 1994) and PGI_2 release (Frangos *et al.*, 1985) are enhanced in isolated preparations by increased flow and perfusate viscosity. The expression of endothelium-derived NOS (eNOS; Section 1.9) mRNA and protein are also upregulated following exposure of endothelial cell cultures to shear stress (Busse *et al.*, 1994). Indeed, a shear stress regulated component has been identified in human eNOS, allowing for a transcriptional regulation of NOS activation *in vivo* (Marsden *et al.*, 1992). Significantly, both EDRFs are implicated in endothelium-dependent hyperpolarisation (Tare *et al.*, 1990; Parkington *et al.*, 1993). NO (Kitamura, 1993) and NO-donor drugs (Haeusleu & Thorens 1976) have been shown to hyperpolarise arterial preparations.

Flow-induced hyperpolarisation can also occur in response to agonists which induce EDRF synthesis (Bolton & Clapp, 1986). This endothelium-dependent hyperpolarisation is thought to reflect a cGMP-dependent protein kinase-mediated phosphorylation of Ca^{2+}-activated K^+ (K_{Ca}) channels (Bolotina *et al.*, 1994) or ATP-sensitive K^+ (K_{ATP}) channels, although the exact mechanism varies between tissues and species (Parkington *et al.*, 1993). Species differences are further suggested by studies using the endothelium-dependent agonist acetylcholine (Ach). In rabbit aortic endothelial cells Ach is thought to cause hyperpolarisation by activating K_{Ca} channels, increasing cytosolic $[Ca^{2+}]$ and stimulating EDRF release (Sakai, 1990). Conversely Ach-induced hyperpolarisation in rat mesenteric artery is not inhibited by oxyhaemoglobin or methylene blue, suggesting an EDRF-independent mechanism (Chen & Suzuki, 1989).

Ach and ATP are synthesised in endothelial cells (Parnevalas *et al.*, 1985) and a number of other EDRF-stimulating agonists are stored, including vasopressin, angiotensin II, substance P and bradykinin (Ralevic *et al.*, 1992). Flow can induce agonist release from endothelial cells (Ralevic *et al.*, 1990) which will potentiate an increase in $[Ca^{2+}]_i$ and the subsequent release of EDRF. By altering membrane potential (ie. hyperpolarisation), agonists upregulate L-arginine transport into endothelial cells which may permit a sustained increase in EDRF synthesis (Bussolati *et al.*, 1993).

Therefore, in summary, the mechanisms employed to sustain Ca^{2+} influx evoked by flow include (1) activation of mechanosensitive Ca^{2+} channels, (2) activation of non-selective cationic channels, (3) activation of an inward rectifying K^+ current involving both K_{Ca} and K_{ATP} channels which may cause hyperpolarisation. Endothelial cells lack voltage-operated Ca^{2+} channels so hyperpolarisation increases the electrochemical gradient for Ca^{2+} entry into endothelial cells (Lückoff & Busse, 1990). Shear stress and endothelium-

dependent agonists initiate a guanine nucleotide (G)-protein-phospholipase C (PPLC) interaction resulting in 1,4,5-trisphosphate (IP_3) formation from phosphoinositides. IP_3 stimulates Ca^{2+} release from internal stores (Prasad *et al.*, 1992). (4) As a byproduct of PPLC activation, 1,3,4,5-tetrakiphosphate (IP_4) may upregulate Ca^{2+} influx by opening Ca^{2+} permeable channels (Lückoff & Clapham, 1992). (5) Adjacent endothelial cells are in intimate contact via gap junctions (Bény & Pacicca, 1994) such that increases in $[Ca^{2+}]_i$ can be propagated from one cell to the next. Similarly this could result in a propagated EDRF release as eNOS is activated in sequence. (6) eNOS is well suited to a Ca^{2+}-dependent activation. Its location in the plasma membrane will favour stress related mechanotransduction and its activity is potentiated by the high intracellular pH induced by agonists such as bradykinin (Fleming *et al.*, 1993).

These mechanisms act to reinforce the Ca^{2+} dependent activation of eNOS and subsequent release of EDRF. Although they may be relevant to both shear stress- and agonist-induced EDRF synthesis, fundamental differences in their use of Ca^{2+} exist. Agonist-induced EDRF synthesis relies on Ca^{2+} derived from internal stores while, in contrast, shear stress primarily utilises cytosolic Ca^{2+} (MacArthur *et al.*, 1993). This is demonstrated using inhibitors of Ca^{2+}-ATPase (the enzyme responsible for Ca^{2+} re-uptake into internal stores) which attenuate agonist-induced EDRF but stimulate shear stress-induced EDRF.

1.5.2 Activation of Guanylate Cyclase

Guanylate cyclase (GC) exists in most cells as a polymorphic enzyme with both cytosolic (soluble) and membrane-bound (particulate) isoforms. NO generally targets the soluble isoenzyme which is a haem-containing heterodimer with α and β subunits of M_r s in the range of 73-88KDa and 70KDa respectively (Waldman & Murad, 1987).

NO released from the endothelial lining of blood vessels permeates through into the neighbouring VSM where it causes vasodilation. This constant or constitutive release of NO from the endothelium is initiated by and helps to counter the vasoconstriction created by Ca^{2+} released both intra- and intercellularly. The biochemical pathway responsible for this vasodilation is initiated by the binding of NO to the haem iron in soluble guanylate cyclase (sGC). This activates sGC which subsequently catalyses the hydrolysis and cyclization of guanosine 5' triphosphate (GTP), forming guanosine 3',5' cyclic monophosphate (cGMP) and pyrophosphate. In its capacity as an intracellular 'second messenger', cGMP functions to alter cellular processes directly, eg. by regulating nucleotide phosphodiesterases or stimulating cGMP-gated ion channels; or indirectly, by activating cGMP-dependent protein kinase. This kinase is then able to phosphorylate key protein substrates which modify cellular function and initiate relaxation (Knowles & Moncada, 1992).

The first conclusive evidence that NO is involved in sGC activation came from studies by Murad *et al.*, (1978). They showed that sodium azide could increase the levels of cGMP in crude tissue extracts via the production of NO. This process required certain haemoproteins to be present and was inhibited by other haemoproteins eg. haemoglobin and myoglobin (Mital *et al.*, 1975). A constituent of the sGC was able to convert the azide, in the presence of oxygen, to NO and so activate the enzyme. This constituent was subsequently identified as haem which forms a very stable bond with NO.

A number of other compounds which are known to release NO including, glycerol trinitrate, sodium nitroprusside (SNP) and amyl nitrite, can also activate sGC from crude extracts (Waldman & Murad, 1987). Because of their ability to release NO and cause relaxation in vascular (and nonvascular) smooth muscle, these compounds are often referred to as 'nitrovasodilators'.

Exposure of sGC (from crude extract) to NO alone also causes activation and results in a characteristic shift in Soret absorption peak from 431 to 398nm. Thus NO appears to be the main mediator of sGC activation (Braughler *et al.*, 1979).

The ability of NO and certain nitrovasodilators to activate sGC is lost in partially purified enzyme preparations but can be restored by adding free haem-containing metalloproteins such as catalase, haematin, cytochrome C or peroxidase (Craven *et al.*, 1978). This suggests two things: Firstly that purification of sGC can result in dissociation of the loosely-bound haem moiety, rendering the haem-deficient enzyme unresponsive to NO. This implies that activation of mammalian sGC by NO (and certain nitroso-compounds) is haem-dependent. Secondly, the haem contained in other proteins can be used by sGC to bind NO and facilitate activation (Craven & DeRubertis, 1978). The formation of the nitrosyl (NO)-haem complex is therefore crucial to the activation of sGC. This is further substantiated using agents which bind haem, eg. sodium cyanide. Cyanide inhibits the activation of sGC by azide, SNP and NO in crude and purified preparations (Kimura *et al.*, 1975).

Preformed NO-haemoproteins (eg. NO-catalase, NO-haemoglobin, NO-cytochrome P450 and NO-myoglobin) can maximally activate haem-containing sGC, as well as partially purified, haem-deficient sGC, more effectively than some nitrovasodilators, although experimental conditions will undoubtedly influence the results as some nitrovasodilators are oxygen dependent (eg. sodium azide) while others are not (eg. SNP) (Waldman & Murad, 1987). Spectrophotometric analysis suggests that an exchange occurs between the NO-haemoprotein and the sGC whereby the NO-haem moiety is transferred from the NO-haemoprotein to the haem-deficient sGC (Ignarro *et al.*, 1986). The sGC is therefore reconstituted with its NO-haem complex. Furthermore, haem transfer does not occur between haem-deficient sGC and haemoprotein suggesting that either NO-haem binds less tightly to haemoglobin, myoglobin and catalase than

haem, so is more easily released, or alternatively, that sGC has a greater binding affinity for NO-haem than haem alone.

Electron paramagnetic resonance (EPR) studies indicate that binding of NO to the haem (ferroprotoporphyrin IX) in sGC weakens the co-ordinate bonds holding iron in the plane of the protoporphyrin ring. This causes the iron to protrude out away from the hydrophobic pocket of the haemoprotein (Ignarro *et al.*, 1992; Morse & Chan, 1980), effectively rearranging the structure of the NO-haem complex to one resembling protoporphyrin IX, activating the enzyme (Wolin *et al.*, 1982). Protoporphyrin IX, the demetalated (ie. without iron) precursor of haem, can activate reconstituted, haem-deficient and haem containing sGC, all in the absence of NO. This, and the fact that by adding iron into protoporphyrin IX inhibits NO mediated activation of sGC (Murad *et al.*, 1979), suggests that formation of this protoporphyrin-like molecule is necessary for sGC activation.

The activation of partially purified enzyme preparations is potentiated by adding reducing agents which generate NO-haem complexes from haemoproteins (but not preformed NO-haemoproteins). This occurs as the haem-iron is maintained in the ferrous (Fe^{2+}) form so its affinity for NO is increased. Oxidising agents also influence sGC activation.

In general thiol-reducing agents (glutathione, ascorbate, dithiothreitol and cysteine) potentiate activation by NO or NO donors and thiol-oxidising agents (hydrogen peroxide, methylene blue and superoxide) inhibit it (Lewicki *et al.*, 1982). Inhibition may be due to the conversion of NO to higher oxides of nitrogen (NO_2) before reaching the active site, or alternatively, oxidation of the recipient haem to its ferric form. Reducing agents will favour activation by preventing these oxidations.

Interestingly, SOD, which protects NO by converting O_2^- to hydrogen peroxide (H_2O_2), can activate partially purified sGC (Mittal & Murad, 1979) an effect which is potentiated by increasing O_2^- concentration (Mittal & Murad, 1977). This suggests that superoxide anions are required for the activation of sGC which seems contradictory to the understanding that they inhibit activation by degrading NO. This may be explained by one of two possibilities, firstly O_2^- and H_2O_2 may act indirectly on sGC by forming hydroxyl free radicals which are known to activate sGC *in vivo* (Murad *et al.*, 1978), or O_2^- may act directly on sGC. The second possibility is that the oxidation of sulfhydryl (SH) groups will promote activation but conversely, 'overoxidation' will inhibit it.

Agents that oxidise or reduce SH groups significantly alter both basal and stimulated sGC activity. Alkylating agents (eg. ethacrynic acid) can covalently modify free thiol groups to inhibit activity (Katuski *et al.*, 1977). Similarly, oxidising agents (eg. oxidised glutathione) which convert free thiols to disulfides, and enhanced mixed disulfide formation with agents such as cystine or cystamine, can also inhibit sGC activity (Katsuki *et al.*, 1977). Inhibition by cystamine is reversible with dithiothreitol in purified rat lung extracts, although this ability very much depends on the oxidation state of sGC (Ignarro *et al.*, 1981). The concentration-dependent nature of these inhibitory agents (Craven & DeRubertis, 1978) implies that the catalytic site of sGC is regulated by one or more SH groups (Braughler Jr., 1983), one dealing with basal (ie. preincubation in oxygen atmosphere) activity and the other with agonist (NO· activated)-stimulated activity.

1.5.3 VSM Contraction

Basal $[Ca^{2+}]_i$ is estimated to be ~100nM in VSM cells, although this varies significantly depending on experimental conditions (eg. stretch, temperature) (Morgan & Sue-Matsu, 1990). An increase in free $[Ca^{2+}]_i$ is a prerequisite for

VSM contraction. This can be established via receptor-or voltage-dependent channels which allow influx of extracellular Ca^{2+} through the sarcolemma (SL) or by Ca^{2+} release from the sarcoplasmic reticulum (SR). The release of Ca^{2+} from the SR is mediated via certain G-protein receptors such as α_1-adrenoceptor and angiotensin II that stimulate phospholipase (PLC) to hydrolyse phosphatidylinositol 4,5, bisphosphate (PI-P_2) to inositol trisphosphate (IP_3; usually the 1,4,5 isomer) and 1,2, diacylglycerol (DG). DG is a potent activator of phosphokinase C (PKC) (Weinheimer *et al.*, 1986) although the consequence of this is poorly understood. It has been suggested that PKC can inhibit EDRF release and also phosphorylate structural components within myosin light chains (Ohanian *et al.*, 1990). This may be important for sustained vasoconstriction.

Ca^{2+} can be pumped into the cell by Ca^{2+}-stimulated ATPases (Ca^{2+}-ATPase) distributed in the SL and SR. IP_3 binds to specific receptors on the SR, releasing Ca^{2+} into the cytosol without influencing SL channel activity (Somlyo *et al.*, 1985).

1.5.3a Contractile Proteins

The trigger for muscle contraction in VSM is the Ca^{2+}-binding protein, calmodulin (CaM), as opposed to troponin C in skeletal and cardiac muscle. Four Ca^{2+} ions complex wth CaM, activating myosin light chain kinase (MLCK; Walter, 1989) to phosphorylate 20KDa myosin regulating light chains (MLC). The phosphorylation of MLC triggers actin-myosin crossbridge cycling and hydrolysis of ATP → ADP+Pi (Moreland, 1990). Ca^{2+}/CaM-stimulated phosphorylation of MLC is associated with the initial phase of VSM contraction (Rasmussen *et al.*, 1987).

1.5.4 Regulation of Myosin Phosphorylation by cGMP- Relaxation

Myosin phosphorylation is mediated by a number of factors including cyclic nucleotides, cAMP and cGMP. However, endothelium-dependent VSM relaxation is preceded by an increase in cGMP but not cAMP (Furchgott *et al.*, 1984). Increases in VSM [cGMP] results in a wide range of target intracellular proteins being phosphorylated by cGMP-dependent kinases (PKGs) (Fiscus *et al.*, 1984) which, under physiological conditions are thought to relax VSM by decreasing $[Ca^{2+}]_i$ (McDaniel *et al.*, 1992). The mechanisms participating in reducing $[Ca^{2+}]_i$ are outlined below.

1) Increased [cGMP] stimulates ATPase-mediated extrusion of $Ca^{2+}{}_i$ and Na^+/Ca^{2+} exchange (ie. Na^+ influx and Ca^{2+} efflux) through the SL (Popescu *et al.*, 1985; Furukawa *et al.*, 1991). Accelerated Ca^{2+} extrusion can be initiated by SNP in the rat aorta (Magliola & Jones, 1990).

2) The level of agonist-stimulated phosphoinositide turnover can be decreased by a downregulation of G-protein activity and uncoupling of activated G-protein to PLC (Hirata *et al.*, 1990).

3) The Ca^{2+} pump in the SR of VSM cells is regulated by protein kinases ie. cAMP-dependent kinase (PKA) and cGMP-dependent kinase (PKG) which phosphorylate certain proteins including phospholamban (Cornwell *et al.*, 1991). Phospholamban is thought to decrease $[Ca^{2+}]_i$ by activating the SR Ca^{2+} pump and sequestering cytosolic Ca^{2+} (Twort & Van Breeman, 1988).

4) Upregulated cGMP, or application of the cGMP derivative 8-bromo-cGMP, inhibits voltage-dependent long-lasting (L)-type Ca^{2+} channels without influencing transient (T)-type Ca^{2+} channels (Bkailey *et al.*, 1988). Receptor-operated channels (ROC) are also inhibited by PKG (Collins *et al.*, 1986). In contrast, Ca^{2+}-dependent K^+ channels are opened by PKG and may explain the resulting membrane hyperpolarisation which often occurs in VSM cells.

5) Changes in membrane potential can interfere with Ca^{2+} motility. Hyperpolarisation closes voltage-operated Ca^{2+} channels (Nelson *et al.*, 1990) and reduces IP_3 formation and the subsequent mobilization of Ca^{2+} from SR stores (Itoh *et al.*, 1992).

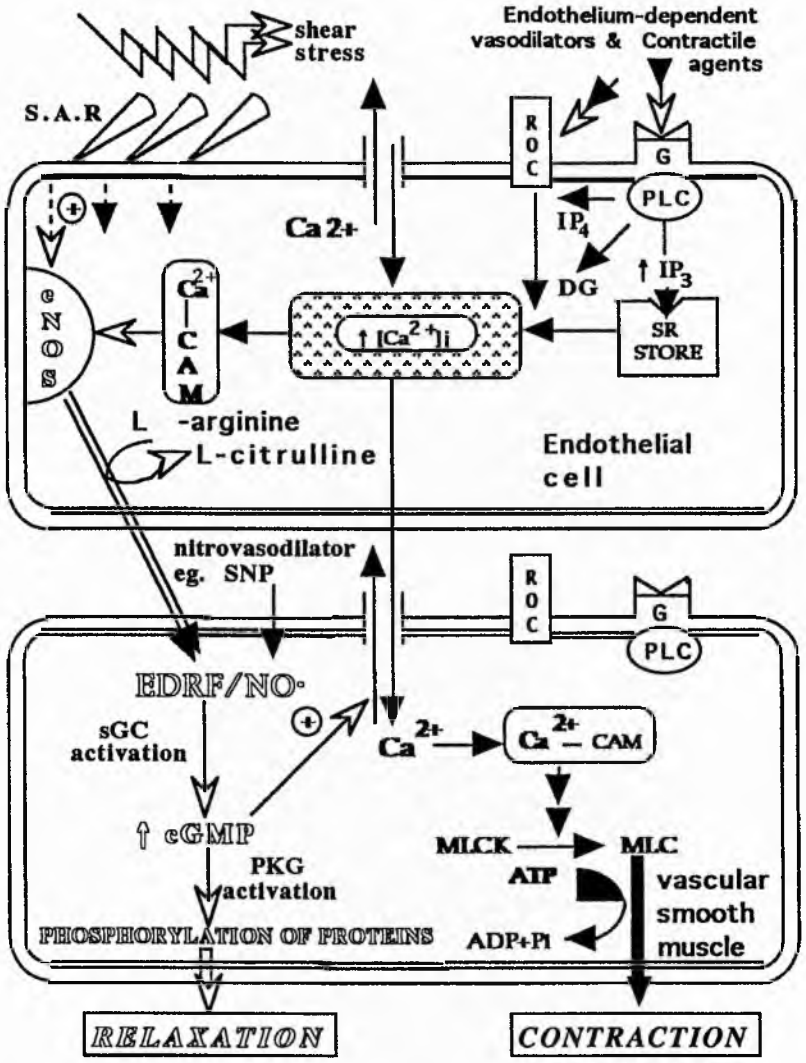


***Fig. 1.1** Diagram of the mechanisms relating to relaxation/contraction in vascular tissues.*

The hydrolysis and inactivation of cGMP and cAMP is performed by specific cyclic nucleotide phosphodiesterases (PDEs). Increased $[cGMP]_i$ leads to inhibition of a cGMP-dependent cAMP-PDE (CGI-PDE), elevating $[cAMP]_i$ in platelets (Maurice & Haslam, 1990) and rat aorta (Lindgren *et al.*, 1990). SNP acts synergistically to isoprenaline-induced increases in cAMP in VSM cells.

CGI-PDE inhibition by increased cGMP is thought to be responsible for this effect (Maurice & Haslam, 1991).

1.6 Other Physiological Roles of NO

NO is involved in numerous processes other than regulation of cardiovascular tone. For the purposes of this introduction only a select few are mentioned.

1.6.1 NO in the Central Nervous System (CNS)

The widespread distribution of NOS within the brain indicates that NO may be involved in most aspects of CNS function. Perhaps of most interest in recent years has been the notion that NO regulates synaptic plasticity. Stimulation of excitatory amino acid receptors eg. NMDA, (the glutamate subtype, N-methyl-D-aspartate) Ca^{2+}-dependently increases NO release to mediate long term potentiation of synaptic transmission (LTP; Schuman & Madison, 1991) or long term synaptic depression (LTD) by supressing the sensitivity of AMPA receptors (the glutamate subtype, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) (Shibuki & Okada, 1991). LTP and LTD are considered elements of experience-driven synaptic network remodelling that may underlie learning and memory.

Under pathological conditions of ischaemia, NOS is no longer exclusive to neurons but can be detected in astrocytes (Wallace & Bisland, 1994) and microglial cells which may promote neuronal cell death (Boje & Arora, 1992). This idea is strengthened by evidence that N^{ω}-substituted L-arginine analogues have been shown to reduce neuronal damage associated with cerebral ischaemic models in the rat (Buisson *et al.*. 1992; Nagafugi *et al.*, 1992; Garthwaite & Boulton, 1995).

1.6.2 NO in the Peripheral Nervous System (PNS)

Two major neurotransmitters in the PNS are acetylcholine and noradrenaline although certain peripheral nerves use neither and are termed nonadrenergic

(NA) noncholinergic (NC) or NANC nerves. NO was discovered to be the neurotransmitter in NANC nerves influencing peristaltic function of the stomach, gastrointestinal tract, respiratory tract and relaxation of the corpus cavernosum to facilitate penile erection. (Rand & Li, 1995).

1.6.3 Immune and Inflammatory Systems

Almost every cell in our body is able to express calcium independent NOS (iNOS) during cell-mediated immune responses (Nathan & Hibbs, 1991). The high levels of NO produced offer a non-specific line of defence against bacteria, parasites and viruses. NO is also thought to suppress T lymphocyte-mediated antigen-specific immune responses (Lepoivre *et al.*, 1995). NO can therefore be regarded as a modulator of the immune response in humans.

NO formation is increased during inflammation (arthritis, colitis, Crohn's disease), although endogenous release appears to have both pro- and antiinflammatory effects. Proinflammatory functions relate to the self-destructive nature of high NO production as demonstrated in autoimmune disease, immune rejection of allografted organs and sepsis. These are off-set by the antiinflammatory effects of NO such as inhibited neutrophil adhesion to vessel walls (Kubes *et al.*, 1991) and cytokine formation.

The role of NO in fighting cancer is discussed in Part C and its role in sepsis is further discussed in Part D.

1.7 Cellular Interactions of NO. Cytotoxic Implications

The interactions of NO with non-haem iron accounts for many of the cytotoxic effects of activated macrophages on tumour cells (Hibbs Jr. *et al.*, 1990) and microbes (Nathan & Hibbs Jr., 1991). Indeed, the non-specific cytotoxicity of

NO can also inhibit the activated macrophages themselves (Drapier & Hibbs Jr., 1988).

High levels of NO potently inhibit mitochondrial respiration and energy metabolism by interacting with non-haem iron in certain vital enzymes, including mitochondrial aconitase (a key enzyme of the Krebs cycle), NADH: ubiquinone oxidoreductase (complex I) and succinate: ubiquinone oxidoreductase (complex II; involved in mitochondrial respiration; Hibbs Jr. *et al.*, 1990). The removal of intracellular iron is critical to this inhibitory effect and is reversible by replacing the lost iron (Drapier & Hibbs Jr., 1986). The profound disruption of iron metabolism stems from NO's ability to complex with transferrin and promote iron exocytosis from the ferritin store (Reif & Simmons, 1990). The resultant loss of intracellular iron causes cytostasis (inhibited cell growth and proliferation) and cytotoxicity.

NO mediated activation of adenosine diphosphate (ADP)-ribosylation may also influence energy metabolism (Zhang *et al.*, 1994). ADP-ribosylation is initiated by the activation of intracellular polyADP-ribosyltransferase which may contribute to NO mediated neurotoxicity *in vitro* by inhibiting the important glycolytic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Zhang *et al.*, 1992). The inhibition of GAPDH decreases ATP formation by glycolysis and reduces the flow of substrates to the electron transport chain, ultimately resulting in cell death. The cytotoxic effects of NO in pancreatic islet cells can be blocked using inhibitors of polyADP-ribosyltransferase such as nicotinamide (NA; Kallmann *et al.*, 1994). NA is also a precursor of NAD^+ (the required substrate of the PolyADP-ribosylation reaction). PolyADP-ribosyltransferase repairs DNA using NADH which is converted to NAD^+. The subsequent depletion of available substrate (NAD^+) severely limits ATP synthesis. Whether NA inhibits NO mediated cytotoxicity by replenishing NAD^+ or preventing ATP

inhibition is not clear. It is also unclear whether GAPDH inhibition is responsible for cytotoxic effects outside the brain.

Ribonucleotide reductase (RNR), the rate limiting enzyme catalysing the synthesis of DNA, is reversibly inhibited by NO in murine adenocarcinoma cells (Lepoivre *et al.*, 1992). Whether other cells apart from macrophages can release sufficient NO to inhibit DNA synthesis is questionable, although VSM cells can be stimulated to release high levels of NO which inhibit mitochondrial respiration (Geng *et al.*, 1992). It has also been suggested that an endothelium-derived factor which is not NO can suppress intra-arterial DNA synthesis (DeMey *et al.*, 1991). This will be important when considering contractile properties of intact and denuded arterial preparations. It is also important to realise that cytotoxicity will also be mediated by other oxides including O_2^- and $ONOO^-$ (Section 1.3.2). This is evident from reports which show that aconitase inactivation is mediated by the above and not by NO (Hausladen & Fridovich, 1994).

Part B

Nitric Oxide Synthase

1.8. Introduction

NO radical is generated by a family of isozymes called nitric oxide synthases (NOSs) which are expressed throughout a diverse range of mammalian cells, including the endothelium (Palmer & Moncada, 1989) and vascular smooth muscle (Busse and Mulsch, 1990), cells in the brain (Bredt *et al.*, 1990a), the liver (Geller *et al.*, 1993), kidneys (Tracey *et al.*, 1994), lungs (Tracey *et al.*, 1994), heart (Schulz *et al..*, 1992), digestive system (Timmermans *et al.*, 1994) and also various cells associated with the immune response, including macrophages (Nathan *et al.*, 1991), Kupffer cells (Billiar *et al.*, 1989), and neutrophils (McCall *et al.*, 1989). In fact NO has been found in nearly every rat or murine tissue so far studied.

The catalytic conversion of L-arginine amino acid to NO and L-citrulline by NOS is thus far thought to predominate amongst mammals. However, within the next few years it is possible that other NOSs will be identified in species further down the evolutionary chain. Animals other than mammals can produce NO but usually do so without the use of NOS: for instance, microbes reduce nitrite or oxidise ammonia to produce NO (Feldman *et al.*, 1993). The many physiological roles governed by NO in mammals may explain why evolution has seen fit to select for the ability to synthesise NO from NOS, which offers a highly flexible and very powerful mechanism for generating NO on demand.

In 1985 mice with particular genetic abnormalities were found to possess macrophages which produced high levels of nitrite and nitrate (Steuhr & Marletta, 1985) and by 1988 it was realised that macrophages could be stimulated to produce NO (Marletta *et al.*, 1988).

Only in the past few years, following the initial purification of NOS from the rat cerebellum by Bredt *et al.* (1990b), has an appreciable understanding and characterization of the enzyme begun to emerge. With improved molecular techniques, more isoforms of NOS have been purified, cloned and in some cases functionally expressed. The purification and cloning procedures applied to these enzymes reveal much about their biochemical composition with regard to the presence of distinct regulatory sites that include 3 cAMP-dependent phosphorylation sites, as well as those for the necessary cofactors: nicotinamide-adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and (6R)-5,6,7,8-tetrahydrobiopterin (BH_4). Molecular cloning of brain (Bredt *et al.*, 1991), macrophage (Lowenstein *et al.*, 1992) and endothelial (Lamas *et al.*, 1992) NOS's has permitted the localization of their respective mRNAs by *in situ* hybridization. Analysis of the precise amino acid sequence in NOS reveals the compartmentalisation that exists in every isoform with the reductase domain at the carboxy-terminal and the oxygenase domain at the amino-terminal. Ultimately, studies detailing the quaternary structure of NOS will help to uncover exactly how it interacts with its substrate and the various prosthetic groups which make up the complete dimeric molecule.

In conjunction with the ability to purify NOS, new technology allowed polyclonal and monoclonal antibodies to be made which are specific to particular isoforms. These antigen-antibody complexes can be visualised immunologically, either histochemically with antibodies conjugated to fluorescent markers, eg. fluorescein isothiocyanate (FITC), or biotinylated to a 2^o antibody which is itself labelled, ie. the avidin-biotin complex (Kobzik & Schmidt, 1996). A more definitive confirmation of antigenic sites can be obtained with Western analysis of protein extracts following electrophoresis (sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SDS-PAGE) onto nitrocellulose membranes (Mayer *et al.*, 1990). A positive band on the membrane can be detected using a

number of methods, including enhanced chemiluminescence (ECL). The activity of NOS can also be assayed directly. This involves using radiolabelled substances, eg. by monitoring the production rate of L-[^{14}C] citrulline from L-[^{14}C] arginine and determining the concentration of L-[^{14}C] citrulline at a given time (Galea *et al.*, 1992). An alternative method for measuring the activity of NOS indirectly involves analysing the levels of nitrite/nitrate production using the Griess reaction (Green *et al.*, 1982).

The ability to purify the different isoforms of NOS has revolutionised the study of NO producing cells and tissues and it is now clear that the use of specific antibodies is far superior to many techniques previously used. Histochemical techniques of detecting NOS activity are still widely used, the most common of which detects NADPH-diaphorase. NADPH-diaphorase staining is a cheaper alternative to antibodies for the detection of NOS activity (Weinberg *et al.*, 1994; Weinberg *et al.*, 1996), although the specificity of NADPH-diaphorase has come under considerable scrutiny since its introduction by Thomas and Pearse in 1961. Much of this scrutiny has been dismissed since the discovery that neuronal NOS (nNOS) is an NADPH-diaphorase (Dawson *et al.*, 1991). Although it is clear that NOS possesses NADPH-diaphorase activity in the presence of the electron acceptor, nitroblue-tetrazolium (NBT), it should be noted that not all NOS isoforms coincide with NADPH-diaphorase staining (Tracey *et al.*, 1993), suggesting that not all diaphorase staining represents NOS activity.

1.9 Isoforms of NO Synthase

Recent biochemical studies and analysis of cDNA amino acid sequences reveal that NOS isoforms are highly conserved (80-94% homology) from one species to the next (Sessa, 1994), a feature that implies the existence of a NOS gene family which, although conserved, are also diverse in both distribution and roles: (vascular signalling:-endothelium, cytotoxicity:- macrophage and neurotransmission:-brain; see Section 1.1-1.7). These regions encode the binding

sites for the oxidative cofactors involved in regulating activity of NOS. All isoforms of NOS so far discovered display binding sites for NADPH, flavin adenine dinucleotide, flavin mononucleotide and (6R)-5,6,7,8-tetrahydrobiopterin (BH_4) (Katusic & Cosentino, 1994).

Type	Comparative Homology	Required Cofactors	Regulators	Potent Inhibitors	M_r (KDa)	Loci	Ref
Constitutive (cytosolic) bNOS- I_a	Significant homology to cytochrome P-450 reductase.	NADPH,FAD, FMN,O_2,BH_4, reduced thiols	Ca^{2+}/CaM, protein kinases	L-NAME, L-NMMA, L-NARG	155-160	Brain, cerebellum, neuroblastoma cells human epithelial-type tumour cells	1
Constitutive (cytosolic) eNOS- I_b	50-60% sequence identity with rat brain and murine macrophage isoforms	NADPH,FAD, FMN,O_2,BH_4, reduced thiols	Ca^{2+}/CaM	L-NAME	-	Vascular endothelial cells (VECs)	2
Constitutive (cytosolic) cNOS- I_c	-	NADPH,FAD, O_2,BH_4	Mg^{2+}, Ca^{2+}. Stimulated by receptor agonists eg. leukotriene B_4	L-NAME	150	Neutrophils	3
Inducible (cytosolic) iNOS- II	50-60% sequence identity with bNOS.	NADPH,FAD, FMN,O_2,BH_4, reduced thiols	LPS, NO, cytokines, IRF-1, NkB. Sensitive to CaM antagonists	Dexamethasone, aminoguanidine, L-NMMA	125-135 150	macrophages, VSM, tumour cells, neurons astrocytes, microglia, kupffer cells, endothelial cells	4
Constitutive (membrane-bound) eNOS- III	95% sequence identity with human VEC eNOS- I_b	NADPH,FAD, FMN,O_2,BH_4	Ca^{2+}/CaM, myristylation for membrane association	L-NAME, L-NMMA, L-NARG	135	VECs	5
Inducible (membrane-bound) iNOS- IV	-	BH_4, NADPH	-	L-NMMA	-	macrophages	6
Inducible (cytosolic) iNOS-V	-	-	IL-1β, Ca^{2+}-dependent	L-NMMA, EGTA	-	Chondrocytes, cartilage	7

Abbreviations; 5,6,7,8-tetrahydrobiopterin (BH_4), flavin-mononucleotide (FMN), nicotinamide-adenine-dinucleotide (NADPH), flavin-adenine dinucleotide (FAD), interleukin-1β (IL-1β), nuclear transcription factor (Nkβ). References; [1]Schmidt *et al.*, 1991, [2]Förstermann *et al.*, 1991, [3]Yui *et al.*, 1991, [4]Lowenstein *et al.*, 1992, [5]Lamas *et al.*, 1992, [6]Förstermann *etal.*, 1991, [7]Palmer *et al.*, 1992.

***Table 1.1** NOS Isoforms*

Three separate genes exist encoding three isoforms of NOS. These genes are distinct from one another. This is manifested by their rather modest (50-60%) comparative homology (Nathan & Xie, 1994). In humans the genes for neuronal/epithelial NOS, cytokine-inducible NOS and endothelial NOS are located on chromosomes 12, 17 and 7 respectively (Förstermann *et al.*, 1994). These three isoforms can broadly be categorised into 2 main groups; a constitutive group (cNOS), containing the neuronal NOS (nNOS) located in the brain together with an endothelial located NOS (eNOS). The second group contains the inducible isoform of NOS (iNOS) which is expressed in macrophages (macNOS).

cNOS isoforms are strictly regulated by binding of the calcium/calmodulin complex (Busse & Mulsch, 1990) and is usually associated with the sustained

release of small (basal) amounts of NO during normal physiological activity (Griffith *et al.*, 1984). Under pathophysiological conditions, such as a stroke, the cNOS isoforms may produce significantly higher levels of NO, enough to become cytotoxic to nearby cells (Nowicki *et al.*, 1991). This increase in cNOS activity is dependent on a stimulus which acts either via a receptor, ie. a receptor-dependent agonist eg. acetylcholine, bradykinin, 5-hydroxytryptamine (serotonin), substance P or thrombin, or alternatively via a receptor-independent mechanism. (Furchgott & Vanhoutte, 1989). These receptor mediated agoinsts bind to the endothelial cell surface causing a rapid increase in intracellular calcium concentration and subsequent NO release. Interestingly the increase in calcium is far more transient than the resultant increase in NOS activity. This may be due to the activation of secondary-mechanisms such as activation of sodium/hydrogen exchange channels which increase intracellular pH levels (Fleming *et al.*, 1994).

Stimulated release of cNOS-derived NO is most commonly associated with the eNOS isoform, where receptor-independent, physical stimuli such as fluid shear stress have been shown to upregulate the expression of NOS activity in cultured endothelial cells (Busse *et al.*, 1994; see Section 1.5.1b). Indeed, the part of the gene involved in this stress-related release of NO has been discovered and clearly shows that shear stress can regulate the transcriptional expression of eNOS *in vivo* (Marsden *et al.*, 1992).

The physical attributes of eNOS which implement shear stress-induced regulation of NO release may be related to the fact that 95% of eNOS is particulate, whilst only 5% is present in the cytosol (Förstermann *et al.*, 1991). Note that although eNOS is found in the vascular endothelium of most organs and tissues (Förstermann *et al.*, 1993), eNOS has also been located outside the vascular endothelium, in CA1 neurons (O'Dell *et al.*, 1994) and kidney tubular epithelial cells (Tracey *et al.*, 1994). The 133KDa eNOS isolated and cloned

from bovine aortic endothelial cells possesses a saturated fatty acid myristate located in its amino terminal (Pollock *et al.*, 1992), either binding to a cellular membrane directly or indirectly via a myristyl protein receptor (Lamas *et al.*, 1992; Sessa *et al.*, 1992). Theoretically, due to the lack of evidence for any transmembrane domain regions, this myristylation binding site may anchor the enzyme to the plasma membrane (Busconi & Michel, 1993) and provide a means for the endothelium to react to small changes in blood flow (ie. fluid shear stress). Subsequent activation of eNOS to release NO into the underlying smooth muscle would enlarge the diameter of vessels and thus reduce the stress induced by blood flow.

Membrane attachment may also improve the coordination between agonists binding to their endothelial receptors and the subsequent agonist-induced release of NO. By minimising the distance between the membrane bound receptors and eNOS the time to react would be reduced. The precise reason for attachment of eNOS to the cell membrane is unclear. It will however, undoubtedly provide an ideal communication between intraluminal and extraluminal environments.

Neuronal NOS and iNOS are completely devoid of any membrane-associating elements and are therefore cytosolic in nature and usually found in the soluble fraction of cell or tissue homogenates (Nathan & Xie, 1994; Stuehr & Griffith, 1992). nNOS was the first enzyme to be purified and fully characterised from rat and purine cerebellum (Bredt *et al.*, 1991). Since then this same isoform has been localised to several cell types including, kidney macula densa cells, peripheral non-adrenergic non-cholinergic neurons, skeletal muscle, pancreatic cells (Bredt & Snyder, 1990; Förstermann *et al.*, 1993; Nathan, 1992) and some forms of epithelial cell (Kobzik *et al.*, 1993). Using sequential affinity chromatographic techniques on 2'5'-ADP sepharose and calmodulin agarose, the denatured monomeric protein displayed a molecular mass (M_r) of 150 to 160 KDa, whilst a molecular mass of around 279 was calculated for the native

protein (Schmidt *et al.*, 1991). This was the first indication that the nNOS enzyme was a homodimer consisting of two equally sized subunits each of ~155KDa. Recently it has been proposed that nNOS may exist as a monomer-dimer equilibrium polypeptide (Masters, 1994).

All NOS isoforms are now thought to be homodimeric in nature, including the inducible isoform which was purified from murine macrophages with a monomeric M_r of 130KDa and was dimeric under native conditions (Hevel *et al.*, 1991; Stuehr *et al.*, 1991a). Subunit dimerization is required to generate active iNOS. Individual subunits cannot synthesise NO but can initiate electron transfer from NADPH to acceptors eg. ferricyanide or cytochrome C (Baek *et al.*, 1993).

The potential to express iNOS is considered ubiquitus among all nucleated cells in the mammal and so fulfills a very general role in non-specific host defense (Karupiah *et al.*, 1993; Nathan & Hibbs Jr., 1991). Thus, a growing number of cells have been shown to express iNOS including human hepatocytes (Geller *et al.*, 1993) human cancerous cells of the brain (Fujisawa *et al.*, 1995) and cervix (Werner-Felmayer *et al.*, 1993), myocardium (Schulz *et al.*, 1992), neurons (Minc-Golomb *et al.*, 1994), glial cells (Simmons & Murphy, 1992; Wallace & Bisland, 1994), human (Sherman *et al.*, 1993) and murine tumour (Bastian *et al.*, 1994) cell lines, human bronchial epithelial cells (Felley-Bosco *et al.*, 1994), human (Janssens *et al.*, 1992) and rodent (Kilbourn & Belloni, 1990) vascular endothelium, human chondrocytes (Charles *et al.*, 1993), Kupffer cells (Billiar *et al.*, 1989), mesangial cells (Pfeilschifter & Vosbeck, 1991), smooth muscle cells (Geng *et al.*, 1994) and neutrophils (McCall *et al.*, 1991).

In contrast to rodent macrophages, the induction of NO from cultured human macrophages is proving curiously difficult, although recently these cells have been shown to express iNOS (Kobzik *et al.*, 1993). As the most prolific producer of NO during certain immunostimulated conditions in the human body (Iyengar

et al., 1987), iNOS is able to kill tumour cells and pathogens (section 1.7). The lack of discrimination by NO means that sometimes, when levels are high enough, more harm than good is done, as vessel walls become excessively dilated, systemic blood pressure drops, and patients can often die. This is thought to underlie the lethality associated with sepsis syndrome (Rees *et al.*, 1990). The expression of iNOS usually occurs in response to various inflammatory cytokines eg. IL-1β, TNF-α, IFN-γ, IL-2 and bacterial lipopolysaccharides (LPS; Chesrown *et al.*, 1994), different combinations of which may result in synergistic enhancement of iNOS expression (Kilbourn & Belloni, 1990). iNOS can also be expressed in some healthy tissues including fetal or adult lung (Kobzik *et al.*, 1993) and during pregnancy (Sladek *et al.*, 1993).

Like all forms of NOS, iNOS also requires the binding of calmodulin (CaM) for activity; however, iNOS CaM is normally tightly bound in a Ca^{2+}-independent and noncovalent manner (Cho *et al.*, 1992). This means the C-terminal domain and the N-terminal domain are always aligned and the enzyme is continually primed for full catalytic activity. Exceptions to the rule do exist with a CaM-free iNOS found in rat liver (Evans *et al.*, 1992) and a calcium-dependent iNOS in rabbit chondrocytes (Palmer *et al.*, 1992).

The activity of iNOS is regulated at many different levels, whether it be transcriptionally eg. nuclear factor (NF)-*K*B (Schreck *et al.*, 1992) which can be stimulated by NO itself (Lander *et al.*, 1993), or post-transcriptionally eg. with protein synthesis inhibitors like cycloheximide which can super-induce a 2.5 to 5 fold increase in iNOS mRNA expression in cultured cells and prolong the life of newly made iNOS mRNA (Oguchi *et al.*, 1994). Alternatively, non-transcriptional factors can regulate iNOS expression eg. cAMP-elevating agents (Gross *et al.*, 1994) or UV-B radiation (Warren, 1994).

The capacity of cycloheximide to super-induce and sustain the expression of iNOS mRNA appears to be unrelated to its ability to inhibit protein synthesis. It

is more likely to be associated with its ability to simultaneously super-induce the NF-*K*B transcription factor and/or the immediate-early genes; c-*fos* and c-*jun* . (Edwards & Mahadevan, 1992). The respective mRNA sequences of these genes, like iNOS mRNA, contain multiple AUUUA destabilizing elements (Asson-Batres *et al.*, 1994). The possibility that cycloheximide interferes with these sequences is becoming increasingly apparent. Conversely, the transforming growth factor (TGF)-β has been shown to infer mRNA instability (Vodovitz *et al.*, 1993) and so inhibits the expression of iNOS in cultured, immunostimulated V.S.M. cells (Durante *et al.*, 1994). It is therefore obvious that mRNA stability maybe the major site for regulating iNOS expression by cytokines. IFN-γ and LPS, like cycloheximide, can also regulate iNOS expression in macrophages both transcriptionally and post-transcriptionally, by increasing the expression of iNOS individually and by prolonging the half-life of iNOS mRNA from 1-1.5 hrs to 4-6hrs synergistically (Weisz *et al.*, 1994). However, the ability to stimulate iNOS expression appears to differ depending on whether the cells are in culture or in *ex vivo* preparation (Sirsjo *et al.*, 1994) and may require the presence of other factors involved in regulating the expression of the iNOS gene. One such factor, termed interferon regulatory factor-1 (IRF-1) is required for iNOS activation in murine macrophages (Kamijo *et al.*, 1994). The associated cytokine, IFN-γ, has also been shown to stabilize iNOS mRNA (Vodovitz *et al.*, 1993) and enhance transcription of the iNOS promotor construct (Xie *et al.*, 1993). Post-transcriptional modification, such as phosphorylation (Michel *et al.*, 1993) and myristylation (Pollock *et al.*, 1992), are likely to influence enzyme activity. However, iNOS is cytosolic in nature and there are no reports of phosphorylation of iNOS either *in vitro* or *in vivo*. It therefore appears that the regulation of NOS activity by phosphorylating calcium/CaM-dependent kinases is solely a property associated with the constitutive isoforms (Bredt *et al.*, 1991). Tyrosine kinases are however thought to be involved in regulating the activation of iNOS (Marczin *et al.*, 1993).

The most important post-translational modification allowing for the activation of iNOS is subunit assembly and the binding of cofactors. Essentially, for full enzymatic activity, iNOS, like the other isoforms, must have the necessary redox cofactors to ensure proper alignment of the monomeric subunits into dimeric formation, which in turn allow the correct electron exchange to occur between the oxidase (amino) and reductase (carboxyl) domains (Stuehr *et al.*, 1991a). Whether the exchange of electrons is between monomeric subunits of the same polypeptide, or between neighbouring proteins in a head to tail arrangement, is still under debate (Feldman *et al.*, 1993). However a recent study characterising macNOS presented evidence for a head to head subunit interaction between oxygenase domains (Ghosh & Stuehr, 1995). The presence of oxygen and adequate L-arginine are also required without which NO cannot be made properly and may result in uncoupled NADPH oxidation (electron loss). The uncoupling of electrons is far more prevelant in nNOS than in iNOS (Abu-Soud & Stuehr, 1993). This may reflect a protective mechanism to reduce the incidence of O_2^- production by iNOS which is more likely to happen with the large amounts of NO it produces compared to cNOS. Studies on substrate levels in plasma samples reveal the concentration of L-arginine to be in the range of 150-200μM (Stuehr & Grffith, 1992), which may appear to be ample, but added exogenous L-arginine can still increase vasodilation suggesting that L-arginine is limiting (Creager *et al.*, 1992). Measures to improve the availability of L-arginine for iNOS-activated NO release are present, as both the L-arginine Y^+ transporter system (Bogle *et al.*, 1992) and argininosuccinate synthetase activity (Hattori *et al.*, 1994) are upregulated with cytokine-induced increases in NO production. The enzyme responsible for the *de novo* synthesis of BH_4, guanosine triphosphate cyclohydrolase 1, is not normally expressed in unstimulated cells but is detectable in endothelial cells and vascular smooth muscle cells following exposure to LPS and IFN-γ (Gross & Levi, 1992) in a time course similar to iNOS expression (Hattori & Gross, 1993). Thus it is becoming increasingly

apparent that several other enzymes are induced simultaneously in a cell after an exposure to cytokines, in order to provide the cofactors required to sustain the high levels of NO associated with iNOS.

The well known affinity of haemoproteins for NO has prompted speculations that a negative-feedback effect of NO on iNOS activity occurs by binding as a sixth ligand to the iron of the heme cofactor (Griscavage *et al.*, 1993). Whether this occurs ***in vivo*** is still a matter of debate. Further characterisation of the stability of NOS-NO complexes is required before this question can be answered. The binding of L-arginine seems to weaken affinity of NO for the ferric heme in NOS but not the ferrous heme (Hurshman & Marletta, 1995).

In general the intra-isoform homology is appreciablely high from one species to the next eg. bovine eNOS displays 95% identity with human eNOS (Lamas *et al.*, 1992), although comparisons between iNOS isoforms from rodents with those from humans reveal significantly lower homology (Marletta, 1994). Surprisingly, several human inducible isoforms of NOS have been cloned, including one from hepatocytes (Geller *et al.*, 1993) and one from a colorectal adencarcinoma cell line (Sherman *et al.*, 1993) and were found to be essentially identical. It seems, therefore, that in general all human isoforms of iNOS are identical regardless of their origin, while significant differences exist when compared with the inducible isoforms of NOS in rodents. This may suggest that the human iNOS gene evolved completely independently from the iNOS gene in other animals. The difficulty in detecting iNOS in human macrophages and monocytes may indicate a reduced tendency to express this isoform in these cells and calls into question the validity of studying iNOS in rodents as a model for iNOS expression in humans.

1.10 Homology

Cloning and expression of the complementary DNA sequence for NOS from rat cerebellum and macrophages reveals a similarity (36% sequence identity) between them and the cytochrome P-450 reductase (CPR) enzymes (Bredt *et al.*, 1991. Indeed bNOS expresses CPR activity (Klatt *et al.*, 1993). The greatest similarity in amino-acid sequence is at the binding sites for NADPH, FAD and FMN, located in the carboxyl (C) terminal. The only other protein displaying this unique binding sequence is the bacterial enzyme, sulfite reductase (32% identity to NOS). The amino (N) terminal of NOS, which contains the heme binding site, shows no significant homology to any other known protein. NOS has a CPR type iron protoporphyric IX heme (White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992; McMillan *et al.*, 1992) and therefore reacts with carbon monoxide in the presence of NADPH to give an absorbance peak characteristic of CPR enzymes (λ_{max} of ~450nm) (White & Marletta, 1992). This spectral signature relegated to substrate-bound, high-spin CPR enzymes (Poules *et al.*, 1985) is conferred by ligation of a cysteine thiolate to the iron (Fe^{3+}) of the heme complex (Renaud *et al.*, 1993). The ten residue consensus sequence encoding for cysteine in CPR is apparently absent in NOS (Marletta, 1993), with the best correspondence seen at residues 409-417 for nNOS and 188-196 for murine macNOS (Poulas *et al.*, 1987). A cysteine thiolate ligand does however exist in native dimeric macNOS (Steuhr & Ikeda-Saito, 1992). The ability of Ebselen to inhibit eNOS (Zembowicz *et al.*, 1993) is thought to relate to its inhibitory effect on the transfer of elecrons from NADPH to FMN and its ability to break a cysteine-thiolate Fe^{3+} bond in some P-450 enzymes (Nagi *et al.*, 1989). This suggests that the presence of the cysteine-thiolate ligand in CPR and NOS is crucial for catalytic activity.

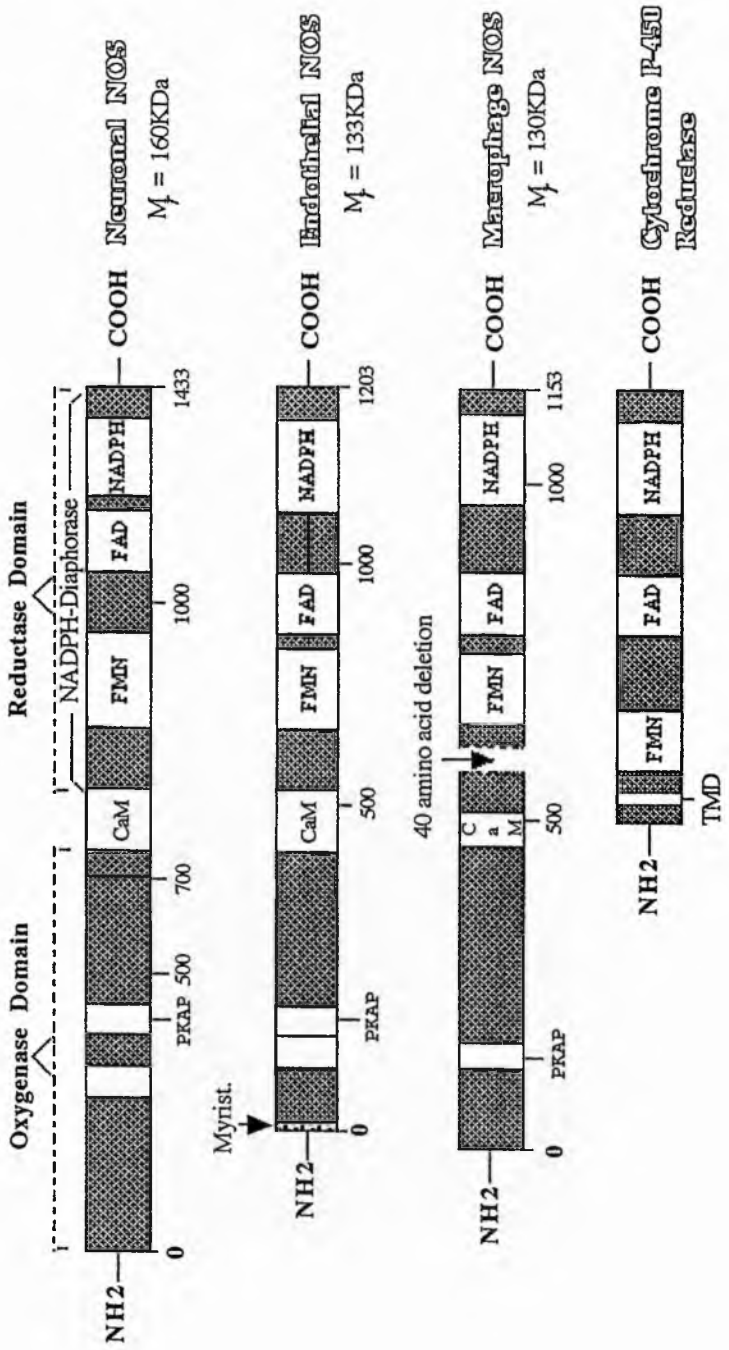


Fig. 1.2 *A schematic model showing the spatial relationships of the cofactor recognition site within NOS and CPR. In humans the genes for iNOS, nNOS and eNOS are found on chromosomes 17, 12 & 7 respectively.* ***Abbreviations:*** *N-terminal myristolation site (Myrist.), protein kinase A phosphorylation site (PKAP), transmembrane domain (TMD).*

The mechanistic roles of NOS and CPR are similar; both enzymes use the same two distinct heme-based oxidants during the course of NO formation and oxygen-activation respectively (Feldman *et al.*, 1993). Both enzymes are involved in single electron transfer during catalysis (Vermilion *et al.*, 1981) and can form superoxide anions under certain conditions. Due to the presence of similar iron-heme centres in NOS and CPR, and considering the high affinity between NO and the iron-heme, it is not surprising that NO can inactivate NOS and CPR to a similar degree (Griscavage *et al.*, 1993; Rogers & Ignarro, 1992; Stadler *et al.*, 1994; Khatsenko *et al.*, 1993). Furthermore, the heme prosthetic group of NOS, like the thiolate-ligated heme of CPR, is presumed to bind and activate molecular oxygen in order to hydroxylate substrates (Kwon *et al.*, 1990), although their respective substrates do differ; CPR substrates tend to be very hydrophobic and their active sites are designed accordingly. L-arginine, on the other hand, is very hydrophilic (Marletta, 1993).

Most CPR are not 'self-sufficient', requiring a flavoprotein reductase and an iron-sulfur protein to allow electron transfer to the heme group (Marletta, 1994). Bacillus megaterum (P-450$_{BM3}$), a fatty acid monooxygenase, is unusual in this respect by having a flavoprotein reductase and a heme group constitutively present (Narhi & Fulco, 1986). P-450$_{BM3}$ is therefore fully functional as a monomer and as such is an example of a 'self-sufficient' P-450 enzyme. The NOS monomeric subunit is not capable of catalytic self-sufficiency as the cofactor binding sites are shared between subunits. NOS must undergo dimerization to become active (Ghosh & Stuehr, 1995). Dimeric NOS contains dual flavoproteins and a heme group and thus could be considered as the first self-sufficient, mammalian, P-450-type enzyme.

The similarities between CPR and NOS led to suggestions that NADPH-diaphorase (N-dia) may be similar to cytochrome P-450 oxidase (Snyder, 1992). Neuronal NOS is identical to neuronal N-dia (Hope *et al.*, 1991), the activity of

which is represented histochemically by a blue/purple colour formed as nitroblue-tetrazolium salt (NBT) is reduced to NBT-formazan (NBTF). Because NOS and diaphorase are identical the substrate NBT can competitively inhibit bNOS activity. Surprisingly, N-dia activity in bNOS is not regulated by calcium/CaM binding (Schmidt *et al.*, 1992), suggesting that the diaphorase activity is not specific to NOS. The same appears to be true for CPR, which as part of the microsomal monooxygenase system in liver, is often associated with mitochondria (Bredt *et al.*, 1991). N-dia is not seen in mitochondria (Mizukawa *et al.*, 1988). NOS contains dihydropterin reductase which converts half quinone-dihydrobiopterin to half tetrahydrobiopterin (BH_4) and may represent the N-dia activity in NOS (Schmidt *et al.*, 1992b). The formation of BH_4 can partially reverse the inhibitory effect of NBT on NOS activity and may explain why iNOS activity, together with the associated increase in BH_4 production (Gross *et al.*, 1994), shows increased N-dia staining compared to cNOS. Rather than the N-dia staining (ie. NBTF formation) being due to the NOS actvity directly, it may be due to the upregulation of dihydropterin reductase and the increased BH_4 production which results. Unfortunately due to the diversity of oxidative-reductive activities that can provide a reduction of NBT, the activity of N-dia is difficult to characterise (Bredt *et al.*, 1991; Snyder, 1992).

In conclusion, much of our understanding regarding the biochemistry of NOS has come from studies involving CPR enzymes. Their roles as electron transferase enzymes are similar and their close homology (~60%) in the C terminal could suggest that in phylogeny CPR may have provided the electron transport necessary for NO activity. Alternatively, considering that the highly conserved motif that denotes a CPR is lacking in NOS (Marletta, 1993) it appears that rather than possessing all the necessary attributes which define a P-450 family member, as well as additional divergent characteristics which make it distinct, NOS may be converging in evolutionary terms towards the P-450 enzyme (Nelson *et al.*, 1993). NOS joins a number of other enzymes, including

thromboxane synthase, chloroperoxidase and allene oxide synthase, as proteins which display similar optical properties to P450-enzymes but are otherwise only distantly related.

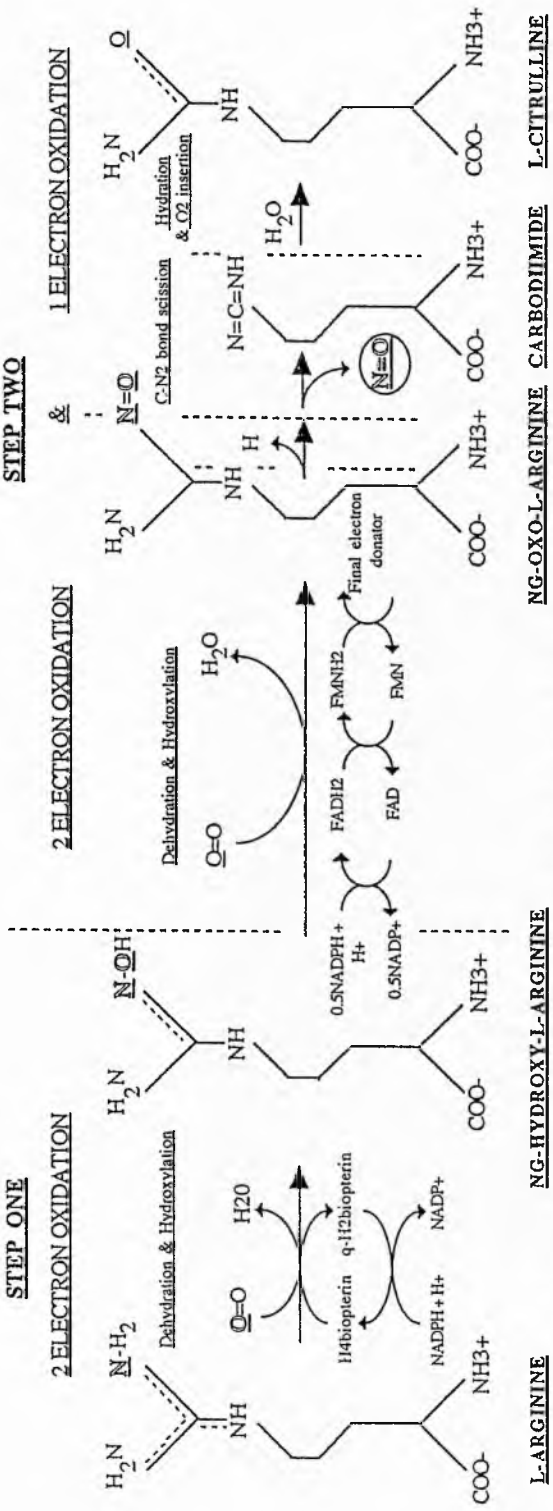
1.11 The L-Arginine/NO Pathway

NOS catalyses the five-electron oxidation of one of two equivalent guanidino nitrogens of the amino acid, L-arginine. This ultimately results in the conversion of L-arginine into equimolar amounts of L-citrulline and NO product (Nathan & Hibbs Jr., 1991; Steuhr & Griffith, 1992). The exact mechanisms of this reaction are not clear, though there are at least two definitive steps involved (see figure 1.3).

The first step involves the hydroxylation of L-arginine into N^{ω}-hydroxy-L-arginine. N^{ω}-hydroxy-L-arginine represents the enzyme-bound intermediate of this reaction (Campos *et al.*, 1995; Stuehr *et al.*, 1991b). This initial step is a two electron oxidation, converting 1 mole of the oxidative cofactor NADPH to $NADP^+$ and the removal of one oxygen atom from molecular oxygen (i.e dioxygen) to form water. The same oxygen atom involved in forming the water molecule is subsequently incorporated into one of the guanidino nitrogens of L-arginine, transforming L-arginine into N^{ω}-hydroxy-L-arginine. This step can therefore be described as an NADPH-dependent, monooxygenase hydroxylation reaction and the dioxygen can in essence be considered a cosubstrate here. (Kwon *et al.*, 1990). The use of N^{ω}-hydroxy-L-arginine intermediate by NOS is an enantiomer-specific reaction, as N^{ω}-hydroxy-D-arginine cannot be substituted as an intermediatory substrate (Campos *et al.*, 1995). Interestingly, N^{ω}-hyroxy-L-arginine is thought to be a potent inhibitor of arginase activity and this may prove crucial in regulating NO synthesis (Daghigh *et al.*, 1994; Boucher *et al.*, 1994). The inhibition of arginase activity would presumably allow more L-arginine for making NO rather than being converted to L-ornithine and urea (see figure 1.5).

The second step involves conversion of N^{ω}-hydroxy-L-arginine to equimolar amounts of L-citrulline and NO. The sequence of reactions is more complicated than the first step and involves a three-electron oxidation, with only 0.5 moles of NADPH being oxidized to $NADP^+$, insertion of oxygen into the ureido group of L-citrulline and finally scission of the carbon-nitrogen bond in N^{ω}-hyroxy-L-arginine. Homolytic cleavage between the oxidized guanidino nitrogen and carbon is thought to result in the release of the previously inserted oxygen atom (step one), together with the hydroxylated nitrogen atom. (Feldman & Griffith, 1993). The nitrogen and oxygen atoms ultimately form the nitric oxide molecule. Therefore L-citrulline and NO derive their respective oxygen atoms from two distinct molecules of oxygen, so the NOS enzyme requires two molecules of molecular oxygen for every catalytic cycle synthesising NO from L-arginine. This explains why the synthesis of NO is severely limited under anaerobic conditions, (>90%) (Steuhr *et al.*, 1991b).

Only recently have the finer details relating to the L-arginine/NO pathway come to light and predictably the intermediatory reactions (steps 1&2) of the pathway are becoming more complicated with time. Indeed, another intermediate is thought to exist called carbodiimide. This amino acid is hydrated to form L-citrulline (Marletta *et al.*, 1988).



Each catalytic cycle oxidizes 1 molecule of L-arginine and requires the cooxidation of 1.5 equivalents of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). This provides 3 electrons to NOS, 2 dioxygen molecules; providing oxygen atoms for the production of equimolar amounts of L-citrulline and NO. Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are thought to transfer and store electrons from NADPH. In theory two flavin nucleotides can store up to four electrons from two molecules of NADPH. FMN is thought to be the ultimate electron donator to the active heme centre of NOS. In vivo the active NOS enzyme is thought to contain one molecule of FAD and FMN and a heme complex (Iron-protoporphyrin IX heme) and a tetrahydrobiopterin complex (BH4) in both subunits. NOS is unique in being the only enzyme known to contain and use all four of these prosthetic groups.

Fig. 1.3 The proposed enzymatic pathway for nitric oxide synthesis from L-arginine.

1.12 Prosthetic Groups

The mechanisms of NO synthase in catalysis are still not fully understood. However, over the past ten years, considerable knowledge as been acquired regarding the role of the various cofactors which ultimately govern the activity of the enzyme.

1.12.1 Flavins

Purified inducible and constitutive isoforms of NOS have been found to contain tightly bound FAD and FMN associated with specific recognition sites encoded in the reductase domain of NOS (i.e. in the carboxyl half of the NOS molecule) (Feldman & Griffith, 1993). Molecular cloning studies reveal that each subunit of the enzyme contains 1 molecule of each of the flavin nucleotides (Stuehr & Griffith, 1992).

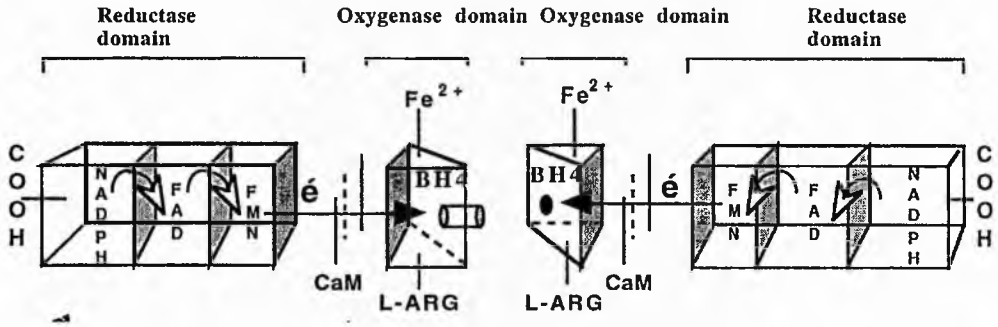


Fig. 1.4 *Schematic diagram of the quaternary structure of activated NOS. The binding of calmodulin (CaM) to cNOS is thought to align the domains, allowing electron transfer from FMN to haem. N.B. CaM is constitutively bound to iNOS.*

By comparing the functions of these flavins in other enzymes which show a striking similarity to NOS (eg. cytochrome P-450 reductase) it is postulated that FAD has the role of storing up to four electrons derived from NADPH (Bredt *et al.*, 1991). From there they are transferred to FMN and subsequently to the catalytic heme centre located within the oxygenase domain. Electrons are delivered one at a time to the heme centre and the flavins can accept one or two electrons from NADPH. The extra NADPH electron is stored from the first

catalytic cycle and is available for the second cycle (Campos *et al.*, 1995). By this process the active NOS enzyme contains a flavin radical (Stuehr & Ikeda-Saito, 1992) undergoing repeated redox cycles (3 cycles/mol. of NO): [FAD, FMNH·] → [FADH·, $FMNH_2$] → [FAD, $FMNH_2$] → [FAD, FMNH·]. Binding FMN and FAD in a single protein is a unique property of NOS and cytochrome P-450 reductase enzymes. This may facilitate the transfer of single electrons between the flavin rings, a requirement in NOS and cytochrome P-450 reductase radical catalysis.

It is worth noting that the consensus recognition sites for the oxidation cofactors are virtually identical in neuronal and macrophage type NOS, and so FAD and FMN may serve similar functions in both isoforms.

1.12.2 Tetrahydrobiopterin (BH_4)

Each subunit of active NOS contains one molecule of (6R)-5,6,7,8-tetrahydrobiopterin (BH_4) and sequence studies suggest that the BH_4-dependent site in NOS, derived from rat brain, is located between the FMN and calmodulin binding sites (amino acid residues 750-769; Uvarov & Lyashenko, 1995). This differs slightly from inducible NOS in which the BH_4 binding site is thought to be nearer the heme binding site, between the N-terminus and the calmodulin (Cho *et al.*, 1995).

Although the exact function of this cofactor remains unclear, both inducible and constitutive NOS have an absolute requirement for BH_4 in order to achieve full activation (Baek *et al.*, 1993). Intact cells use BH_4 to regulate NOS activity and therefore the levels of NO being produced (Werner-Felmayer *et al.*, 1993). This opens up the possibility of using BH_4 inhibitors to regulate NO production. Indeed, NO synthesis by the immune activated NOS can be inhibited dose-dependently by 2,4, diamino-6-hydroxypyrimidine (DAHP), a selective inhibitor of GTP-cyclohydrolase 1, the rate-limiting enzyme for *de novo* BH_4 synthesis

(Gross & Levi, 1992). The latter study also showed that BH_4 was limiting the rate of NO synthesis in V.S.M: by adding excess BH_4 the rate and onset of NO production could be considerably increased. Similarly, cytokine-activated murine brain endothelial cells require *de novo* protein synthesis for BH_4 production and increased NO synthesis (Gross *et al.*, 1991). The same appears to be true of constitutive NOS (Werner-Felmayer *et al.*, 1993). Not surprisingly, the induction of NO synthesis in these cells was inhibited by the protein synthesis inhibitor, cycloheximide. This suggests that guanosine triphosphate (GTP) is the main biosynthetic precursor to BH_4 involved in basal and inducible NO production in both endothelium and V.S.M. (Gross *et al.*, 1994). Cytokines, including IFN-γ and TNF-α, together with LPS can induce up to a 100 fold increase in the levels of GTP-cyclohydrolase 1 (Werner-Felmayer *et al.*, 1993). This LPS-induced increase in GTP-cyclohydrolase 1 and BH_4 in rat tissues correlate directly with increased levels of plasma nitrogen oxides and systemic vascular hypotension, indicative of increased NO production.

Cytokine-induced production of NO is suppressed by selective inhibitors of sepiapterin reductase, although the constitutive NO production associated with endothelial cells remains unaffected (Schoedon *et al.*, 1994). This implies that constitutive production of NO by endothelial cells derives much of its BH_4 from the GTP precursor, while iNOS requires both the pterin-salvage pathway and the GTP precursor. This may provide a means of selectively inhibiting the high levels of inducible NOS often associated with certain pathophysiologial conditions, without compromising constitutive NOS necessary for normal physiological function.

Although cytokines can induce BH_4 biosynthesis in human vascular endothelial cells most (up to 90%) of the BH_4 produced by the endothelium is not used locally but is directed at the underlying V.S.M. (Schoedon *et al.*, 1993). The resultant increase in cytokine-inducible NOS activity and NO release in the

V.S.M. facilitates vasodilatation. This means that in human studies of inducible NO release by V.S.M. the endothelium is required to be intact inorder for full iNOS activity in V.S.M. to be expressed. The same may not be true of non-human endothelial cells, but may reflect an inherent inability of human type endothelial cells to express an inducible, high output NOS (Schoedon *et al.*, 1993). If it is discovered that a similar situation exists in non-human vasculature (eg. rodent), then subsequent studies of NO release by V.S.M. would require the use of intact vessels.

Much of the work concerning BH_4 production has been carried out in animals, most commonly the rat or mouse, so it is important to realise that results obtained from these studies may not always correlate with those found in human studies.

Cultured rat aortic smooth muscle cells exposed to LPS can produce BH_4 via *de novo* synthesis of GTP (Gross *et al.*, 1994), although NOS activity is superinduced in these cells following inhibition of the GTP converting enzyme, GTP cyclohydrolase 1. This apparent contradiction may relate to a balance or interplay between two transcriptional processes associated with LPS induced NO production. The first involves the induction of NOS and GTP cyclohydrolase 1: If BH_4 synthesis is prevented by inhibiting the GTP *de novo* synthetic pathway (using DAHP), together with the pterin salvage pathway (using methotrexate), then NO synthesis is almost completely inhibited. Neither of these inhibitors interferes with the induction of iNOS. This situation results in a pool of newly formed iNOS which is unable to produce NO due to the lack of BH_4 and can be rectified by replacing BH_4.

A second process may exist by which GTP regulates NOS activity directly. This would conform with both situations. The ability to induce NOS activity in a given cell and limit BH_4 availability to determine when and how much NO is

produced will undoubtedly offer scope for clinical application. NO could be released as a cytotoxic agent against tumour cells where and when it was needed.

Exactly how BH_4 determines NOS activity, and to what extent this is due to a limited catalysis or a change in its structural configuration, remains elusive. NOS is unique in forming a stable bond with BH_4 (Schmidt *et al.*, 1995). In most other enzymes which require BH_4, like aromatic amino acid hydroxylases (*eg.* phenylalanine hydroxylase, where it serves to provide the 2 electrons necessary for oxygen reduction), the prosthetic group is freely diffusible in and out of the enzyme, allowing its interaction with any number of enzymes (Giovanelli *et al.*, 1991). Whether this property of BH_4 in NOS bears any relevance to its mechanism is still unclear, but a more stable bond between BH_4 and NOS may allow for a more permanent and definitive change in the shape of NOS. This would seem less likely if the BH_4 was diffusing freely between other enzymes. BH_4 may therefore serve to regulate NOS activity by rearranging the enzyme from its inactive monomeric state to its dimeric active state (Baek *et al.*, 1993). Indeed spectral analysis has revealed a significant shift in peak Soret absorbance when comparing BH_4-deficient NOS (λ_{max} = ~418nm) with BH_4-saturated NOS (λ_{max} = ~400nm) (Hevel & Marletta, 1992). This suggests a structural effect that is mediated by BH_4. It was subsequently found that the majority of pterin-deficient NOS exists in the inactive monomeric state, while NOS with added BH_4 is shifted to the dimeric state (Hevel & Marletta, 1992). However, the process of dimerization cannot be accredited to BH_4 alone, since it also requires the binding of L-arginine and heme (Baek *et al.*, 1993). Interestingly, NOS deficient of BH_4 is unable to couple the electrons continuing to be released by NADPH (Heinzel *et al.*, 1992). This suggests that electrons cannot flow through NOS without BH_4.

As previously mentioned, aromatic hydroxylases require BH_4 to carry out redox reactions. It has recently been suggested that BH_4 in NOS may also participate in

redox reactions (Mayer *et al.*, 1995), this theory is however controversial (Giovanelli *et al.*, 1991).

1.12.3 Heme Group

Heme (iron-protoporphyrin IX), like other prosthetic groups, is an integral part of the enzyme and perhaps the role most commonly attributed to this cofactor is the final electron acceptor during NO synthesis (Abu-Soud & Stuehr, 1993). The heme centre receives NADPH-derived electrons from FMN and so completes the transfer of electrons from the COO^- terminal (reductase domain) to the NH_2-terminal (oxidase domain). This transfer of electrons results in the reduction of heme: Fe^{3+}(ferric) $\rightarrow$ Fe^{2+}(ferrous) and is regulated by calmodulin (Abu-Soud & Steuhr, 1993). The flavin-to-heme electron transfer also requires NOS to be assembled into a dimeric conformation, which for the macrophage derived NOS (mac-NOS) may involve two monomeric subunits interacting at their NH_2, catalytic terminals (Ghosh & Stuehr, 1995). This process requires L-arginine, BH_4 and heme, with stoichiometric amounts of BH_4 and heme remaining bound after dimerization in order to bind the subunits together (Baek *et al.*, 1993).

Deficiences in any one of the cofactors necessary for dimerization may lead to electron uncoupling (Pou *et al.*, 1992). In such cases, electrons are not transported to the heme centre, but may instead be sequestered by electron acceptors eg. cytochrome C, ferricyanide or more commonly oxygen (Schmidt *et al.*, 1992a). The acceptance of stray electrons by oxygen can result in the production of superoxide anions (Saran *et al.*, 1990) which can then react with NO to form peroxynitrite.

With sufficient cofactors, the activated heme iron is able to bind and reduce O_2 (at the sixth ligand), facilitating both the oxidation of L-arginine to hydroxylated L-arginine (NOH-Arg) and ultimately the conversion of NOH-Arg to citrulline and NO (Pufahl & Marletta, 1993, Marletta, 1993). Corroborative evidence for this

comes from studies using carbon monoxide, which binds the active heme giving an absorbance maximum of ~446nm. This is also a feature of reduced CO complex formation found in cytochrome P-450 type hemes (Stuehr & Ikeda-Saito, 1992). Also, in common with P-450 enzymes, NOS hemes are bound to a cysteine (Cys) thiolate and recently the putative binding sites of heme in the N-terminal region have been identified with the fifth ligand attaching to Cys 194 (mouse iNOS), Cys 415 (rat nNOS) and Cys 186 (bovine eNOS) (Renaud *et al.*, 1993).

The affinity of hemoproteins to bind NO is well established (eg. the high affinity associated with the NO-mediated activation of gyanylate cyclase), so the possibility that NO may bind to heme and regulate NOS activity is not altogether surprising. What is surprising, however, is the lack of rapid self-in-activation related to NOS. NO can potentially form unstable nitrosyl complexes with both ferric and ferrous NOS hemes in the presence of O_2 (Marletta, 1994) and in this way inactivate NOS. The presence of the cysteine ligand in NOS may confer instability in these complexes, as hemoproteins with ligands other than cysteine (eg. haemoglobin, which has a histidine ligand), form stable heme-NO complexes under aerobic conditions. Exactly how this works is not known. In *vitro* assays reveal that substrate binding also influences the affinity of NO for NOS heme. L-arginine binding in iNOS, although not necessary for nitrosyl complex formation, reduces the affinity of NO for the ferric heme but not ferrous heme (Hurshman & Marletta, 1995). Similar results have been found for cNOS (Wang *et al.*, 1994). This suggests that L-arginine offers a measure of protection from NO binding in place of O_2, with less protection after the enzyme has been activated. Further analysis of the intermediate steps involved in NO synthesis will undoubtedly reveal other mechanisms designed to protect NOS activity from NO-mediated inactivation.

1.12.4 Calmodulin

Recognition sites for calmodulin (CaM) are present in neuronal NOS (nNOS) (Zhang & Vogel, 1994), endothelial NOS (eNOS) (Lamas *et al.*, 1992), and the inducible NOS in actived macrophages (macNOS) (Lyons *et al.*, 1992; Xie *et al.*, 1992). However, there are several differences in the consensus binding sites and proposed functions of CaM in these isoforms of NOS (Abu-Soud & Stuehr, 1993).

CaM is a single polypeptide with a molecular weight of 16,700 and is thought to be present in all eukaryotic cells so far studied. In mammalian tissues the concentration of CaM is thought to vary between 2 and 30μM (Carafoli, 1987). CaM has been found in a wide range of phylogenetically diverse species, yet the primary structure of CaM is so highly conserved that antibodies from bovine brain can cross-react with CaM in cotton seed (Wallace & Cheung, 1979). This suggests that CaM is one of the oldest members of the calcium-binding protein family. As a Ca^{2+} receptor, CaM is an integral part of many intra- and intercellular mechanisms that exist within any given tissue.

In NOS, CaM serves to regulate NO synthesis and does so through a variety of means (Schmidt *et al.*, 1991). The cNOS isoforms in endothelial cells (Busse & Mülsch, 1990) and neuronal cells (Bredt & Snyder, 1990), have an absolute requirement for Ca^{2+} and CaM. In contrast, inducible macNOS is apparently not regulated by intracellular Ca^{2+} although it does contain CaM (Cho *et al.*, 1992), and is regulated instead at the level of gene transcription (Xie *et al.*, 1992). The activity of purified macNOS is therefore neither enhanced by exogenous Ca^{2+} and CaM, nor inhibited by chelators of divalent cations or drugs that block the binding of CaM to its targets (Stuehr *et al.*, 1991; Yui *et al.*, 1991a). Constitutive NOS is inhibited by such inhibitors (Bredt & Snyder, 1990; Busse & Mülsch, 1990). Not all inducible forms of NOS conform to this rule; Ca^{2+}-dependent

(Palmer *et al.*, 1992) and CaM-dependent (Iidas, 1992) isoforms of inducible NOS also exist.

Polyacrylamide gel electrophoresis has revealed that all cNOS peptides bind to CaM with 1:1 stoichiometry (Zhang & Vogel, 1994). On binding the CaM takes on an amphipathic helical conformation, similar to the CaM involved in binding to the domains of myosin light chain kinases (Olwin *et al.*, 1984), ensuring an adequate interaction with its receptor proteins. The ability of CaM to assume a tertiary structure and display high flexibility may relate to the fact that NOS associated CaM does not contain cysteine or hydroxyproline (Cheung, 1980). Cysteine would restrict these conformational changes. Whether this binding pattern also exists for inducible NOS has not been addressed.

Urea gel electrophoresis and fluorescence spectroscopy have been used to identify the putative high affinity CaM binding sites associated with the NOS enzymes. The CaM binding domain is conserved in cNOS enzymes as a stretch of basic and hydrophobic amino acids and for nNOS this sequence exists between the 725-754 peptide residues (Vorherr *et al.*, 1993). The eNOS consensus binding site is situated between the peptide residues 493-513 (Lamas *et al.*, 1992). The eNOS consensus binding site is located at a similar amino acid sequence number to macNOS; the macNOS consensus binding site is at residues 503/4-532 (Lowenstein *et al.*, 1992) however 16 out of the 29 residues in the eNOS CaM binding site are different to macNOS. This suggests that the CaM binding sequences are genetically distinct from one another and will most probably display different binding affinities for CaM. The cNOS binding site is thought to share the same or similar region on CaM as a CaM-dependent phosphodiesterase (Zhang & Vogel, 1994). This is evident by the competitive inhibition that exists between these two enzymes.

nNOS does not exist as an enzyme bound to CaM. It is therefore inactive, but can be constitutively activated following binding of CaM with transient

increases in intracellular Ca^{2+} levels. Spectral anaysis indicate that this binding is not a prerequisite for L-arginine binding to nNOS (Abu-Soud & Stuehr, 1993). In contrast CaM is very tightly bound to macNOS (Stuehr *et al.*, 1991a). macNOS is one of the only four enzymes known to date to bind CaM in an apparently Ca^{2+}-independent manner (Vorherr *et al.*, 1993) and does not require the elevation of $[Ca^{2+}]$ above levels found in resting cells (400-1100 nM) (Olwin & Storm, 1985). Indeed, CaM is so tightly bound to macNOS that they copurify (Cho *et al.*, 1992), which would explain why inhibitors of CaM are unable to detach or interrupt CaM's interplay with macNOS. Three other enzymes which also display this characteristic are the *Y*-subunit of phosphorylase kinase (Picton *et al.*, 1980), a cyclic nucleotide phosphodiesterase (Laemmli, 1970) and the adenylyl cyclase of Bordetella pertussis (Ladant, 1988).

Cho *et al.*, (1992) were the first to suggest a role for CaM in regulating electron transfer and this was confirmed the following year by Abu-Soud & Stuehr, (1993). The idea is not altogether a novel one, nor is it unique to NOS. Evidence implies that the multimeric heme protein, sulfite reductase, may also use CaM to regulate electron transfer. As CaM is constitutively bound to macNOS it is continually primed to transfer NADPH-derived electrons onto its heme, requiring only the addition of NADPH to enable spontaneous reduction of the macNOS ferric-state heme (Abu-Soud & Stuehr, 1993). The addition of L-arginine to macNOS significantly increases the NADPH oxidation, however, in nNOS L-arginine binding slightly decreases NADPH oxidation. This suggests that electron transfer from NADPH in macNOS is dependent on substrate, in contrast to that of nNOS. This may enable nNOS to limit the amount of superoxide production in the absence of substrate. The substrate-dependent heme reduction in macNOS is also seen in cytochrome P450 enzymes (Gonzalez, 1989).

When considering the respective interactions of CaM with the different isoforms of NOS it is clear that the constitutive nature of CaM in macNOS is suited to release high levels of NO over prolonged periods of time, while the Ca^{2+}/CaM regulated cNOS is better suited to release short bursts of NO in response to humoral signals mediated by Ca^{2+} transients.

CaM will undoubtedly influence pathways related to NO synthesis. For instance, it can stimulate phospholipase A_2 activity in human platelets and may also regulate both the synthesis and degradation of 3',5'-cyclic adenosine monophosphate (cAMP) in the brain (Cheung, 1980). Agonist-induced increases in cAMP can inhibit LPS-induced NO synthesis (Hon *et al.*, 1995). CaM may also have an important role in cellular proliferation, as preventing it from binding and activating its target enzymes can attenuate the growth of malignant cells (Hait *et al.*, 1995).

1.13 L-Arginine and its Analogues

1.13.1 L-Arginine

L-arginine is an essential amino acid involved in a number of pathways relating to the metabolism of nitrogen, including the urea cycle and amino acid metabolism. L-arginine is required for polyamine biosynthesis, a process required for cell replication, and it also regulates a number of hormones in the body including insulin (Dupre *et al.*, 1968), pancreatic somatostatin (Utsumi *et al.*, 1979), adrenal catecholamines (Imms *et al.*, 1969) and growth hormones (Merimee *et al.*, 1965). L-arginine is therefore important for growth and development of the body, so requires rapid synthesis and recycling to satisfy demand.

In mammals the synthesis of L-arginine occurs predominantly in the liver from L-glutamate, although synthesis also occurs in proximal tubules of the kidney and is supplemented by an exogenous supply of L-arginine in the diet.

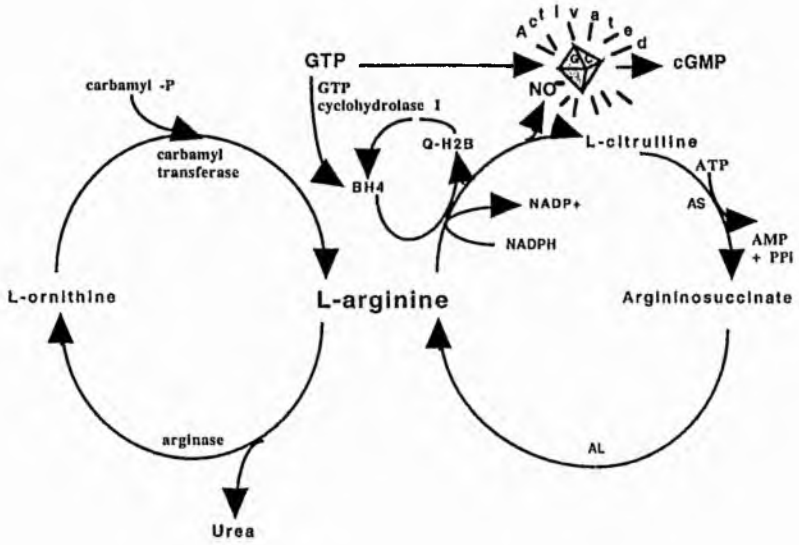


***Fig. 1.5** Schematic diagram of L-arginine metabolism.*

The biosynthesis of L-arginine in the liver is catalysed by two cytosolic enzymes: argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) and due to the high levels of arginase most of the arginine is quickly converted to urea and ornithine and so is not released into the circulation (Meijer *et al.*, 1990) (see figure 1.5 above). The complete urea cycle exists solely in the liver, and though the kidney also synthesises L-arginine from AS and AL, its incomplete urea cycle allows L-arginine to be released into the circulation for use elsewhere (Morris *et al.*, 1994).

AS and AL also function to recycle arginine from citrulline for the sustained high level NO production associated with inducible NOS. Immunostimulants can induce AS activity and mRNA expression in cultured vascular smooth muscle cells, macrophages (Hattori *et al.*, 1994) and vascular endothelial cells (Morris *et al.*, 1994). These cells also express iNOS and the timecourse for AS induction is similar to that for NOS (Morris *et al.*, 1994), with peak activity occuring around 24hrs after the initial stimulation. The ability to regenerate L-arginine offers an important means for providing adequate substrate during immune-activated high NO production. The potential for supressing 'overproduction' of NO, in

conditions such as septic/cytokine-induced shock, by regulating AS activity is still an avenue to be explored. However, the development of a specific inhibitor to inducible AS (iAS) may prove invaluable in discerning the relative importance of this specialised arginine-citrulline cycle in disease. N^G-monomethyl-L-arginine (L-NMMA) does not inhibit the LPS and/or IFN-γ-mediated expression of iAS in cultured aortic smooth muscle cells (Hattori *et al.*, 1994), suggesting that depletion of L-arginine is not a prerequisite for iAS expression. Indeed, excess L-arginine was found to potentiate iAS activity. It appears therefore, that AS joins an ever increasing group of enzymes which become active during the immune response.

Cycloheximide potentiates the induction of iNOS mRNA, but attenuates the induction of AS mRNA (Hattori *et al.*, 1994) which may result in a pronounced substrate deficiency and perhaps invoke an upregulated transport of L-arginine into the cells from the surrounding milieux to compensate.

A number of cell types when activated express iAS and AL *in vitro* and are also capable of transporting extracellular L-arginine, including murine macrophages (Bogle *et al.*, 1992), vascular endothelial cells and vascular smooth muscle cells (Stoclet *et al.*, 1994). The uptake of L-arginine is thought to be facilitated by the cationic amino acid transporter, system y^+, which is characterised by being a single/saturable receptor (k_m= 0.11-0.14mM; Sato *et al.*, 1992). Cytokines and endotoxin also potentiate the uptake of L-NMMA NOS inhibitor into human vascular endothelial cells (Bogle *et al.*, 1993). L-arginine competitively inhibits this uptake, suggesting that L-NMMA may enter cells via system y^+. In contrast N^ω-nitro-L-arginine methyl ester (L-NAME) probably enters via another route as its uptake is only slighty inhibited by L-arginine.

Why these cells should require increased uptake of L-arginine together with an increased synthesis of L-arginine is not clear. In macrophages, the L-arginine may be rapidly converted to urea and ornithine by arginase, thus limiting its use

for NO production (Chen & Broome, 1980). Whether extracellular L-arginine is required to replace recycled L-arginine, or whether the cycled L-arginine is part of a closed pathway is not known. Either way, the fact that two systems are adapted to provide L-arginine during cytokine or bacterial-induced activation highlights its importance and potentially limiting effect on the high output NO pathway.

The notion that arginine may influence the progression of cancer is by no means new. Since the beginning of this century investigators have looked at the effects of altered arginine uptake on the growth of experimental tumours in animals and found that arginine could either stimulate (Gilroy, 1930) or inhibit (Beard, 1942) growth, depending on experimental protocol. Further studies suggested that perhaps the inhibitory effects of arginine were specific to certain types of carcinogenically-induced tumour (Weisburger *et al.*, 1969; Takeda *et al.*, 1975). With a better understanding of the many physiological roles involving arginine it is becoming increasingly apparent that this 'double-edged sword' may reflect an imbalance in two metabolic pathways in which arginine is an intermediate (ie. the urea cycle and the L-arginine/NO pathway). The urea cycle involves the enzymatic conversion of arginine to ornithine and urea by arginase, while NOS converts L-arginine to L-citrulline and NO. The competition for arginine which exists between these pathways is evident by the increased urea synthesis which occurs in the presence of N^G-monomethyl-L-arginine (NOS inhibitor). High levels of NO are known to have cytotoxic/cytostatic effects on tumour cells (Hibbs *et al.*, 1987) and will therefore suppress tumour growth, while ornithine promotes cellular proliferation, DNA synthesis and tissue regeneration, so will favour tumour growth. (Haddox & Russell, 1981). Consequently, it is thought that malignant cells require a higher concentration of arginine than normal cells to maintain their high cellular turnover (Currie & Basham, 1978).

Immune-activated macrophages, which are known to express iNOS and arginase activity (Albinar *et al.*, 1988), also require high levels of arginine, and are thought to be instrumental in modifying tumour growth (Mills *et al.*, 1992). It appears a rather unfortunate quirk of nature perhaps, that arginine should be required by tumour cells as well as by the macrophages which are designed to kill them.

1.13.2 L-Arginine Binding to NOS

On binding to the NH-terminal of NOS, L-arginine facilitates a stiochiometric activation of the enzyme by assembling the dimeric structure and allowing electrons to flow through the reductase terminal onto the heme group (McMillan & Masters, 1993). As with BH_4, the binding site for L-arginine within NOS remains unidentified, although both appear to be close to the ferric iron of the heme group (Wang, 1994) between the NH-terminal and calmodulin. This may include residues 194 to 503 in the macrophage associated NOS (Ghosh & Stuehr, 1995) although this varies between isoforms. The region thought to contain the binding sites for L-arginine, BH_4 and heme is, however, highly conserved, with 65-71% sequence identity between the three main isoforms of NOS (Hurshman & Marletta, 1995).

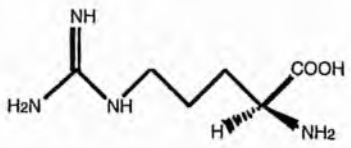
Similarly, all isoforms of NOS display enantiomer-specific binding of arginine, such that L-configuration at the α-carbon is required for substrate activation (Moncada, 1989). D-arginine is therefore not a substrate for NOS. Although L-arginine is the only 'true' physiological substrate for NOS, the enzyme displays considerable flexibility to accomodate a number of different L-arginine-containing peptides which can substitute for L-arginine. These include L-homoarginine, L-arginamide and L-arginyl-L-aspartate (Hibbs *et al.*, 1987). With plasma levels ranging from 150 to 200μM, L-arginine is by far the most abundant of these molecules (Stuehr & Griffith, 1992). If L-arginine is deficient,

the biosynthetic intermediate N^G-hydroxyl-L-arginine can also act as substrate for both iNOS and cNOS (Hecker *et al.*, 1991).

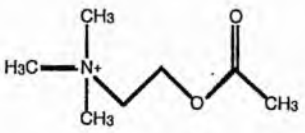
1.13.3 Analogues of L-Arginine. NOS Inhibitors

The discovery in 1988 that NO is synthesized in porcine vascular endothelial cells from the terminal guanidino nitrogen atom(s) of L-arginine (Palmer *et al.*, 1988) led to the idea of using substituted analogues of L-arginine to regulate NO production.

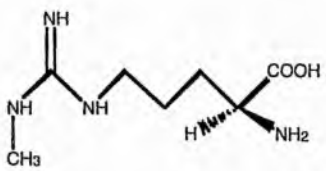
L-ARGININE



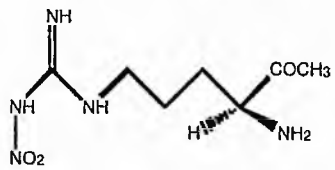
ACETYLCHOLINE



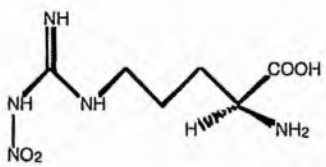
L-NMMA



L-NAME



L-NA



AG

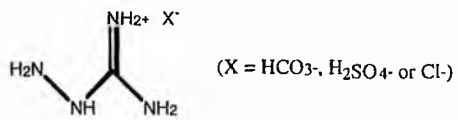


***Fig. 1.6** Some analogues of L-arginine.*

The biological actions of NO can be regulated either directly, by interfering with the synthesis of NO from the NO-generating cell itself, or indirectly by inhibiting the influence of NO on its target cell. Direct inhibition of NO synthesis by the generator cell is usually achieved by disabling NOS, while indirect inhibition involves binding the NO released to a compound. For example haemoglobin (Hb) scavenges NO from the surrounding milieu to form nitrosylated-haemoglobin (HbNO) and thus prevents it from interacting with its target cell.

Examples of generator-target cell 'couples' include endothelium with VSM and macrophages with tumour cells. In the first, endothelial-derived NO diffuses into the VSM where its molecular target is gyanylate cyclase (GC) and by binding to GC, initiates VSM relaxation. In the second, activated macrophages release high levels of NO which can enter tumour cells and cause an array of metabolic dysfunction eg. inhibition of mitochondrial respiration, cellular iron loss, inhibition of DNA synthesis (Hibbs *et al.*, 1988). In cases where the tumour cells are unable to synthesise NO, nitrosylation of iron proteins by macrophages illustrates a paracrine interaction between the two cell types.

With the increasing number of inhibitors available which display modest selectivity towards either the constitutive or inducible isoforms of NOS (Marletta, 1994), regulation of NO activity by inhibiting its synthetic enzyme promises to be of considerable therapeutic advantage. High output NO derived from iNOS is implicated in a number of pathological conditions such as arthritis (Stefanovic-Racic *et al.*, 1993; Ialenti *et al.*, 1993), diabetes (Kolb & Kolb-Bachofer, 1992) and septic shock (Ochoa *et al.*, 1991). In such conditions selective inhibition of iNOS-derived NO, without interfering with basal EDNO, would eliminate the compromise which usually results from treatment using non-selective inhibitors (Robertson *et al.*, 1994). Isoform-selective inhibition of NO is not usually a characteristic of NO scavengers. In a condition such as septic

shock, the aim would be to administer a drug which rapidly removes the potentially cytotoxic, high output NO released by immune-activated cells and discharges it equally rapidly from the body with no harmful side effects. Recently Johnson Matthey have developed a number of compounds which may be suitable in treating septic shock (Fricker *et al.*, 1995; see chapter 6).

Direct inhibition of NO synthesis results from using L-arginine analogues as they compete with L-arginine for the same binding site on NOS. N-alkyl derivatives were first used as NOS inhibitors by Hibbs *et al.*, (1987). Since then, numerous derivatives of L-arginine have been tested which offer varying degrees of selectivity, potency and reversibility: two of the most widely used are the methyl ester analogue of N^G-nitro-L-arginine, N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA). Both dose-dependently inhibit endothelial-derived NO (EDNO) *in vitro* and *in vivo*, although L-NAME takes almost twice as long to to have its effect (Rees *et al.*, 1990). The latter study also suggested that L-NAME is ca. 100x more potent against acetylcholine (Ach)-induced EDNO release as compared to L-NMMA *in vitro*, while a more recent study, also carried out in the rat, found that L-NMMA (up to 1mM) cannot inhibit agonist-stimulated NO production (Frew *et al.*, 1993). The situation is further confused by evidence that L-NMMA can inhibit agonist-stimulated relaxations in a number of unrelated tissues (Crack & Cocks, 1992; Liu *et al.*, 1991). It appears therefore that L-NMMA inhibits basal EDNO release independent of the tissue type but only inhibits agonist-induced NO in certain types of tissue. The same is also true for L-NAME which displays tissue-specific inhibition and tissue-specific reversibility of agonist-stimulated NO *in vitro* (Randall & Griffith, 1991; Martin *et al.*, 1992). This infers mechanistic differences in basal and agonist mediated NO release between tissue types and/or differences in analogue binding sites between tissues.

The hypertensive response induced by L-NAME *in vivo* indicates that it is approximately 10x more effective at inhibiting basal NO synthesis in the rat than L-NMMA (Rees *et al.*, 1990), although the metabolite of L-NAME, N^G-nitro-L-arginine, is reported to be ca. 87x more potent than L-NMMA (Aisaka *et al.*, 1989). At a supramaximal dose (4.8×10^{-5} mol/Kg, i.v.), the effects of L-NMMA and L-NAME are comparable; however at this dose L-NAME only inhibited Ach-induced depressor response by ca. 50% *in vivo* (Wang *et al.*, 1993). Again, this reveals differences between basal and agonist NO inhibition, and suggests a difference in L-NAME's ability to supress Ach-induced vasodilation *in vitro* and *in vivo*. *In vivo*, L-NAME and L-NMMA both cause an L-arginine-reversible bradycardia (dose; < 300mg/Kg i.v.) (Rees *et al.*, 1990) and since NO is involved in C.N.S. neurotransmission (Knowles *et al.*, 1989), NO levels may also regulate heartrate. Regional differences in NO-mediated neurotransmission may also reflect differences in pressor responses to L-NAME and L-NMMA amongst different vascular beds in the rat (Gardiner *et al.*, 1989).

In addition to the constitutive enzyme (cNOS) associated with basal and agonist-induced NO release, a calcium/calmodulin independent isoform of nitric oxide synthase (iNOS) can be induced following exposure to bacterial endotoxins (Rees *et al.*, 1990; see Part D of Introduction). The subsequent expression of iNOS in cultured endothelial cells (Radomski *et al.*, 1990) and homogenised vascular smooth muscle cells (Knowles *et al.*, 1990) of the systemic vasculature results in vasodilatation, hyporesponsiveness to constrictors and, in extreme cases, cardiovascular collapse and septic shock. It is perhaps surprising that expression of iNOS in systemic vessel walls has so far not been shown convincingly using histological techniques. A sustained period of excess NO release is implicated in the pathogenesis of septic shock by the fact that NOS inhibitors can restore vascular responsiveness *in vitro* (Schott *et al.*, 1993) and *in vivo* (Thiermermann & Vane, 1990). Results from *in vitro* studies suggest that L-

NAME and L-NMMA are equipotent in reversing the endotoxin-induced vascular hyporesponsiveness to phenylephrine in rat aorta (Joly, *et al.*, 1994).

The effects of L-NMMA and L-NAME on immunologically-activated NO production is discussed in chapter 4.

1.13.4 Non-Specificity of NOS Inhibitors.

Much of our knowledge regarding the physiological and pathophysiological roles of NO is based on experimental observations using L-NAME or L-NMMA. The term 'specific' NOS inhibitor is often used to define them, implying that any haemodynamic effects caused by these arginine analogues results from inhibition of NO synthesis. Increasing evidence suggests that this may not be the case.

Homeostasis requires that any alteration in physiological state will be compensated by changing some other physiological parameter. This will also apply to altered NO production following treatment with NOS inhibitors and may partly explain why the levels of prostaglandin (PG), a potent vasodilator, are increased following NO inhibition. (Salvemini *et al.*, 1995). The increased levels of PG may in effect compensate for the reduced NO levels. Endogenous inhibitors of NOS, like N^G, N^G-dimethylarginine (DMA), could theoretically be regulated in accordance with NO levels in human plasma (Moncada *et al.*, 1991). If NO levels increase, DMA could convert from its inactive isomeric form to its active form and temporarily inhibit NOS activity. L-NMMA is also present in the body, although in smaller quantities (Vallance *et al.*, 1992) and studies carried out on isolated rings of vasculature suggest that L-NMMA can be metabolized to L-citrulline and ultimately L-arginine (Archer & Hampl, 1992). Indeed, cultured bovine aortic endothelial cells deprived of L-arginine express an enhanced ability to metabolize L-NMMA to L-arginine (Hecker *et al.*, 1990). It is not known whether this also occurs *in vivo*.

The structural properties of L-NAME and L-NMMA can also result in various non-specific effects, unrelated to the competitive inhibition of L-arginine binding. For instance, L-NAME and L-NMMA both contain iron binding groups (eg. alcohols, amines and carboxylates) which could interfere with electron transfer through iron centres in a number of enzymes directly relevant to NO-mediated relaxation (Peterson *et al.*, 1992). Soluble gyanylate cyclase (sGC) and NOS both contain iron, so it could be said that the ability for L-NMMA and L-NAME to inhibit NO-mediated relaxation may result from inhibited NO synthesis and/or the inactivation of sGC.

Similarly the ability for L-arginine derivatives to modulate adrenergic neurotransmission may reflect this non-stereospecific interaction with iron (Peterson *et al.*, 1992) or equally may be due to the decreased NO levels resulting in altered release of noradrenaline (Yamamoto *et al.*, 1993).

Analogues of L-arginine often have altered carboxyl groups which in itself can impart distinct nonspecific interactions. Analogues with alkyl or aryl esterification of the carboxyl group eg. L-NAME and Nα-benzoyl L-arginine ethyl ester (BAEE) respectively, are competitive antagonists of muscarinic receptors, an effect which is not reversed by added L-arginine (Buxton *et al.*, 1993). This suggests that antagonism is not related to the inhibition of NOS. Analogues with free α-carboxyl groups (eg. N^G-nitro-L-arginine) can also bind to arginase (Robertson *et al.*, 1993). This may result in elevated extracellular [L-arginine], as L-arginine transport into cells may be inhibited (Westergaard *et al.*, 1993), NOS activity is inhibited and arginase activity is inhibited.

As more structural analogues of L-arginine are introduced into experiments, so there will be a growing number of nonspecific anomalies associated with them. It is important to be aware of these when interpreting and evaluating NOS inhibitors *in vitro* and *in vivo.*

Table 1.2 Analogue inhibitors of NOS: comparative effects in rats.

Inhibition of vascular NO synthesis

	IN VITRO	IN VIVO	SPECIFICITY	OTHER Effects
L-NMMA	Reversibly inhibits basal EDNO release, maximally effective at >50μM, 15minutes incubation. Does not inhibit agonist-stimulated EDNO release from the rat aorta (300μM) but does in the rabbit aorta(by 64%). This effect is reversible with a 5 fold [L-arginine]. Is a potent reversible inhibitor of LPS-induced NO production. Uptake in human V.E.C's is potentiated by LPS.	At 3mg/Kg i.v. produces a small (13+/-5mmHg), transient (<20 mins.) increase in M.A.P. At 300mg/Kg the increase in M.A.P. is maximal (45.6+/-4.4mmHg) with rapid onset, <5 mins. and returns to control levels within 1hr. At >100mg/kg inhibits the hypotensive response to Ach and Bradykinin. Moderate dose of 30mg/Kg is most effective against endotoxin-induced hypotension. Also decreases heart rate (~25 b.p.m.). Equimolar [L-arginine] required to reverse effects.	L-NMMA metabolized to L-citrulline by E.Cs may enhance NO production (rat aorta).	Maximal inhibition of NANC relaxation is 50%. Has ability to modify electron transfer through iron centres which may extent its inhibitory effects beyond NOS.
L-NAME	Is 2-3x more potent at inhibiting basal and >100x more potent at inhibiting Ach-induced EDNO release compared to L-NMMA. Ach relaxations in the rat aorta are completely abolished by 10μM L-NAME, an effect which is reversible with 300μM. Is less potent than L-NMMA at inhibiting LPS-induced NO release at low concentrations but equipotent at >50μM. Is an irreversible inhibitor of bNOS.	10x more potent than L-NMMA at increasing blood pressure but response slower to plateau (~10 mins) and longer (>2hrs). A bolus injection 10mg/Kg (i.v.) increased b.p. by 43.4%, decreased heart rate and cardiac output by 28% and 39% respectively (all within 10 mins.). >1000mg/Kg causes partial inhibition of Ach-induced responses (by ~53+/-4%). Partially reverses the hypotension in LPS -treated rats. A 10 fold dose of L-arginine is required to fully reverse effects.	L-NAME is thought to be metabolised to L-NARG (~10 mins. *in vivo*, 30mins.*in vitro*). Differential effects on regional vascular conductances reflects varying degrees of neural intervention, or different interactions between L-NAME and the NO pathway.	Has the ability to modify electron transfer through iron centres. Is a muscarinic receptor antagonist.
L-NARG	A reversible inhibitor of basal and agonist (Ach)-stimulated EDNO release (approx. 5 and 30x more potent than L-NMMA respectively). Reversibily inhibits bNOS (>5x more potent than L-NMMA). Equipotent with L-NMMA and AG at inhibiting LPS-induced NO production in rat aorta. Maximally effective at >10μM, 15 mins. incubation and requires a 10 fold [L-arginine] to ensure full reversal within 1hr. D-NARG also inhibits Ach-induced relaxations in aortic rings.	Approx. 87x more potent than L-NMMA (i.v.). Maximal change in M.A.P. within 6-8 mins. Sustained elevations in M.A.P. at ≥ 0.6mg/Kg (i.v.) and at 2mg/Kg significantly inhibits Ach-induced vasodilations. No significant effect on heart rate (<21.9mg/Kg i.v.). A 3-4 fold dose of L-arginine is required to reverse effects; (6.5mg/Kg (i.v.) L-NARG: +40-45mmHg M.A.P.).	One of the most potent inhibitors of EDNO production. Due to its relative insolubility in water is less frequently used than other analogues.	Decreases N.A. outflow in rat isolated perfused mesenteric vasculature. 30x more potent than L-NMMA at inhibiting NANC transmission. Inhibits rat liver arginase (Ic50= 27+/-4 mM).
AG	Approx. 15x less potent than L-NMMA on bNOS and does not inhibit basal or agonist-induced EDNO release. Is a potent reversible inhibitor of LPS-induced NO production. AG is maximally effective at >100μM, 1hr incubation and requires equimolar [L-arginine] for full reversal within 1hr.	40x less potent at increasing M.A.P. than L-NMMA in anaesthetized rat. Effective doseage,100-300mg/Kg/day (orally), >300mg/Kg/day may lead to behavioural side effects.	Is approx. 10-100x more potent as an inhibitor of iNOS activity compared to cNOS activity.	Inhibits histamine catabolism. Affects polyamine degradation. Inhibits oxidative modification of LDL proteins. Inhibits RNR and nonenzymatic glycosylation.

Abbreviations : LNMMA = L-N^G-monomethylarginine L-NAME= L-N^G-nitro-arginine methyl ester L-NARG= L-N^G-nitro-arginine AG= Aminoguanidine

Part C
NO and the Growth and Maintenance of Solid Tumours.

1.14 Introduction

Mortality caused by the four most common solid tumours found in humans, (ie. lung, colorectal, breast and prostate) has not significantly decreased in recent years (Boring *et al.,* 1992). The reason for this disappointing statistic is due to the relative insensitivity of these tumours to many of the therapeutic strategies presently used, including radiation therapy, chemotherapy, hormone therapy and certain immune therapies (Jain, 1994; Denekamp, 1993). This section of the introducion deals with newly-acquired knowledge concerning the role of NO in regulating tumour growth.

1.14.1 The Solid Tumour Solid tumours are composed of two distinct but interdependent compartments: maligant cells and the stromal matrix that they induce and in which they are dispersed (Dvorak, 1994). It is now clear that much of the inefficiency of current treatments is due to the inability of systemically administered drugs, or in the case of immune therapy, cytotoxic antibodies and circulating cytotoxic inflammatory cells, to penetrate the stroma and reach the tumour cells in sufficient quantities to be effective (Jain, 1994). The stroma commonly consists of laminin, a dense lattice of collagens and interstitial connective tissue containing a fibrin-fibronectin gel, new blood vessels and cells derived from tissue (eg. fibroblasts and mast cells) and blood (eg. monocyte-derived macrophages, platelets and lymphocytes). Therefore, although stroma represents the 'lifeline' to solid tumours, providing a vascular supply carrying nutrients, allowing gas exchange and waste disposal, it also forms an effective barrier between the tumour cells and the surrounding host tissue.

1.14.2 Angiogenesis Tumour vascularization involves two types of vessels: those recruited from normal host tissue and newly formed vessels (neovasculature). The term 'angiogenesis' was first used by Hertig (1935) to describe the development of new blood vessels in the placenta. However, the mechanisms of angiogenesis have been described most extensively in studies involving solid tumours. Tumour angiogenesis refers to the directional sprouting of new vessels toward a solid tumour (Baillie *et al.*, 1995) and an increasing number of factors which stimulate or inhibit this process have been identified both *in vitro* and *in vivo* (Klagsbrun & D'Amore, 1991; Folkman, 1985). By targetting these factors it is hoped to render the prevascular solid tumour dormant, both in terms of growth and metastases. The potential for this so called 'anti-angiogenic strategy' was highlighted by the discovery that solid tumours are dependent on angiogenesis to grow beyond a diameter of ~2-3mm (Folkman, 1972). However, the ability to arrest tumour growth, by interfering with the angiogenic processes, is complicated by the multitude of factors released within a single tumour. Inhibition of a single factor is unlikely to be effective and only through a better understanding of the synergism that may or may not exist amongst them, will the key ones be identified and targetted.

1.14.3 Metastasis The formation of secondary tumours at anatomical sites away from the primary tumour requires that tumour cells successfully negotiate a number of barriers, including extracellular matrix, intravasation and dissemination through the bloodstream or lymphatic channels and finally extravasation through the vessels and surrounding interstitial connective tissue. This process is referred to as metastasis and underpins the deadly nature of cancer. A tumour cell undergoing metastasis can release degradative enzymes to aid its passage through extracellular matrix (Tryggvason *et al.*, 1987). Furthermore, tumour-activated host cells are also thought to release similar enzymes (Gabbert, 1985). Specific adhesion molecules have been identified mediating the adherence of tumour cells to the vascular endothelium (Alby &

Auerbach, 1984) and underlying basement membrane (Haberern & Kapchik, 1985). These can also be induced by immune-activated cytokines (Rice *et al.*, 1988). It appears, therefore, that the immune system may in some cases promote metastasis rather than inhibit it.

1.14.4 Tumour Vasculature In practice, the ability to identify and treat solid tumours before they establish their blood supply (2-3mm diameter) is not always possible and they often remain undiagnosed until such time as they have undergone metastases. This fact led to research into 'vascular targeting' as a potential therapy with the premise that destruction, occlusion or altered blood flow of vessels would result in the death of tens of thousands of tumour cells that were totally dependent on them (Denekamp, 1982, 1984, 1986). So, in contrast to anti-angiogenesis, which aims to induce tumour stasis, vascular targeting aims to provide partial or complete regression of the solid tumour (Denekamp, 1993). The rationale for targeting tumour vasculature has been further strengthened by the discovery that they possess distinct anatomical, physiological and immunological characteristics that distinguish them from normal vessels of the host (Baillie *et al.*, 1995; Buttery *et al.*, 1993; Chaplin, 1991; Kennovin *et al.*, 1994).

The hallmarks of tumour neovasculture include their chaotic, tortuous nature, often twisting into corkscrew-like coils (Peterson, 1991), their sustained hyperpermeability to plasma proteins and their propensity to collapse as the interstitial pressure increases within the growing tumour (Jain, 1994). These characteristics are due to the frail architecture of the vasculature which consists of only a single, often incomplete, endothelial lining (Peterson, 1991) with tumour cells sporadically integrated between them (Konerding *et al.*, 1989). They lack adrenergic innervation and contractile elements associated with the VSM (Peterson, 1991). These features are usually retained in normal vessels incorporated into the tumour (Peterson, 1991). The incomplete structure inherent

to tumour neovasculature has been exploited recently with the introduction of specific antibodies which target the exposed subendothelial matrix (Epstein *et al.*, 1995). Studies have shown that by conjugating these antibodies to vasoactive agents (ie. IL-2, TNF and IL-1ß), a more specific and efficient uptake of tumour-specific radiolabeled antibodies can be afforded. It is hoped that the same principal may also extend to the delivery of cytotoxic reagents in highly vascularised tumours.

A vascular permeability factor (VPF) has been isolated from tumourigenic cells (Senger *et al.*, 1990) and may be responsible for the hyperpermeability of tumour vessels (Dvorak *et al.*, 1991). Furthermore hypoxia, due to central vascular collapse, induces VPF which can promote angiogenesis (Schwelki *et al.*, 1992) and antibodies directed against VPF have been shown to significantly reduce tumour growth (Kondo *et al.*, 1993). VPF gene expression in response to hypoxia is thought to be regulated by NO (Tuder *et al.*, 1995) yet the vascular hyperpermeability in solid tumours is inhibited by NOS inhibitors and NO-scavengers (Maeda *et al.*, 1994). This evidence suggests that while both NO and VPF enhance vascular permeability, NO may act to counter the angiogenic stimulus of VPF. The continued expression of VPF may be crucial when defining the difference between the processes underlying tumour growth and wound healing. Vascular permeability in wound healing is transient; after about a week any remaining blood vessels are reabsorbed and a scar is formed. In contrast, the persistant hyperpermeability associated with tumour vessels allows new provisional stroma to be laid down at the periphery. It is for this reason that solid tumours are often referred to as 'wounds that don't heal' (Dvorak, 1994).

1.14.5 Tumour Blood Flow Recent approaches to tumour therapy are based on the concept that tumours can be starved of nutrients and oxygen, which are necessary for growth, by restricting their blood flow (Denekamp *et al.*, 1983). A novel pharmacological approach has potential value only if a difference exists

between the responses to vasoactive drugs by normal vessels and by tumour-supplying vessels. This difference has been established using a number of vasoactive agents (Hirst & Wood, 1989).

Fully-differentiated tumour vessels, which possess VSM, constrict with systemically administered vascontractile agents (eg. phenylephrine), but are unresponsive to vasodilatory drugs (eg. hydrazaline; Chan *et al.*, 1984; Mattisson & Peterson, 1981), while poorly-differentiated vessels, which lack VSM, are relatively unreactive to drugs which act on smooth muscle, and so tumour blood flow varies in concert with local blood flow. LPS-induced systemic hypotension in a tumour-bearing animal would therefore be expected to reduce blood flow through a tumour. This phenomenon where blood is diverted from the tumour to normal tissue is often referred to as the steal effect. The diminished response to vasodilatory drugs prompted the idea that tumour vessels reside in a state of near maximal dilatation, although the factor mediating this effect has yet to be identified.

More recently, a study by Stücker *et al.*, (1991) found that host-derived arterioles, feeding into a chemically-induced (Meth-A) tumour in mice, were significantly more sensitive to i.v. injections of serotonin when compared to control arterioles not associated with the tumour. Furthermore, the feeding arterioles were also of a larger diameter than the controls, although the vasocontractile effect of serotonin was sufficient to reduce tumour growth and prolong survival in these animals. The effects of these agents are transitory and may prove to be more effective when used in combination with other more conventional therapies. Vasodilators could improve the delivery of drugs for chemotherapy or increase oxygen supply to radioresistant, hypoxic cells, or in some instances, actually increase the hypoxic cell fraction due the steal effect, which can then be targeted using bioreductive agents (Brown, 1991).

More 'long-term' effects on tumour blood flow are possible using other agents including, flavone acetic acid (FAA) and TNF, although as with other therapies which mediate their effects by induction of ischaemia (eg. photodynamic therapy; PDT), their mechanism of action remains unclear. Recently, however, NO has been implicated as a mediator in PDT (Gilissen *et al.*, 1993) and FAA therapy (Li *et al.*, 1991; Thomsen *et al.*, 1990). Indeed the haemorrhagic necrosis produced in subcutaneous tumours by FAA is similar to that produced by endotoxin, which is known to induce NO production (Parr *et al.*, 1973).

For a number of years now, the pulsatile pressure of arterioles within tumours and feeding vessels located in the surrounding host tissue has been documented (Peters *et al.*, 1980; Intaglietta *et al.*, 1977; Kennovin *et al.*, 1994), but again, the mechanisms underlying this are not altogether clear. Fluctuations in pressure are thought to reflect spontaneous vasomotion within these vessels, resulting in intermittent blood flow through them. Large subcutaneous tumours are more prone to intermittent blood flow than small tumours (Chaplin *et al.*, 1987) which may indicate that increased interstitial pressures can potentiate intermittency as vessels that constrict become crushed by the surrounding tumour cells (Jain, 1994). Interestingly, the radiosensitizer, nicotinamide, has been shown to reduce pulsatile pressure in host-derived tumour vessels (Hirst *et al.*, 1995) which may indicate that its ability to re-oxygenate hypoxic cells reflects an improved blood flow through these vessels.

1.14.6 The Role of NO in Solid Tumour Growth Human and animal cancer cells grown in culture are capable of expressing NOS genes at the mRNA level (Amber *et al.*, 1988; Radomski *et al.*, 1991) and of generating NO (Jenkins *et al.*, 1994). Furthermore, *in situ* studies indicate a correlation between the levels of NOS protein within tumour cells and the grade of malignancy of the tumour (Thomsen *et al.*, 1994; Cobbs *et al.*, 1995). Tumour-infiltrating macrophages can also be activated to release high levels of NO, as well as IL-1 and TNF (Brunda

et al., 1991), although Thomsen *et al.* (1994) determined that they did not provide a significant contribution to tumour NOS activity in human gynecological tumours.

The production of NO within tumour vasculature and its role in tumour biology remains ill-defined, however, increasing evidence indicates that the neovasculature and incorporated host vessels of some tumours express iNOS activity (Buttery *et al.*, 1993; Kennovin *et al.*, 1994). Specific inhibitors of NOS have been widely used to assess the consequence of a sustained, high level NO release within these vessels which has led to the conclusion that it is involved at all stages of tumour growth; by regulating angiogenesis (Pipili-Synetos *et al.*, 1993, 1994), and the subsequent blood flow through these newly formed blood vessels (Andrade *et al.*, 1992; Tozer *et al.*, 1995) and also in the processes associated with metastasis (Dong *et al.*, 1994).

Ultimately, a growing number of NOS inhibitors have been shown to suppress tumour growth (Thomas *et al.*, 1986; Freemantle *et al.*, 1994; Kennovin *et al.*, 1994) although, the multiple roles of NO, ie. as a vasodilator, an inhibitor of platelet adhesion and aggregation (Hogan *et al.*, 1988), a cytotoxic agent or as an inducer of apoptosis in tumour cells (Jonathan *et al.*, 1994), has made it difficult for such studies to clearly identify which inhibited effect(s) of NO is responsible for reduced growth. Clearly any one of these effects could be instrumental in determining the growth of a tumour; enhanced platelet aggregation associated with NO inhibition could promote microthrombus formation in tumour feeding vessels and so contribute to tumour vascular stasis. A reduced vasodilation of these vessels may also increase the likelihood of vascular stasis as they constrict and obstruct blood flow. Regardless of the mechanism involved, the main effect of NOS inhibitors is a reduced blood flow (Andrade *et al.*, 1992; Tozer *et al.*, 1995). Using sponge implants in mice, Andrade *et al.* found that L-NAME was ~2 fold more potent than L-NMMA at reducing blood flow and that this effect

was not seen until after day 7 post-implantation. This suggests that NOS is not active by day 7. This was confirmed in a more recent study using sponge implants in mice (Buttery *et al.*, 1993). Visual analysis of altered blood flow in tumour-associated vasculature has been carried out using tumours (R3230Ac mammary carcinomas) implanted into subcutaneous window chambers (Meyer *et al.*, 1995). The diameter of post capillary venules and red blood cell flux were measured using video microscopy and the effects of L-NMMA (50μM) and L-arginine (200μM) determined. L-NMMA decreased tumour blood flow by 43% and by 17% in normal vessels close to the tumour. Control (non-tumour) vessels displayed an 83% decrease in blood flow which was subsequently restored by L-arginine. Interestingly, L-arginine had little effect in tumour containing chambers, suggesting that a reduction of tumour NO, combined with normal tissue rescue with L-arginine, may provide a means for selective tumour therapy. The ability of L-NMMA to reduce blood flow in tumour vessels correlated with an increase in the number of vessels showing intermittent flow, which suggests that L-NMMA potentiates spontaneous pulsatile pressure by inhibiting NO synthesis. This study also highlighted the fact that normal vessels in the vicinity of a tumour respond to NO inhibition in a similar manner as tumour vessels. This implies that a growing tumour continually releases factors into the surrounding milieu which can alter the physiological characteristrics of nearby host vessels. The extent of this 'sphere of influence' is not known.

The consequences of NO inhibition have also been studied using magnetic resonance spectroscopy which allows the ratios of high to low energy phosphates within the growing tumour to be assessed in response to the altered nutrient availability which results (Wood *et al.*, 1993). When administered to tumour and normal tissue, nitro-L-arginine (10mg/Kg; i.v.) caused a tumour selective 2-4 fold increase in the ratio of inorganic phosphate to total phosphate, implying that the tumour was being starved of essential metabolic substrates eg. oxygen. From this study and those mentioned above it is clear that NOS inhibitors promote

hypoxia in solid tumours. It follows that NOS inhibitors may prove beneficial when applied in combination with bioreductive alkylating agents, eg. mitomycin C, which target oxygen-deficient cells (Sartorelli, 1988). Considerable research now focuses on this area of tumour therapy.

Recently, the increase in NO concentration within peritoneal carcinomas, induced in syngeneic BD 9 rats, has been shown to parallel tumour growth (Lejeune *et al.*, 1994). This study also found that NO production was effective at supressing the proliferation of tumour-infiltrating T-lymphocytes, an effect which was reversible with L-NMMA. It could be suggested from this evidence that NO may potentiate the growth of certain solid tumours by supressing the host's immune response, which counteracts the cytotoxic function normally associated with high level NO release (Stuehr *et al.*, 1989). The immunosuppressive effect of NO is further implicated by the work of Orucevic & Lala (1995), who showed that NO interferes with lymphocyte-activated killer cell activation *in vivo*, an effect which could be abolished by L-NAME. This study also showed that although the high output NO induced by IL-2 is inhibited by L-NAME, the anti-tumour effect of IL-2 *in vivo* is potentiated. This suggests that NOS inhibitors may be effective when used in combination with certain immunostimulants, for instance LPS. LPS is known to induce the expression and synthesis of a great many factors which will undoubtedly influence tumour growth (including TNF, IL-1ß and IFN-gamma) and because the effect of LPS on vascular resistance in tumours is not inhibited by specific scavengers of NO (Tozer *et al.*, 1995), it appears that NO itself is not responsible. It is conceivable that bioadducts of NO (ie nitrosothiols) are involved, although this view is still controversial (Feelisch *et al.*, 1994). The potential for combined therapy of LPS + selective iNOS inhibitors is particularly relevant in view of recent clinical trials which show that the efficacy of LPS against cancer is limited by its toxic side effect, most noteably the sustained hypotension associated with high NO production (Engelhardt *et al.*, 1991).

It has yet to be determined if the presence of NO within tumour vasculature represents a homeostatic response by the tumour to an inadequate supply of nutrients, in an attempt to increase blood flow through the few unoccluded vessels which remain, or whether it is simply due to the large number of immune-activated host cells releasing high levels of NO inside the tumour. Until this ambiguity is resolved, the true function of NO in tumours, whether it is induced by the tumour to assist in its growth or as part of the immune response to attack it, will remain undefined and the intervention of therapies which modify NO levels may not attain their full potential.

The opposing effects of NO on the immune system, and hence tumour growth, is determined largely by the amount of NO indigenously present (Jenkins *et al.*, 1995), with higher concentrations of NO providing anti-tumour activity and lower concentrations promoting tumour growth. Hopefully, a better understanding of the host's immune response to solid tumours will make it possible to manipulate factors, including NO, within tumours to be released at the right concentration, in the right place and at the right time, and by applying this strategy in combination with therapies which already exist, ultimately achieve complete regression of the tumour.

Part D

1.15 Endotoxic Shock

Septic shock is a life-threatening condition manifested by cardiovascular collapse and is estimated to affect up to a quarter of a million people in the U.S.A. every year (Kilbourn *et al.*, 1990b). It is defined as a bacterial infection resulting in a potentially fatal condition. The syndrome is associated with haemodynamic dysfunction, most noteably a sustained drop (> 40mmHg) in systolic blood pressure (Glauser *et al.*, 1991). It is only since the 1950's that the condition has progressed from obscurity, behind a number of other better known conditions such as typhoid fever, also caused by gram-negative pathogens, to become one of the most studied and money-invested areas in medical research today. Unfortunately, the treatment of septic shock in humans is not straight forward as it is commonly contracted during surgery when bacteria are able to enter the body unimpeded by the ususal barriers. Therefore septic shock is often the cause of death in patients being treated for conditions which may not at first be considered life-threatening.

The clinical hallmarks of septic shock are peripheral arteriolar vasodilation, resulting in low systemic vascular resistance, high cardiac output, hypotension and inadequate tissue perfusion. The mechanisms underlying these symptoms are not fully understood, although many of the pathophysiological reactions occuring in septic shock patients can be mimicked and studied in laboratory animals by infusion of live bacteria or endotoxin (Suffredini *et al.*, 1989; Kilbourn & Billiar, 1996). Endotoxin is the lipopolysaccharide (LPS) component of the bacterial cell walls and is the prime initiator of gram-negative bacterial septic shock (Glauser *et al.*, 1991). From studies such as these the role of some potential mediators of endotoxic shock is gradually emerging and animals pre-treated with anti-tumour necrosis factor antibodies (Kilbourn *et al.*, 1990a),

platelet activating factor receptor antagonists (Doebber *et al.*, 1985) and prostacyclin inhibitors (Halushka *et al.*, 1983) are significantly protected against septic shock. However, the relative importance of these mediators in the pathophysiology of septic shock remains unclear.

Increasing evidence from more recent studies suggests that NO is at least partially responsible for the sepsis syndrome (Stoclet *et al.*, 1993). Indeed, the afore-mentioned factors and others, including the monocyte/macrophage-derived cytokines, interleukin 1 and 2 (IL -1, IL-2) are now thought to be involved in regulating NO production (Thiermermann *et al.*, 1993; Szabó *et al.*, 1993; Salvemini *et al.*, 1995; Beasley, 1990; Kilbourn *et al.*, 1994).

As outlined in section 1.13.3, NO is synthesised from the substrate L-arginine by a group of isoenzymes, NOSs, which are specifically inhibited by L-arginine analogues such as N^{W}-nitro-L-arginine methyl ester (L-NAME) and N^{G}-monomethyl-L-arginine (L-NMMA). LPS is known to induce a calcium-independent NOS (iNOS) resulting in overproduction of NO as part of the host-defense mechanism (see chapter 3). Unfortunately, high levels of NO not only have a cytotoxic effect on bacteria and tumour cells but can also be cytotoxic to host cells as well and this is thought to be responsible for the severe hypotension associated with endotoxic shock. This is certainly the case in animal models, as initially indicated from studies showing that the diminished pressor response to catecholamines in aortas from endotoxin-treated rats could be reversed by L-NMMA (Julou-Schaeffer *et al.*, 1990) or L-NAME (Schott *et al.*, 1993). These findings were further confirmed by similar studies carried out *in vivo* (Gray *et al.*, 1991; Thiermermann & Vane, 1990).

With the purpose of investigating the clinical potential for targeting NO in humans, recent research has focused on using L-NMMA or L-NAME to treat the condition. Results have been mixed, with L-NMMA able to reverse the hypotension in septic shock both in animal models (Kilbourn *et al.*, 1990b) and

humans (Petros *et al.*, 1994; Lin *et al.*, 1994), but only at the expense of other haemodynamic parameters which are altered and may ultimately be just as harmful. In a canine model of endotoxic shock, for instance, L-NMMA causes a large decrease in cardiac output for up to 2hrs after administration (Klabunde & Ritger, 1991). This will obviously impair regional blood flow and is therefore of limited clinical worth. Similar detrimental effects accompany L-NAME administration, most noteably pulmonary hypertension (Robertson *et al.*, 1994) and in one study, using female Swiss-webster mice, L-NAME was found to increase fatality by 87.5% (Minnard *et al.*, 1994).

The factors governing whether L-NMMA and L-NAME have a beneficial or adverse effect on blood pressure may include the dose and method of administration and/or the resulting increase in tumour necrosis factor (TNF) and interleukin-6 (IL-6) (Nava *et al.*, 1991; Tiao *et al.*, 1994). Nava *et al* (1991) using anaesthetised rats found that a high dose of L-NMMA (300mg/Kg, i.v.) potentiated the LPS-induced hypotension while an intermediate dose of 30mg/Kg, i.v. was sufficient to prevent it, resulting in a 100% survival. Tiao *et al* (1994) concluded from their rat studies that NO inhibition was detrimental due to the loss of NO-mediated suppression of TNF and IL-6 levels. Hepatotoxic levels of IL-6 following treatment with a NOS inhibitor have previously been observed (Frederick *et al.*, 1993). The idea that NO does not mediate the toxic effects of TNF contradicts several studies which suggest that it does (Estrada *et al.*, 1992; Drapier *et al.*, 1988). Cytotoxic levels of superoxide anion following NO inhibition may also be involved.

Detrimental effects most likely arise because these inhibitors do not discriminate between cNOS and the inducible isoform. By inhibiting cNOS, a basal release of NO is prevented and numerous physiological functions will be affected, including vasomodulatory activity in the lungs. Increasing evidence suggests that non-specific inhibition may not explain the increased mortality as cNOS activity

is down-regulated in aortic rings isolated from guinea pigs (Parker & Adams, 1993), but whether this also occurs *in vivo* remains to be elucidated. It was recently shown using a porcine model of endotoxic shock that some of the detrimental haemodynamic effects caused by L-NMMA can be prevented by combined inhalation of NO (Klemm *et al.*, 1995).

A specific inhibitor to iNOS may prove to be more valuable as therapy for septic shock and recent studies using a compound called aminoguanidine (AG) in the rat have shown partial reversal of LPS-induced vascular hyporeactivity *in vitro* (Joly *et al.*, 1994) and ex vivo (Wu *et al.*, 1995b), with improved survival following LPS treatment (10mg/Kg, i.v.) *in vivo* (Wu *et al.*, 1995b). Although AG inhibits iNOS more potently than cNOS (Misko *et al.*, 1993), it also inhibits copper-containing diamine oxidases, which may ultimately affect vascular tone (Bieganski *et al.*, 1983). Several other compounds are thought to inhibit iNOS although, the majority of these are not selective and their mechanism of inhibition has yet to be clearly defined (see Fig. 1.7).

Other methods of manipulating NO production and/or levels may include agents which inhibit essential cofactors like BH_4 or L-arginine transport (see Part A, section 1.12.2).

A different approach to the same problem is being exploited by chemists and biochemists using compounds which selectively and irreversibly bind NO, so removing the influence of excess NO, without compromising the activity of cNOS. A team from Johnson Matthey Technology Centre in Reading, England have synthesised a number of ruthenium-based NO binding compounds. The leading compound to date is called JM1226, a polyaminocarboxylate complex. JM1226 (100mg.Kg, i.p.) has been shown to fully reverse the LPS-induced (4mg/Kg, i.p.) hypotension (~30% decrease in mean arterial blood pressure) in unanaesthetised Wistar rats over a period of 4-19hrs (Fricker *et al.*, 1995).

Scavengers of NO will also have the added advantage of removing NO produced non-enzymatically although, it is unclear whether or not this mode of NO production is increased during septic shock.

Sepsis and endotoxaemia are associated with increased production of NO as evident by the elevated circulating levels of nitrite and nitrate. Nitrite and nitrate are the stable end products of NO's oxidation by haemoglobin (See section 1.3.4). In *vivo* this conversion usually occurs fairly rapidly (3-5 seconds) which suggests that NO is not wholly responsible for the sustained haemodynamic alterations associated with sepsis. The role of iNOS-derived NO in LPS-mediated mortality has been studied using mice which lack the gene for iNOS (Wei *et al.*, 1995; Laubach *et al.*, 1995). It appears that resistance to LPS-induced mortality is dependent on the type of LPS used, with those derived from *E. coli* causing death independently of iNOS.

1.15.1 LPS LPS from the cell wall of gram -ve bacteria consists of a polysaccharide and lipid domain (Hitchcock *et al.*, 1986). From the lipid layer in the cell wall the polysaccharide domain projects out into the extracellular environment and is thought to be responsible for the differences in serotype amongst gram -ve bacteria. Exposure to LPS results in a wide spectrum of physiological effects associated with the immune response, not least of all septic shock. LPS injectioned intraperitoneally into a rat is cleared from the circulation via the portal vein into the liver. Clearance is usually complete within 72hrs (Freudenberg *et al.*, 1984), which is in accordance with the time course of nitrite and nitrate accumulation, which generally returns to control levels 72hrs after LPS exposure (this study; Tracey *et al.*, 1995).

Macrophages are an important component of the immune system and have a role in receptor-mediated LPS detoxification (Hampton *et al.*, 1991). *In vitro* studies implicate other receptors on macrophages, not involved with LPS detoxification, that provide a specific interaction with cell-free LPS and may instead be

involved with tumouricidal activity (Chen *et al.*, 1990). The receptor-mediated activation of macrophages by LPS may therefore initiate the sequence of events (ie. second-messenger systems) which culminate in altered gene expression and the subsequent release of various inflammatory mediators, including NO, certain ILs and TNF-α (Rees *et al.*, 1990; Libby *et al.*, 1986; Zuckerman *et al.*, 1989). Altered gene expression will also change the expression of cell surface molecules on a number of cell types, most notably endothelial cells. LPS increases T-cell (Yu *et al.*, 1986), leukocyte (Bevilacqua *et al.*, 1987) and granulocyte (Yamada *et al.*, 1981) adhesion to vascular endothelial cells with deleterious effects. The perturbed endothelial lining may become increasingly permeable (Hutcheson *et al.*, 1990) and promote easy access for inflammatory mediators going to the subjacent VSM. It is clear, therefore, that LPS-induced endothelial cell injury is important in the pathogenesis of septic shock.

Many of the pathophysiologic features resulting from exposure to LPS *in vivo* can be attributed to NO. Several studies using the rat model of endotoxic shock have shown that LPS induces high levels of iNOS in many tissues including, lung, liver, spleen, kidney, heart, brain and skeletal muscle (Hom *et al.*, 1995; Liu *et al.*, 1993; Buttery *et al.*, 1994). Significant discrepancies are evident when comparing these studies and may relate to the different serotypes of LPS used. For instance, the expression of iNOS mRNA induced by *Samonella enteritidis* is sensitive to the glucocorticoid, dexamethasone (Liu *et al.*, 1993), while that induced by *Escherichia coli* is not (Hom *et al.*, 1995).

Subsequent chapters report on the findings of this study, comparing some of the physiological characteristics of vessels which previously supplied solid tumours and vessels isolated from rats in endotoxic shock.

NAME OF COMPOUND	INHIBITORY ACTION	REFERENCE(S)
N^G-nitro-L-arginine methyl ester	Replaces L-arginine substrate; inhibits activity of NOS in vascular tissues and macrophages.	Ayotunde S.O. et al 1993
N^G-mono-methyl-L-arginine	Replaces L-arginine substrate; inhibits activity of NOS in vascular tissues and macrophages.	Gross S.S. et al 1990, Julou-Schaeffer G. et al 1990, Kilbourn R.G. et al 1990.
Aminoguanidine	Replaces L-arginine substrate; inhibits activity of iNOS in vascular tissues.	Misko T.P. et al 1993, Griffiths M.J.D. et al 1993, Joly G.A. et al 1994.
L-canavanine	Replaces L-arginine substrate; inhibits Induction / Activity of iNOS in vascular tissues.	Umans J.G. et al 1992
Dexamethasone	Inhibits the induction of iNOS at a transcriptional level in vascular tissues and macrophages.	Rees D.D. et al 1990, Smith R.E.A. et al 1991.
Diphenyleneiodonium (and its analogues).	Acts by inhibiting NADPH-dependent flavoproteins, which are required for NO synthase activity in macrophages.	Stuehr D.J. et al 1991.
Nicotinamide	Inhibits the induction of iNOS at a transcriptional level in activated macrophages. May act by inhibiting poly(ADP-ribosyl)- ation.	Pellat-Deceunynck C. et al 1994.
TGF-β_1,$PDGF_{AB}$, $PDGF_{BB}$	Inhibits the induction of iNOS in VSMC.	Schini V.B. et al 1992.
Thaliporphine	Inhibits the induction of iNOS in activated macrophages via a mechanism that may involve its inhibitory effect on IL-1β.	Yu S.M. 1994.
Macrophage-stimulating protein	Inhibits the induction of iNOS	Wang M-H. et al 1994.
Glibenclamide	Inhibits the induction of iNOS in cultured macrophages.	Wu C-C. et al 1995.
Phorbol esters	Inhibits the induction of iNOS at the transcriptional level in VSMC[1] but not macrophages[2], via a mechanism that may be governed by activation of protein kinase C.	[1]Geng Y. and Hansson G.K. 1994, [2]Severn A. et al 1992.
Colchicine	Inhibits the induction of iNOS in VSMC, suggesting that microtubules may be involved in regulating this enzyme.	Marczin N. et al Mar.1993.
Cycloheximide	Inhibits the synthesis of the iNOS protein in VSMC[1], but potentiates the expression of the iNOS mRNA.[2]	[1]Koide M. et al 1993, [2]Sirsjo A et al 1994.
Angiotensin II	Inhibits the induction of iNOS through the activation of the AT1 receptor in VSMC.	Nakayama I. et al 1994.
Thrombin	Inhibits the induction of iNOS in VSMC.	Schini V.B. et al 1993.
Tyrosine Kinase inhibitors	Inhibits the induction of iNOS in VSMC.	Marczin N. et al Sept. 1993.
Curcumin	Inhibits the induction of iNOS in activated macrophages via a mechanism that may involve its inhibitory action on several transcription factors or PKC and TPK.	Brouet I. and Ohshima H.,1995.

Abbreviations: **iNOS** = inducible nitric oxide synthase, **VSMC** = vascular smooth muscle cells, **TPK** = Tyrosine protein kinase, **PKC** = protein kinase C.

***Fig. 1.7** Table of compounds which are known to inhibit iNOS.*

Chapter 2
Scope of the Present Study

The present study follows on from that of Kennovin *et al.* (1993, 1994) and examines the role of nitric oxide in the unusual hyporesponsiveness to vasoconstrictors (ie. phenylephrine; PE) displayed by vessels isolated from animals in endotoxic shock as compared to those which formerly supplied solid tumours (Stoclet *et al.*, 1993; Kennovin *et al.*, 1993). It was postulated at the outset that the former might serve as a suitable model for predicting the therapeutic potential of novel drugs to retard solid tumour growth: agents which most effectively reverse the hyporesponsiveness associated with endotoxaemia may sufficiently constrict tumour-supply vessels so as to reduce blood flow into the tumour and thereby limit its growth (Andrade *et al.*, 1992).

A single injection of bacterial lipopolysaccharide (LPS) induced a form of endotoxaemia in the rat, as defined by five separate parameters (see Chapter 3). A novel NO scavenging compound was found to alleviate the severe hypotension resulting from LPS treatment, highlighting NO as the primary mediator of this effect (see Chapter 6). Using this model, experiments were conducted *ex vivo* with NOS inhibitors to confirm the involvement of the L-arginine/NO pathway in the hyporesponsiveness of vessels to PE. Parallel experiments were conducted on isolated epigastric arteries which previously supplied a solid tumour (see Chapter 7). It is clearly of more therapeutic value for an inhibitor to selectively constrict vessels which supply nutrients to a tumour without unduly compromising the function of normal (non-tumour associated) vessels in the host. With this in mind, experiments were performed using a selective NOS inhibitor aminoguanidine, and the results were compared with those obtained for L-NMMA and L-NAME, and also for a number of novel NO scavenging drugs (see Chapters 4 & 6).

Kennovin *et al.* (1994), also showed that cycloheximide administered on its own could reverse the hyporesponsiveness associated with isolated tumour-supply vessels and they postulated that this effect was due to inhibition of iNOS. Similar experiments were therefore performed using vessels isolated from LPS-treated animals. However, because cycloheximide indiscriminately inhibits protein synthesis, its effects can seldom be attributed to a single mediator. For this reason, indomethacin was used to see if vasoactive products derived from the cyclooxygenase pathway are also involved (see Chapter 5).

Intermittent blood flow into the tumour has been suggested to occur as a consequence of vasomotion in feeding vessels (Intaglietta *et al.*, 1977) Increasing this intermittent flow could sufficiently augment the fraction of hypoxic tumour cells as to significantly improve the efficacy of bioreductive agents (Chaplin *et al.*, 1987). The present study (Chapter 7) looks at the effects of NOS inhibitors and L-arginine on spontaneous pulsatile pressures in isolated supply arteries and raises the possibility that NO is involved in this phenomenon.

Finally, having established that these NOS inhibitors have a greater constricting effect on tumour-associated vessels as compared to normal (control) vessels, studies were then conducted *in vivo* to confirm their ability to suppress tumour growth (see Chapter 8). The effects on tumour growth of LPS on its own, or in combination with a NOS inhibitor, were also addressed. A discussion of the results is included at the end of each Chapter and the overall conclusions are considered in the final Chapter (Chapter 9).

Chapter 3

Part A

A Model of Endotoxic Shock in the Male Wistar Rat

3.1 Introduction

The most obvious similarity between vessels which previously supplied solid tumours and those removed from animals in endotoxic shock is their marked hyporesponsiveness to vasconstrictor agents, like phenylephrine (PE; Kennovin *et al.*, 1994; Julou-Schaeffer *et al.*, 1990; Schott *et al.*, 1993). The underlying factor which appears to be common to both and is likely be responsible for this phenomenon is iNOS. This study was undertaken to investigate whether the expression of iNOS in vessels from endotoxic animals and tumour supply vessels and their relative insensitivity to PE is sufficient to justify using one as a model of the other. The inherent technical difficulties associated with isolating tumour-associated vessels, their susceptibility to damage during the experimental procedure and the fact that only one tumour vessel is obtained from each animal, all conspire to compromise the acquisition of reliable data. A relatively straight forward surgical dissection of the rat tail artery (RTA) that provides 3-4 preparations per animal per day is clearly of more value. It was therefore postulated that this model could provide a more reliable alternative for studying the potential of NOS inhibitors to selectively abolish this abnormal hyporesponsive effect.

Materials & Methods

3.2 Methods

3.2.1 Preparation of Isolated Rat tail Artery. Male Wistar rats (350-500g) were killed by cervical dislocation and their tails amputated by cutting through the intervertebral disc. The skin was reflected around the proximal 2 -7 cms of the tail and a longitudinal incision made through the midline of the fascia on the underside of the tail. The tail artery was exposed using blunt forceps and cleaned of fat and connective tissue. Following insertion of a polyethylene cannula, arteries were mounted in a perfusion apparatus, schematically shown in fig. 3.1. Vessels were incubated at 37°C (ambient temperature was ~20-21°C) in the organ bath, perfused internally (flow rate; 2mls.min^{-1} driven by a Gilson minipuls 3) and superfused (flow rate; ~8mls.min^{-1} driven by a flow inducer, Watson-Marlow LTD, England) with prewarmed, oxygenated Krebs solution (composition (mM); NaCl 118, KCl 4.7, $NaHCO_3$, 25, NaH_2PO_4 1.15, $CaCl_2$ 2.5, $MgCl_2$ 1.1, glucose 5.6, gassed with 95% O_2 / 5% CO_2 to maintain pH at 7.4). The internal perfusion rate was gradually increased over 1hr to allow the arteries to adjust and stabilize to the new perfusion pressure. Once a stable passive pressure had been attained drugs (ie. PE or SNAP) were injected (10µl, bolus) through a resealable rubber septum directly into the path of the internal perfusate just proximal to the cannula. A differential pressure transducer (P; Sensym type SCX 150NC, Farnell Electronic Components, Leeds) detected perfusion pressure determined by arterial tone. Changes in perfusion pressure reflected changes in the resistance to flow: vasoconstriction of the arteries resulted in an increased perfusion pressure and vasodilation caused a decrease in pressure.

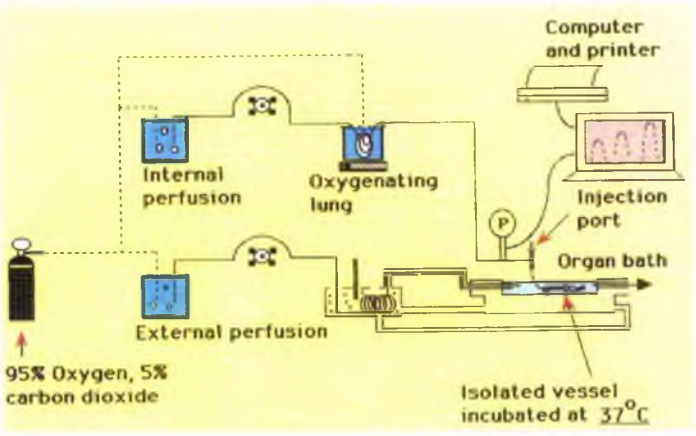


***Fig. 3.1** The apparatus used for perfusing isolated vessels.*

3.3 Evaluation of a rat model of endotoxic shock. Male Wistar rats were given a single injection of bacterial lipolysaccharide (4mg/Kg; i.p.; *E. coli* serotype 055:B5; Sigma Ltd). Similar doses of LPS have been shown to induce iNOS activity in the rat (Knowles *et al.*, 1990). In order to characterise this model, five parameters were monitored to indicate the extent of endotoxaemia.

3.3.1 Measuring Blood Pressure. Haemodynamic parameters in unrestrained, unanaesthetised animals were measured using the tail cuff method (Model 179 Blood Pressure Analyzer; IITC Life Science, Calif., USA.). Animals were familiarised to the procedure by a 'training' regimen (daily for 3 to 4 weeks) before commencing an experiment so as to avoid undue stress. Recordings of systolic (S.b.p.), mean (M.b.p.) and diastolic (D.b.p.) arterial pressures were made at the following times relative to the time at which animals were injected with LPS (t=0hr) : t= -96, -48, 0, 6, 12, 18 & 24hrs. Before each reading, the

animals were placed in a warmed environment (26-29°C) for 15 mins after which time they were cradled under one arm and comforted. The cuff was carefully placed on the tail about 2-3 cm from the body and pressure pulses were monitored on an oscilloscope as the cuff was inflated. Handling proved to be far less stressful to the animals than the perspex holders, with consistent readings usually attained within 5 mins. Analysis of digital traces (as outlined by Mr. Tom Delahanty, Model 179 Engineer, personal commun.), showing the tail pulses on one channel and corresponding cuff pressure on the other (see fig. 3.2), was carried out manually to avoid false readings which often occured as the computer detected movements of the animal causing artifacts on the trace.

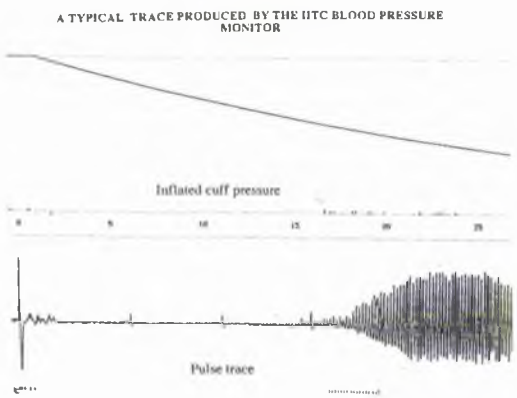


Fig. 3.2 *A trace of arterial pulse pressure obtained using the tail cuff method. The pulse pressure returns when the cuff pressure is equal to systolic b.p. (**S b.p**; indicated by a tall tick mark above the cuff pressure trace baseline). The point at which the pulse amplitude is maximal represents the mean b.p. (**M b.p**; small ticks above the cuff pressure trace baseline indicate updates throughout the reading cycle), and diastolic b.p. is calculated using the equation below:*

Diastolic b.p. = Mean b.p.- 1/2 (Systolic b.p. - Mean b.p.)

The heart rate was estimated from the pulse trace by measuring the time (each grid spacing = 1s) from the first of a series of sixteen small ticks to a final taller tick (ie. seventeen ticks in all) as shown above the pulse pressure baseline (see fig. 3.2). This value was multiplied by 60 to estimate beats /min. Ticks appearing

below either trace after this series of seventeen ticks indicated an interruption in the automatic reading cycle and the computer generated results were ignored.

3.3.2 Measuring Nitrate and Nitrite levels in blood samples The second parameter involved monitoring accumulated plasma nitrate and nitrite levels (=NO_X) at various times (6, 12, 18, 24, 48 & 72hrs) after LPS injection. Animals were sacrificed by cervical dislocation and exsanguinated. A modified assay was employed, since the main oxidation product of NO in blood is nitrate (Wennmalm *et al.*, 1993) and this must be quantitatively reduced to nitrite before performing the Griess test (see Section 1.4.1 and Appendix 3.5).

Blood samples (5-8mls) were obtained from the thoracic cavity of each animal and immediately centrifuged at 15,000g for 10-15mins at room temperature to separate out the plasma, which was then freeze-dried overnight. Thereafter, plasma was kept frozen (-20°C) in small vials until use. Samples containing low NO_Xs (from control animals and LPS-treated animals at t= 6, 12 & 72 hrs were re-constituted in 0.5ml de-ionised water (4 x concentration) and those with high NO_Xs (t= 18, 24 and 48hrs) were re-constituted in 8ml of de-ionised water (4 x dilution; deionised water: Milli-Q+ deionised water filtered through a 0.2µm pore membrane; Millipore Bedford, MA., USA.).

Test solutions, containing blood plasma, were spiked with known dilutions (mM) of nitrate (TNO_3^-) or nitrite (TNO_2^-) and standard (control) solutions, containing de-ionised water instead of plasma were also spiked with nitrate (CNO_3^-) or nitrite (CNO_2^-; see fig. 3.3). Two sets of five tubes containing test solution (sets 1&2: TNO_3^- & TNO_2^-) and two sets of five tubes containing standard solution (sets 3&4: CNO_3^- & CNO_2^-) were used, each of which contained either plasma (127µl; sets 1&2) or de-ionised water (127µl; sets 3&4), to which the following were added (final concentrations): FAD (48.2µM), NADPH (482µM), phosphate buffer (pH 7.4; 40mM), nitrate reductase enzyme isolated from *Aspergillus* species (70mU; added to all of the solutions containing

nitrate; from Boehringer Mannheim GmbH, Germany) and, in tubes 1-5 respectively, the following quantities (nmoles) of Analar grade authentic NO_2^- or NO_3^- as 'internal' standards: 45, 22.5, 15.75, 11.25 and 2.25 nmoles.). Tubes were incubated at 37°C for 2hrs before the Griess test was performed. Absorbance was read at 540 nm and a linear graph of the results plotted with absorbance (Y axis) against [nitrate/nitrite] (X axis; see fig. 3.3). The intercept along the X axis represents nanomoles of nitrate/nitrite in the serum sample, (for control, t= 6, 12, & 72 hr samples this value was divided by four and for t= 18, 24 and 48 hr samples this value was multiplied by four). A correction was made to allow for the efficiency of enzymatic reduction of NO_3^- to NO_2^- (average conversion ~93%) and the results expressed in µmoles/l plasma.

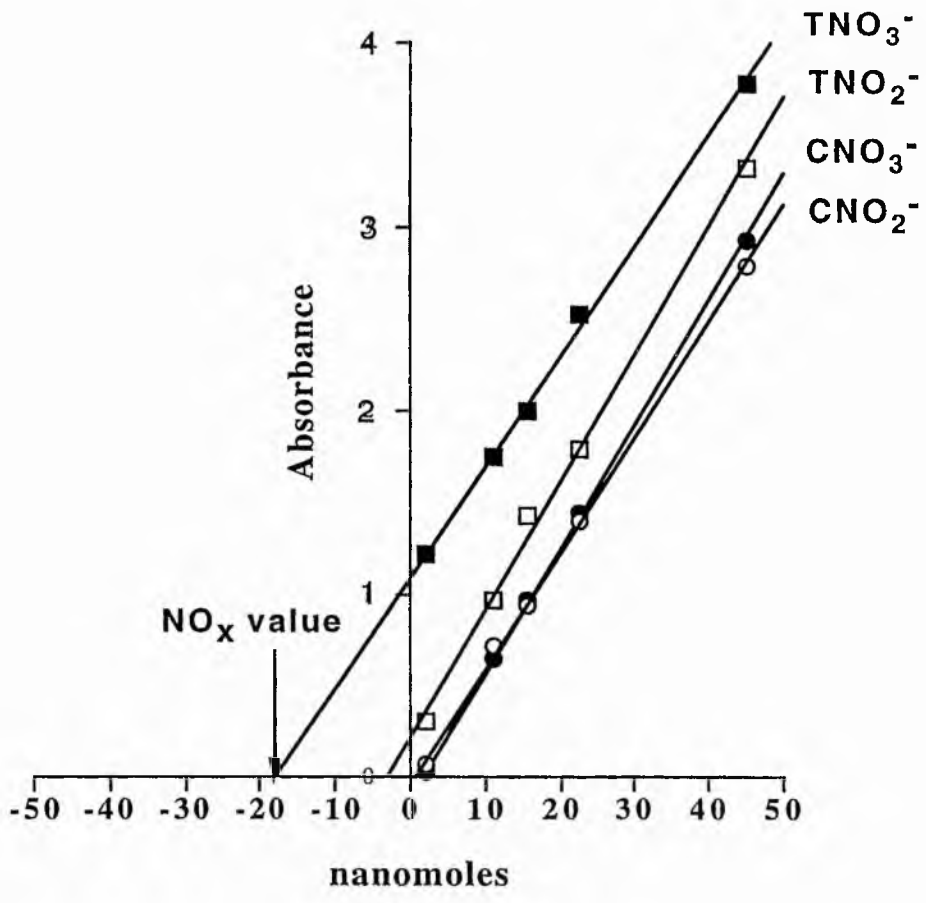


Fig. 3.3 *An example of a graph used to calculate to the levels of nitrate and nitrite in blood plasma. NO_X = TNO_3^- (closed squares) and total nitrite = TNO_2^- (open squares).The gradients for CNO_3^- (closed circles)and CNO_2^- (open circles) are used to calculate the % conversion of nitrate to nitrite by NR (for explanation see text).*

3.3.3 The hyporesponsiveness to PE of isolated, internally-perfused tail arteries (see Section 3.2.1) from LPS-treated rats. PE (10^{-5}M-10^{-2}M) was injected into internally-perfused RTAs taken from animals treated with LPS (*viz*: at t= 6, 12, 18, 24, 48 & 72hrs). PE produced transient vasoconstrictor responses (see fig. 3.4). Peak pressures were recorded on a computer and a print out was obtained at the end of the experiment. These values were used to construct log dose-response (D.R.) curves.

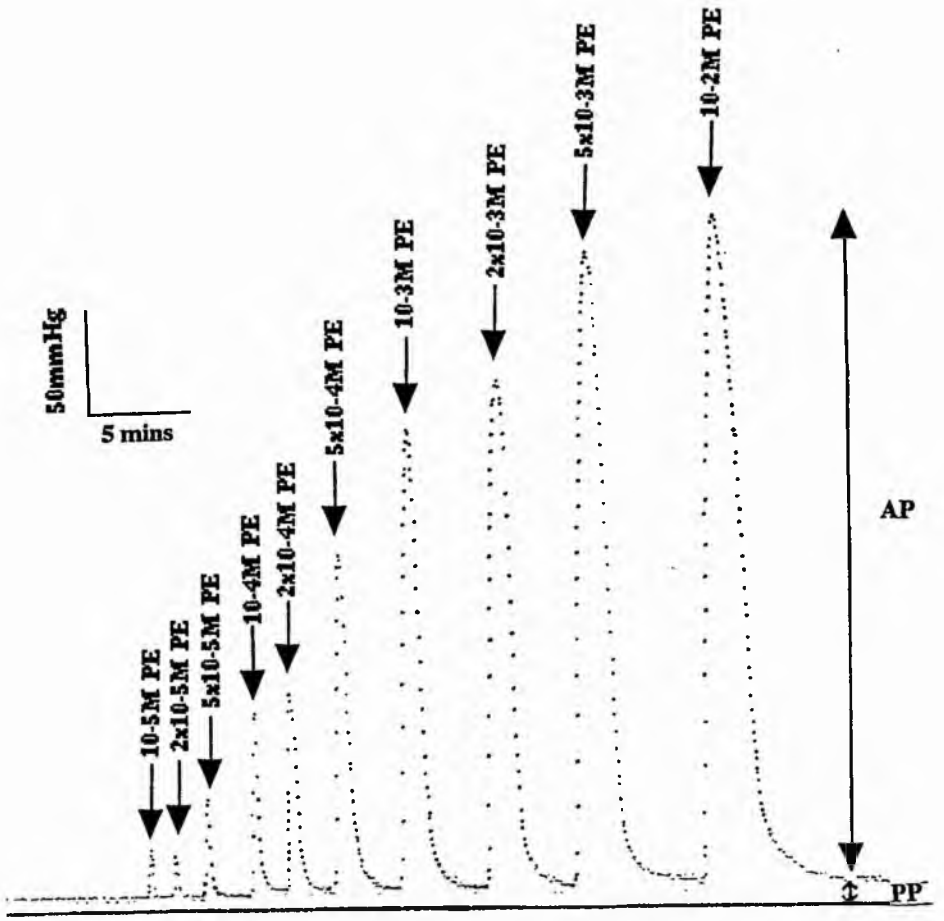


Fig. 3.4 *Pressure recording of RTA to bolus injections (10µl) of PE (10^{-5}M-10^{-2}M). The pressure induced by the internal perfusate alone is called 'passive pressure' (PP) while that induced by PE is 'active pressure' (AP). Passive pressure was restored before subsequent injections of PE were administered.*

3.3.4 The hyporesponsiveness of pre-contracted, internally-perfused tail arteries to bolus (10µl) injections of SNAP. Control and LPS-treated (t= 24hrs) RTAs were precontracted to ~100mmHg perfusion pressure with PE (2-5µM - 10-12µM respectively) and exposed to increasing concentrations of the

endothelium-independent vasodilator, SNAP (10^{-7}M-10^{-3}M). The responses to SNAP are expressed as a percentage of the active perfusion pressure (mmHg) and plotted as a function of log[SNAP] (see fig. 3.8).

3.3.5 The expression of NOS Antibodies specific to eNOS (# 2361) and iNOS (# 2464) were used for immunohistochemical analysis in various tissues including, lung, liver, heart, RTA, tumour-supply arteries and tumour tissue. A number of methods were employed to indicate the expression of NOS: NADPH diaphorase staining, indirect immunofluorescence, avidin-biotin-immunoperoxidase complex (ABC) method and Western blot analysis. The protocol for Western blotting is briefly oulined below with a more detailed account given in Appendix 3.1. Essentially, Western blots were made from extracts of lung, heart, liver and RTAs taken *post mortem* from animals sacrificed at different times after LPS injection (t= 4, 6, 12, 24, & 72hrs).Tissues were weighed and homogenised for 3-5 mins at 24,000rpm (Ultraturrax T25 homogeniser) in 5 volumes ice cold buffer (see Appendix 3.1.2 for composition). The resulting lysate was centrifuged (100,000g, 1hr at 4°C) and the protein content of the supernatent estimated using the Bradford assay. Samples containing 2mg protein/ml (lung, liver and heart) or 1mg protein/ml (RTAs) were mixed with 0.5x volume of laemmli buffer (see Appendix 3.1.5) (3x strength) and β-mercaptoethanol added to a final concentration (v/v) of 15%. Samples were boiled and then subjected to SDS-PAG electrophoresis (15μl per well; 7.5% SDS). Proteins were transferred onto a nitrocellulose membrane, probed overnight with a rabbit polyclonal antibody (#2464; 1:1000 dilution) and then treated with goat anti-rabbit IgG-horseradish peroxidase secondary antibody (GARP; 1:3000 dilution). Finally, the secondary antibody was detected by enhanced chemiluminescence (ECL). Molecular weight markers (*ca.* 26-170KDa) were used to estimate apparent molecular weights of iNOS-positive bands.

3.4 Materials

3.4.1 Drugs In general these were prepared immediately before each experiment.

Lipopolysaccharide purified from *Escherichia coli* serotype 055:B5, (dissolved in 0.9% sterile saline (NaCl) solution), **N^{w}-nitro-L-arginine methyl ester hydrochloride** (L-NAME hydrochloride; M_r 269.7), **L-Phenylephrine hydrochloride** (PE; M_r 203.7), **aminoguanidine hemisulfate salt** (AG; M_r 123.1), **L-arginine hydrochloride** (L-Arg; M_r 210.7), **cycloheximide** from *Streptomyces griseus* (M_r 281.4) and **indomethacin** (M_r 357.8); 10^{-3}M stock solution (dissolved in 5% sodium carbonate), were all obtained from Sigma Ltd and added to the internal perfusate at the appropriate concentration.

L-N^{G}-monomethyl-L-arginine hydrochloride (L-NMMA hydrochloride; M_r 266) was a gift from Dr. H. Hodson, Wellcome Res. Labs., Kent.

S-nitroso-N-acetylpenicillamine (SNAP; M_r 220) prepared by Dr. A.R. Butler, Dept. of Chemistry, St. Andrews University. Solutions were kept on ice during the experiment and administered as a bolus injection (10µl) into the internal perfusate at concentrations of 10^{-7}M-10^{-3}M.

3.4.2 Antibodies All **primary** antibodies are diluted and then stored at -20°C in aliquots of 2-5µl. Dilutions were made in PBS_C (complete phosphate buffered saline) containing bovine serum albumin (BSA; 0.1% (v/v). See Appendix Section 3.2.3). Further dilutions made before Western blot analysis were made in 1% skimmed milk (Boots PLC).

Primary antibodies. Specific to iNOS - # 2464 (Rabbit polyclonal raised against a murine macrophage iNOS polypeptide). Stored at 1:10 dilution and used at 1:200 for indirect immunofluorescence (IF), 1:750-1:1000 for avidin-biotin-complex-immunoperoxidase (ABC-IP) method and 1:1000-1:2500 for

Western blot analysis. **Specific to eNOS - # 2361** (Mouse monoclonal raised against bovine aortic cell extract). Stored at 1:100 dilution and used at 1:1000 for IF, 1:10,000 for ABC-IP and 1:10,000 for Western blot analysis. Both were a gift from Dr. Lee Buttery, RPMS, Hammersmith Hospital, London.

Conjugated Secondary antibodies. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate (**FITC**; GAMF, diluted to 1:200 for IF on histological sections), **goat anti-rabbit IgG** conjugated to either fluorescein isothiocyanate (**FITC**; GARF, diluted to 1:200 for IF on histological sections), or tetramethylrhodamine isothiocyanate (**TRITC**; GARR, diluted to 1:80 for IF on histological sections) were obtained from Sigma Ltd. **Donkey anti-rabbit IgG** conjugated to horseradish peroxidase (**HRP**; DARP, diluted to 1:1000 for Western blot analysis using enhanced chemiluminescence detection) and **sheep anti-mouse IgG** conjugated to horseradish peroxidase (**HRP**; SAMP, diluted to 1:1000 for Western blot analysis using enhanced chemiluminescence detection) were obtained from SAPU (Scottish Antibody Production Unit), Lanarkshire, Scotland. All secondary antibodies were stored neat in 10µl aliquots at -20°C before use.

3.4.3 Anaesthetic

A general anaesthesia ($90mgKg^{-1}$ Ketamine, $10mgKg^{-1}$ Xylazine; i.p.) was administered during tumour implantation and excision of inferior epigastric arteries.

3.4.4 Animals

Male Wistar rats (350-550g; John Tuck Ltd) were used for the model of endotoxic shock and all RTA experiments. Male, isogeneic BD9 rats (350-450g; from the CRC Gray Lab., Mount Vernon Hospital, Middlesex, England) were used for all tumour experiments.

3.5 Results

3.5.1 The Time course of LPS-induced hypotension. Four haemodynamic parameters were measured using the tail-cuff method; systolic, mean and diastolic b.p's (mmHg) and heart rate (beats/min). A group of ten rats was trained for approximately 1 month and b.p. was monitored at 4 and 2 days (-96hrs, -48hrs; see fig. 3.5) prior to a single bolus injection of LPS (4mg/Kg; i.p.) at t =0. Thereafter, b.p. and heart rate were monitored every 6hrs for a total of 24hrs with an average of four readings recorded per animal at each time point to improve accuracy and ensure that the results were consistent.

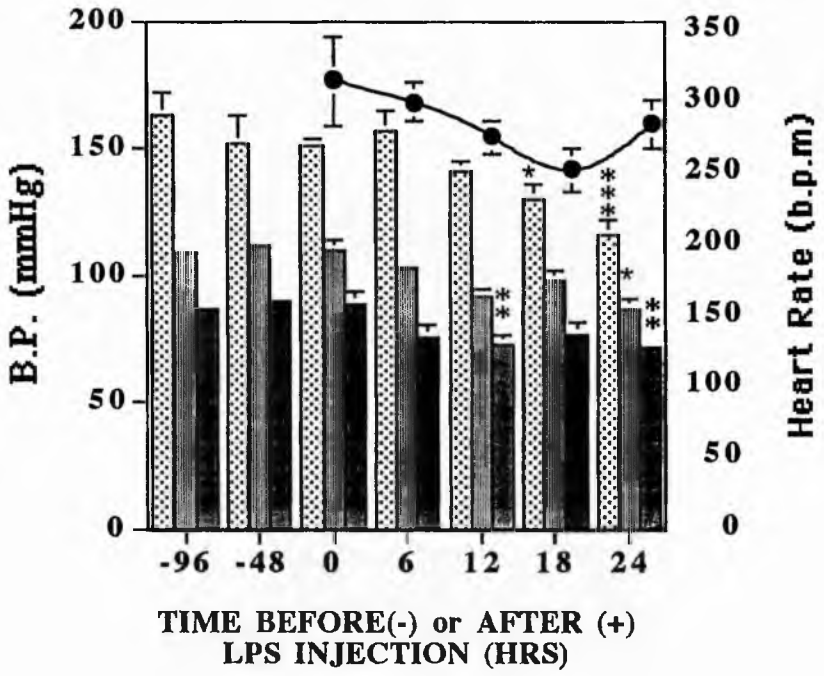


Fig. 3.5 *A graph showing the effect of LPS treatment on* systolic (stippled column), mean (light grey column) and diastolic (dark grey column) b.p. *recorded using the tail-cuff method, where (*) indicates a significant ($p< 0.05$) drop in b.p. compared to t= 0hrs. Filled circles denote meaned heart rate.*

Animals were observed throughout the experiment and visual symptoms of LPS treatment were noted in conjunction with b.p. measurements. Within 30-60mins. after LPS injection, animals exhibited erectae pilae, reddening of the eyes, cold feet and became very sluggish. Rectal temperatures varied from 36.5-37°C.

These symptoms were thought to reflect the onset of fever characteristic of endotoxic shock. During this period animals were kept warm by increasing R.T. from 21°C to 25°C. At t= 6hrs animals displayed no obvious symptoms of fever, although a marked darkening of faeces was noted. Haemodynamic parameters were not significantly altered from t= 0hrs (see fig. 3.5 & appendix table 3.1). At t=12hrs the symptoms of fever had returned in a few animals despite having been kept at a R.T. of 25°C. Systolic b.p. decreased from 150.3 ±3.59mmHg to 140.3 ±4mmHg and diastolic was significantly reduced from 89 ±4.09mmHg to 73 ±3.6mmHg. Warming the animals immediately prior to measuring b.p. consistently reversed the obvious symptoms of fever at every time point. At t= 18 and 24hrs more than half of the animals appeared to be dehydrated, although they remained active and alert. All three parameters of b.p. were significantly reduced by t=24hrs with a noteable 23.5 ±6.2% drop in systolic b.p. from 150.30 to 115.5 ±5.76mmHg.

3.5.2 The Time Course of Enhanced Nitrite and Nitrate accumulation following LPS exposure. The levels of nitrite and nitrate in blood plasma were measured using the Griess reaction after conversion of nitrate to nitrite using the enzyme nitrate reductase. Nitrite and nitrate represent the main oxidative products of NO metabolism and can therefore be used to indicate the accumulated NO levels within the bloodstream at any given instant in time. In order to monitor the levels of NO being produced *in vivo* following LPS treatment, blood samples were collected from animals sacrificed at t= 6, 12, 18, 24, 48 & 72hrs after LPS injection (control animals (0hrs) received 0.9% sterile saline solution and samples were taken at t= 24hrs). Results are expressed as mean (±SEM) of the combined amounts of nitrite and nitrate; (NO_X: μmoles/l) plotted as a function of time post-LPS injection (hrs; see fig. 3.6 & appendix table 3.2).

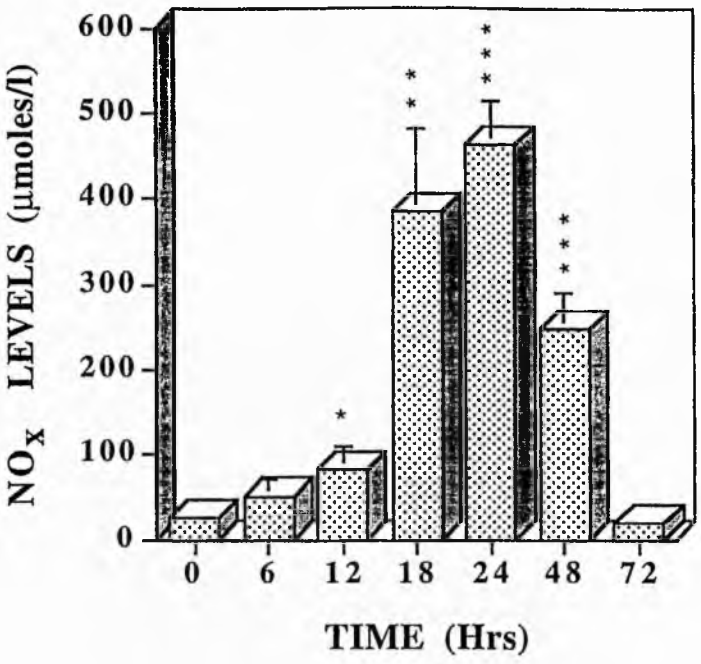


Fig. 3.6 *Time-dependent increase in NO_X levels at depicted timepoints post-LPS treatment. NO_X levels are also shown for control animals (0hr).*

Student's t-test analysis found levels of NO_X in blood plasma significantly increased from 26.35 ±6 µmoles/l to 81.8 ±27.9 µmoles/l by t= 12hrs as compared to controls, rising to a maximum of 463.1 ±50.3 µmoles/l by 24hrs (~18 fold increase) and returning to control levels by 72hrs (19.7 ±6.28µmoles/l; see appendix table 3.2).

3.5.3 LPS-induced Hyporesponsiveness of RTAs to PE. Administration of *E. coli* LPS to rats caused a time-dependent hyporesponsiveness to PE. This effect has previously been attributed to LPS-induced activation of the inducible NOS and the subsequent high levels of NO produced (Schott *et al.*, 1993). Fig. 3.7 shows dose response curves to PE for RTAs from animals receiving 0.9% saline injection (controls) compared to those obtained using vessels isolated from animals sacrificed at 6, 12, 18, 24, 72 hrs post-LPS injection.

Group	Control	6hr	12hr	18hr	24hr	48hr	72hr
% Con at ($5x10^{-3}M$)	100.0 ±4.4	112.8 ±7.8	89.1 ±7.7	83.1 ±9.4	56.9 ±8.1	101.4 ±11.7	107.0 ±15.2

Table 3.1 *Shows the perfusion pressures produced by $5x10^{-3}M$ PE in control and LPS treated RTAs (removed 6, 12, 18, 24, 48 and 72hrs after LPS treatment) expressed as a % of that for controls. [PE]= $5x10^{-3}M$ was chosen as a maximum so as to include all the data.*

The LPS-induced hyporesponsiveness in RTAs becomes apparent at t=12hrs with ~11% decrease in sensitivity compared to the control at $5x10^{-3}M$ [PE] (see table 3.1). However, the curve is not significantly different from the control curve (2 way ANOVA; F= 0.56, df= 9, 183_e, p= 0.827). At t= 18hrs the contractile responses are significantly diminished compared to the control (2 way ANOVA; F= 2.29, df= 9, 256_e, p= 0.017) and the response at $5x10^{-3}M$[PE] is reduced by ~17%. The hyporesponsiveness to PE is further accentuated by t=24hrs with responses now highly significantly as compared to controls (2 way ANOVA; F= 15.74, df= 9, 289_e, p <0.001) with ~43% decrease in sensitivity at $5x10^{-3}M$[PE]. At 48hrs (not shown in Fig. 3.7) and 72hrs post-LPS treatment, the contractile responses are no longer reduced and now resemble those obtained with control vessels. Neither are significantly different from the control (see appendix table 3.3).

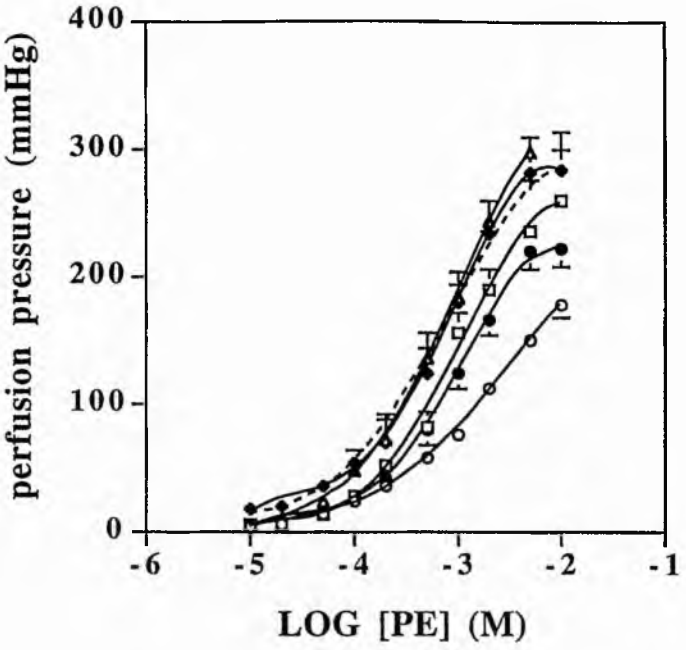


Fig. 3.7 Shows the contractile responses for RTAs from control animals (dotted line; n= 18 RTAs), LPS treated animals after 6hrs (open triangles; n=16 RTAs), 12hrs (open squares; n=4 RTAs), 18hrs (closed circles; n= 13 RTAs), 24hrs (open circles; n= 15 RTAs) and 72hrs (closed diamonds).

These results confirm that LPS treatment results in a transient decrease in sensitivity to PE with time after treatment.

3.5.4 Diminished Sensitivity to Bolus Injections of SNAP.

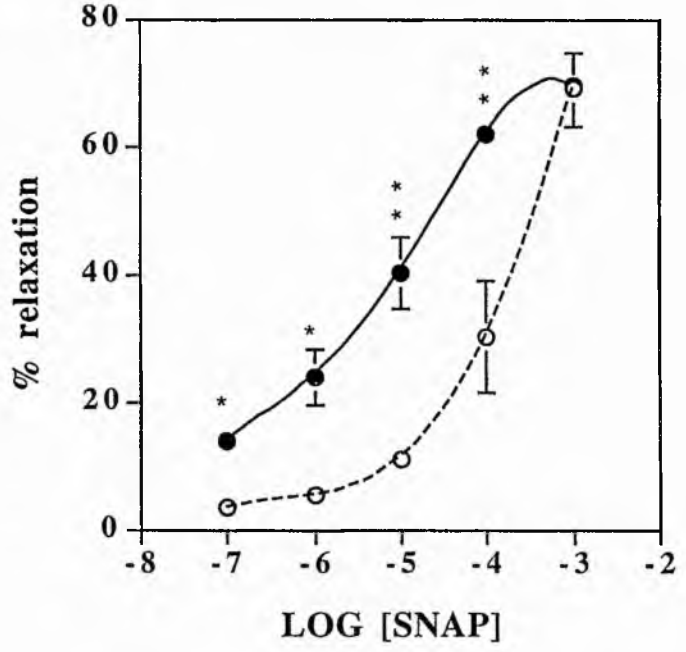


Fig. 3.8 Dose-response curves to increasing concentrations of SNAP for control (closed circles, n= 8) and LPS-treated (24hrs; open circles, n= 4) RTAs. Asterisks= unpaired t-test.

Pre-contracted, LPS-treated vessels displayed a highly significant hyporesponsiveness to SNAP at concentrations < 10^{-3}M (see fig. 3.8 & Appendix table 3.4). The ED_{50} values reveal a 26.7 fold rightward shift in [SNAP] for LPS-treated responses compared to their respective control values. At 10^{-3}M [SNAP] both groups displayed similar vasodilator responses to SNAP (~70%).

3.5.5 Time Course of iNOS Expression after LPS Treatment. The expression of iNOS protein was detected by Western blotting in extracts of lung, heart, liver and RTAs from *post-mortem* control and LPS-treated animals (t= 4, 6, 12, 24, 72hrs; see Appendix 3.1). Fig. 3.9 shows lung extract from t= 24hr LPS-treated rats expressing high levels of iNOS as evident by the densely staining band at 130KDa. Lung extract from t= 24hr LPS-treated rats was used as a positive control for iNOS expression until the commercially available positive cell lysate arrived. The validity of the lung extract was confirmed with both bands detected at 130KDa (see fig. 3.10). Fig. 3.10 also reveals a time course of iNOS expression in LPS-treated RTAs, with a maximum at t= 4-6hrs, gradually declining until it was barely detectable by t= 24hrs and undetectable by t= 72hrs. Dot blot analysis further revealed that liver extracts from t= 24hr LPS-treated rats also expressed iNOS (see fig. 3.11).

Several histochemical and immunohistochemical techniques were used to detect eNOS and iNOS expression in tissue sections (see figs. 3.12-3.18 and Appendix 3.2). Immunoflourescent labelling revealed intensely stained macrophage-like cells scattered throughout the lung tissue of t= 24hr LPS-treated rats (fig. 3.12). Intensely fluorescent cells were often seen in lung tissue from control animals but not to the same extent.

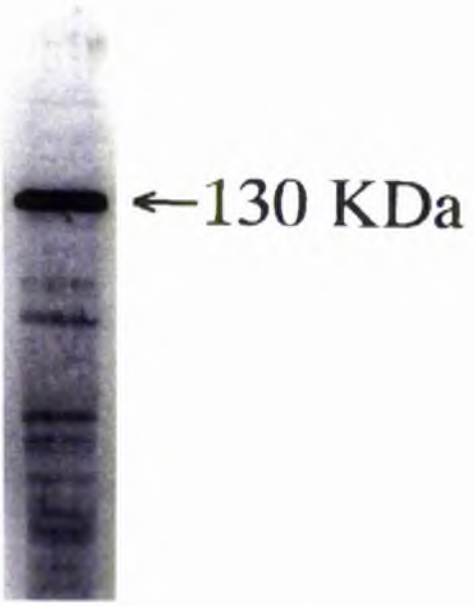


Fig 3.9 *Western blot analysis using a specific antibody to iNOS enzyme of lung extract from t= 24hr LPS-treated rat reveals an intensely staining band at 130 KDa, suggesting that iNOS is being expressed.*

1 2 3 4 5 6 7

Fig. 3.10 *Western blot analysis using a specific antibody to iNOS of LPS-treated RTAs at t= 4hrs (lane 1), 6hrs (lane 2) 12hrs (lane 3) 24hrs (lane 4) and 72hrs (lane 5). Lung extract from t= 24hr LPS-treated rats and activated-macrophage lysate are used as positive controls (lanes 6 & 7 respectively). iNOS expression is visibly reduced at t= 24hrs after LPS treatment.*

Fig. 3.11 shows a dot blot of lung and liver extracts from t= 24hr LPS-treated rats demonstrating a more intense signal for iNOS with increasing protein concentration.

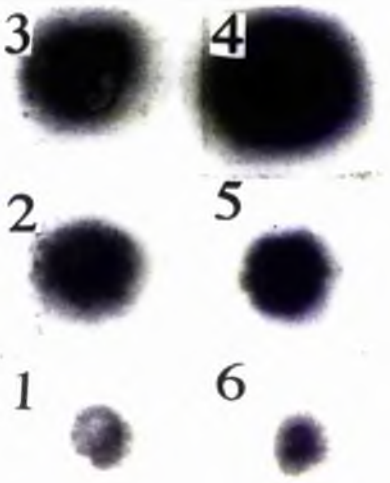


Fig. 3.11 *Dot blot analysis with anti-serum to iNOS on lung ([protein]s, 1.7, 6.6, 7.6g/l; Nos 1-3 respectively) and liver ([protein]s, 1, 7.6 and 30g/l; Nos 6-4 respectively) extracts from t= 24hr LPS-treated rats.*

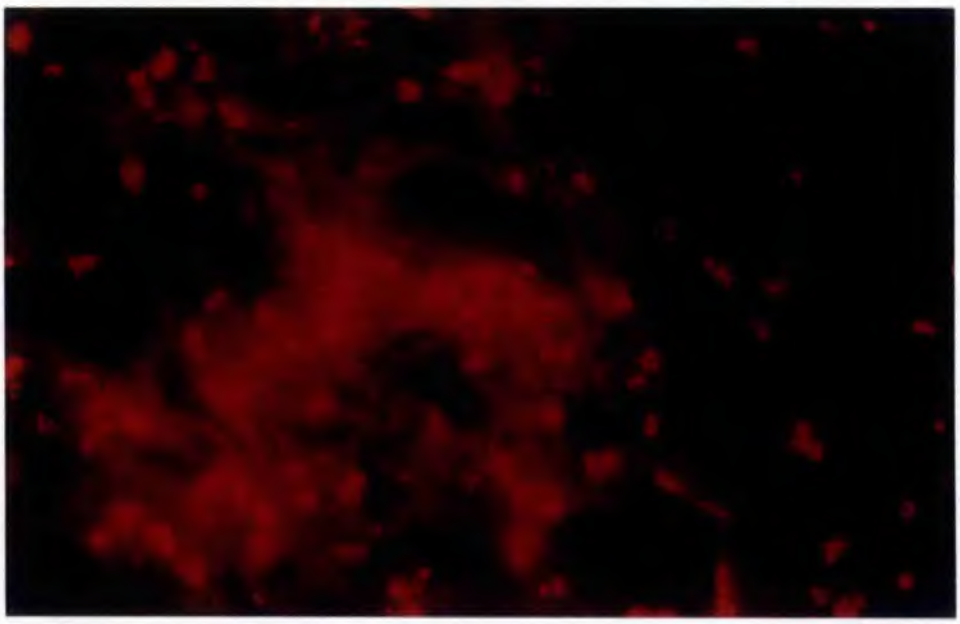


Fig. 3.12 *Cryostat section (5-7μm) of lung tissue from a t= 24hr LPS-treated rat immunostained with fluorescently-labelled (TRITC) 2º antibody. Intensely stained macrophage-like cells are scattered throughout the lung tissue (x400).*

Fig. 3.13 shows that iNOS positive cells were not seen within bronchioles or their associated epithelial lining but were instead interposed between the alveolar network in the surrounding visceral pleura.

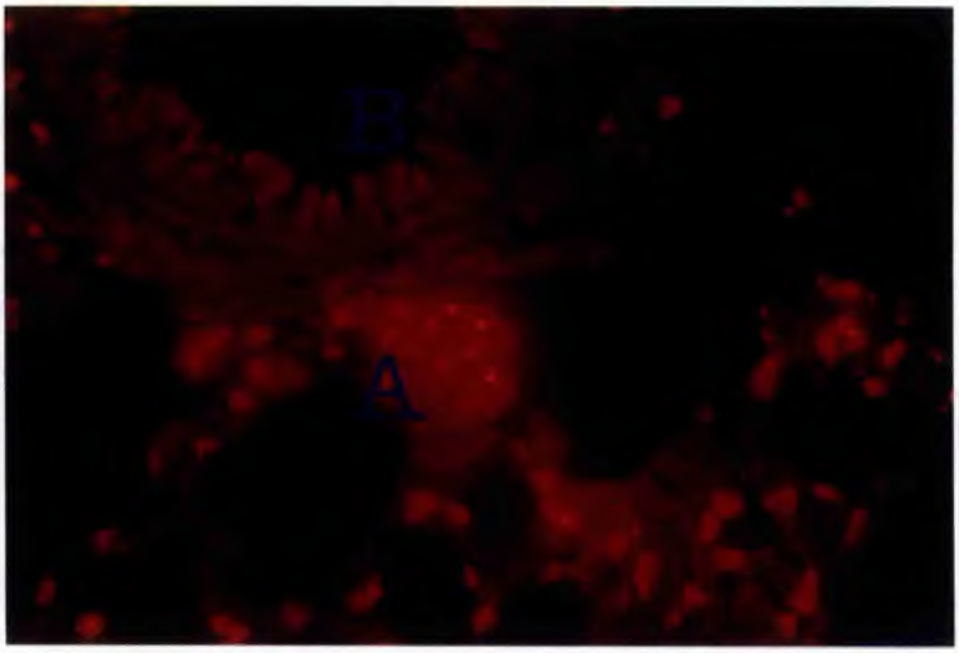


Fig. 3.13 *TRITC-labelled cryostat section of lung from t= 24hr LPS-treated rat showing the intensely fluorecent cells in the visceral pleura (**A**) but absent from the nearby bronchiole (**B**; x400).*

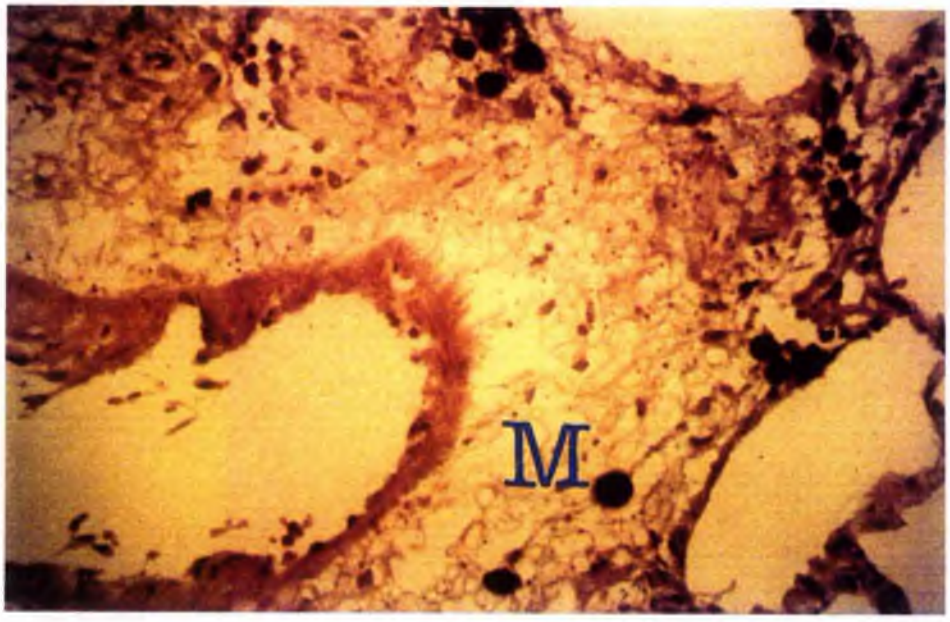


*Fig. 3.14 Similarly, immunostaining by the ABC method on sectioned lung tissue from a t=24hr LPS-treated rat showed iNOS positive cells (**M**) as large rounded black dots within the visceral pleura (x400).*

Cryostat sections of the same area in the lung as shown in fig. 3.14 were also stained for NADPH-diaphorase activity (see Appendix 3.4) and resulted in a large number darkly stained cells within the visceral pleura and attached to the epithelial lining of a bronchiole (**B**; fig. 3.15).

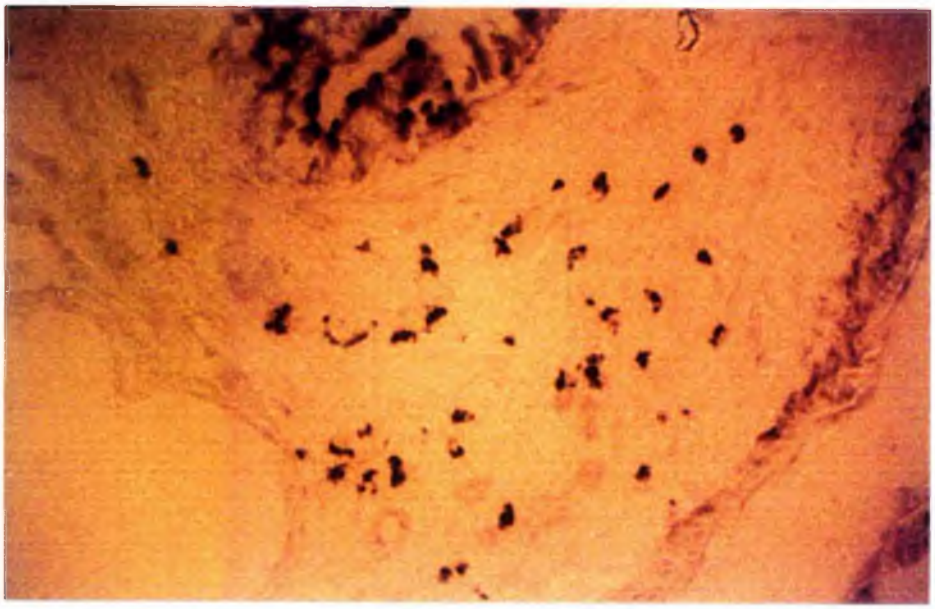


Fig. 3.15 *Cells within lung tissue from t= 24hr LPS-treated rat stain intensely for NADPH-diaphorase. NADPH-diaphorase-Positive cells are also evident on the epithelial lining of a bronchiole (**B**; x400).*

LPS-treated RTAs at t= 6, 24 and 72hrs were immunostained with anti-serum to eNOS to determine whether eNOS expression persisted throughout the LPS-mediated induction of iNOS (see figs. 3.16-3.18). Fig. 3.16 shows an LPS-treated RTA at t= 6hrs immunostained using the ABC method. iNOS is highly expressed at t= 4-6hrs and it is clear from fig. 3.16 that eNOS is also present at this time.

Fig. 3.16 *Paraffin-sectioned (5-7μm) RTA from an LPS-treated rat at t= 6hrs immunostained with anti-serum to eNOS using the ABC method. Endothelial cells (**E**) which express eNOS are seen here as a thin, darkly stained layer facing towards the lumen of the vessel (**L**; x1000).*

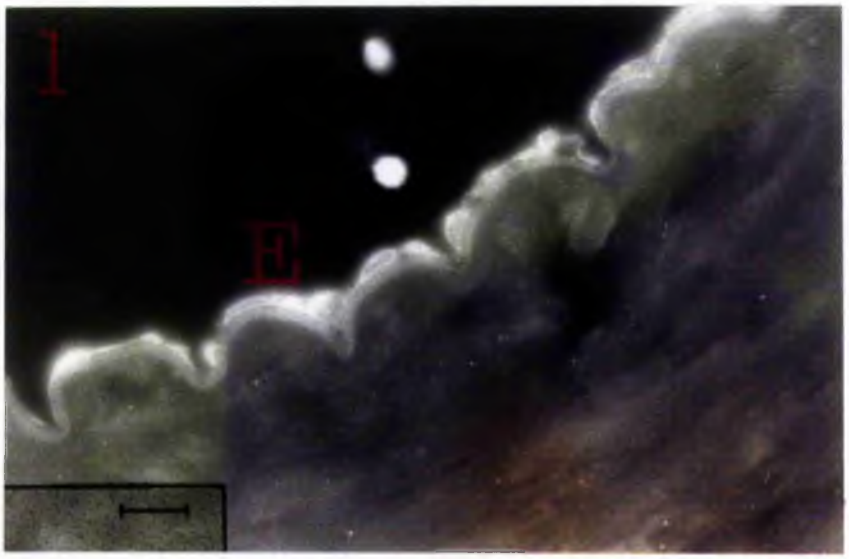


Fig. 3.17 *Paraffin-sectioned (5-7μm) RTA from an LPS-treated rat at t= 24hrs immunostained with anti-serum to eNOS and detected using a fluorescently-labelled (FITC) 2º antibody. Endothelial cells (**E**) which express eNOS are seen here as a thin, brightly stained layer facing towards the lumen of the vessel (**L**; x1000).*

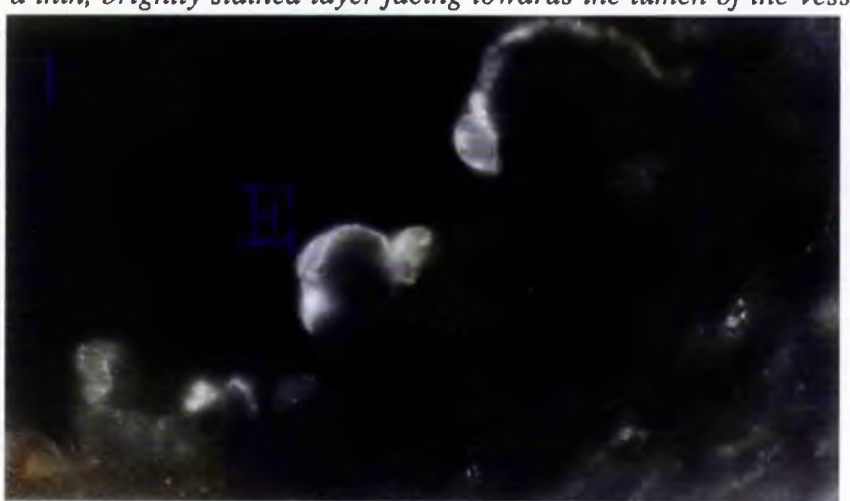


Fig. 3.18 *Paraffin-sectioned (5-7μm) RTA from an LPS-treated rat at t= 72hrs immunostained with anti-serum to eNOS and detected using a fluorescently-labelled (FITC) 2º antibody. Endothelial cells (**E**) which express eNOS are seen here as a thin, brightly stained layer facing towards the lumen of the vessel (**L**; x1000).*

It is important to realise that little quantitative information regarding the relative expression of eNOS at t= 6, 24 and 72hrs post-LPS is provided by these sections as staining will depend very much on **a**) the type of detection method used **b**) the condition of the RTA, which will vary from one preparation to the next; and finally **c**) the quality of the photograph. It can however be concluded that eNOS is expressed in RTAs at t= 6, 24 and 72hrs post LPS.

Part B

3.6 Quantitative analysis of hyporesponsive vessels isolated from animals in endotoxic shock.

It will be seen later (Chapter 7) that isolated vessels that previously supplied a solid tumour exhibit a similar diminished sensitivity to PE when compared to their respective controls: superficially, at least, the hyporesponsiveness shown by LPS-treated vessels and tumour supply vessels appears very similar. However, a more detailed analysis (below) suggests that there may be qualitative differences between the two, a point which is discussed again later (Chapter 9).

3.6.1 Theory.

The diminished sensitivity to PE shown by LPS-treated vessels and tumour supply vessels could in theory take one of two forms: (a) one resembling a competitive-type of inhibition, where the log dose response curve is simply shifted to the right along the abscissa, with no change in the maximum response attainable at the higher doses; or (b) one resembling a non-competitive type of inhibition, where there is a decrease in the maximum response even at the highest doses. Of course, in practise, a given set of experimental results may only permit a restricted *part* of each curve to be constructed and this makes it difficult to decide to which type of inhibition the data belongs.

The two situations outlined above are illustrated diagramatically in Figure 3.19 using simulated data. The analysis proceeds as follows. First, the 'test' values are expressed as a fraction of the corresponding 'control' values. The results obtained ($b/a = f$) are then plotted as a function of $\log_{10}$[PE]. Theoretically, if the response to PE were not altered the log dose response curves would be identical and all f values = 1.0. Plotting the latter as a function of [PE] would therefore yield a horizontal line with a slope (m) of zero. Similarly, a horizontal

line would also be obtained if all points on the curve were reduced by the same fraction ($f < 1.0$) at each [PE]. Fig 3.19 (1b & 2b) show that the simulated data, representing the two types of inhibition, give qualitatively different results.

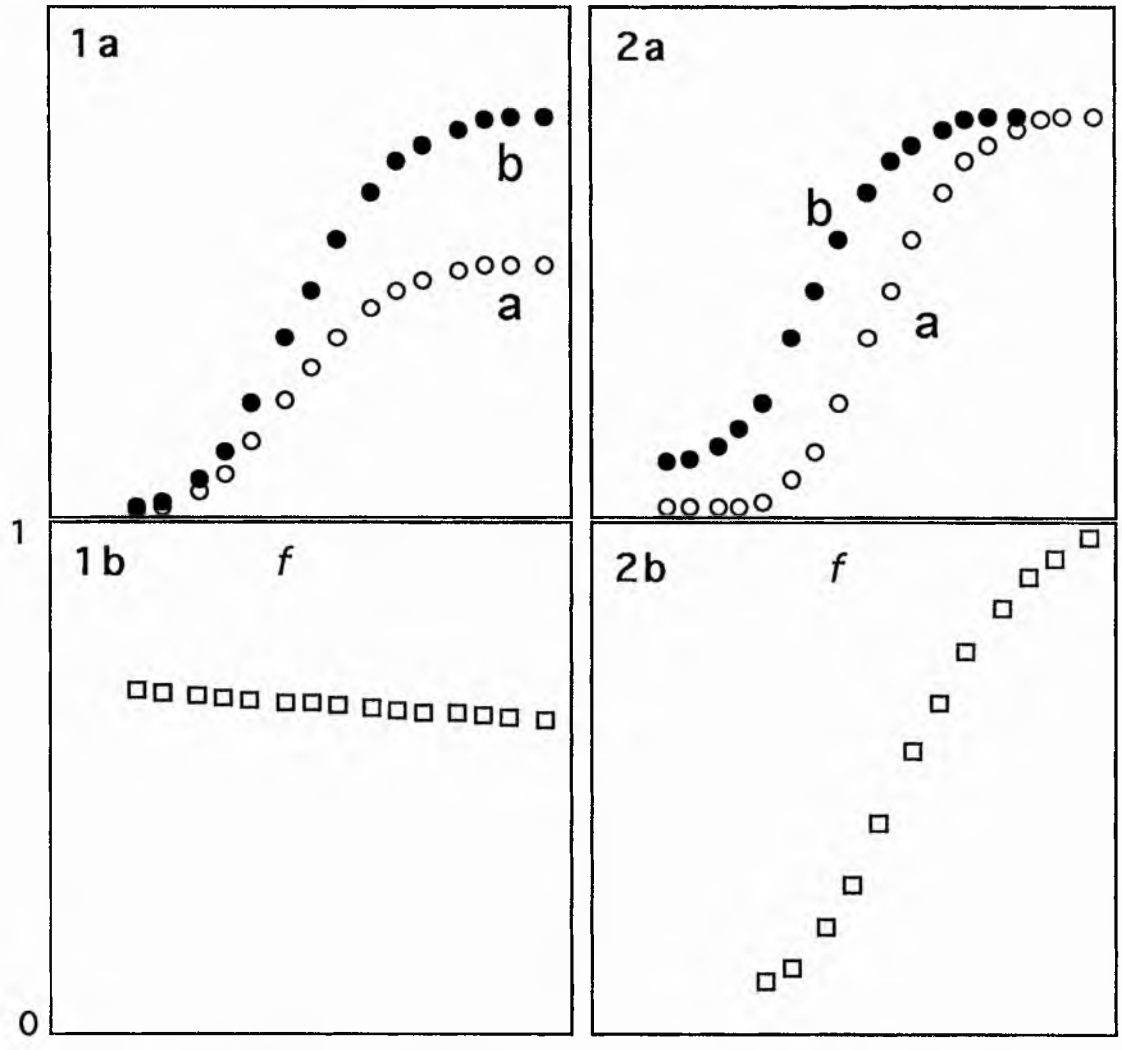


Fig. 3.19 *The difference in sensitivity to PE shown by LPS-treated RTAs and tumour supply vessels (line a; open circles) compared to their respective control (line b; closed circles) can theoretically resemble a non-competitive-type (fig.**1a**) or competitive-type (fig. **2a**) shift with f values (b+ a; open squares) resulting in a negative slope (m; fig. **1b**) and a positive slope (fig. **2b**) respectively.*

3.6.2 Results of analysis. Fig 3.20 shows the results obtained by applying this method of analysis using the actual experimental data obtained with vessels taken from animals previously treated with LPS, presented earlier (t = 18, 24 and 72 hr; Fig. 3.7). The values for f vary approximately linearly with the [PE], revealing a progressive increase in the *relative* sensitivity of LPS-treated vessels

with increasing [PE]s: in other words, the administration of a sufficient [PE] is able to counteract, to some extent, the effects of LPS treatment.

Significantly, the slopes of the lines, *m*, derived by linear regression analysis, vary with time after LPS treatment. Table 3.2 shows a gradual reduction in the value of *m* between t = 6 - 24hr (from 0.29-0.10), falling to 0 at t = 48 and 72hr. The values of *f* obtained for the latter two time points approximate to 1.0: the means (+/- SEM) were found to be 0.96 ±0.18 and 1.00 ±0.20 respectively. The 72hrs data is shown in Fig 3.20 (open circles).

Group	Control	6hr	12hr	18hr	24hr	48hr	72hr
m	0.00	0.29	0.10	0.18	0.10	0.00	0.00
1/m	0.00	3.44	5.13	5.71	10.41	0.00	0.00
r^2	-	0.879	0.851	0.960	0.887	-	-

Table 3.2 *Showing the 'm' values and the respective r^2 coefficients for the linear curve fit.*

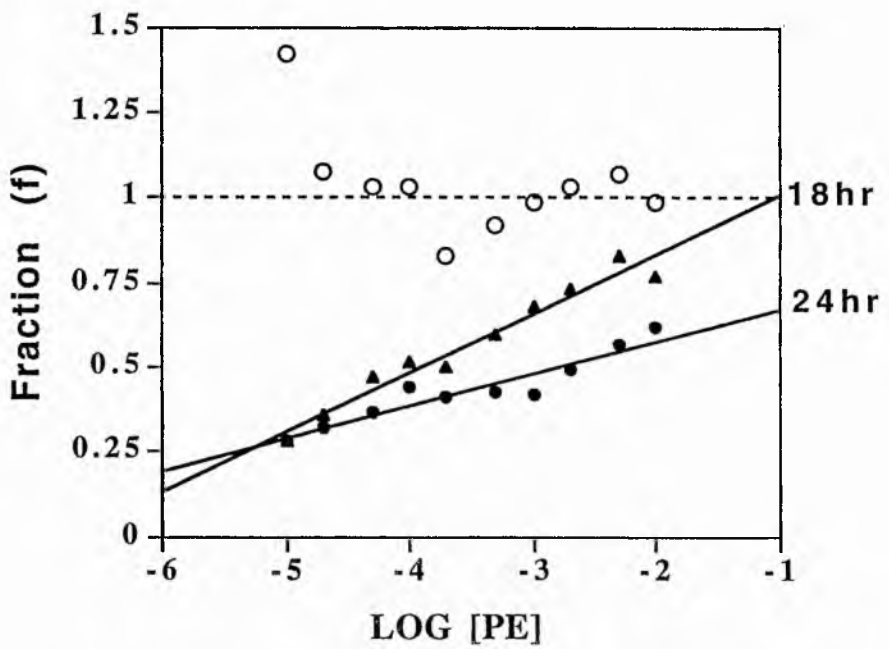


Fig. 3.20 *Shows the LPS log D.R.s at 18hrs (closed triangles), 24hrs (closed circles) and 72hrs (open circles) expressed as a fraction (f) of control values plotted against log[PE]. The dashed line represents the theoretical result for values identical to the control (see text for further details).*

The hyporesponsiveness to PE clearly varies with time after LPS, increasing up to t = 24 hr and then decreasing thereafter. The time course of the development of the hyporesponsive state is therefore better described by plotting the *reciprocal* of *m* as a function of time after LPS treatment. The result of doing this using the values shown in Table 3.2 appears in Figure 3.21. This shows that the extent to which responses are impaired in LPS treated vessels rises to a maximum at t = 24 hr and then declines, so that by t = 48 and 72 hr, vessels show the same sensitivity to PE as control vessels. This compares closely with the time course for the increased accumulation of plasma NO_X levels, shown in Figure 3.7.

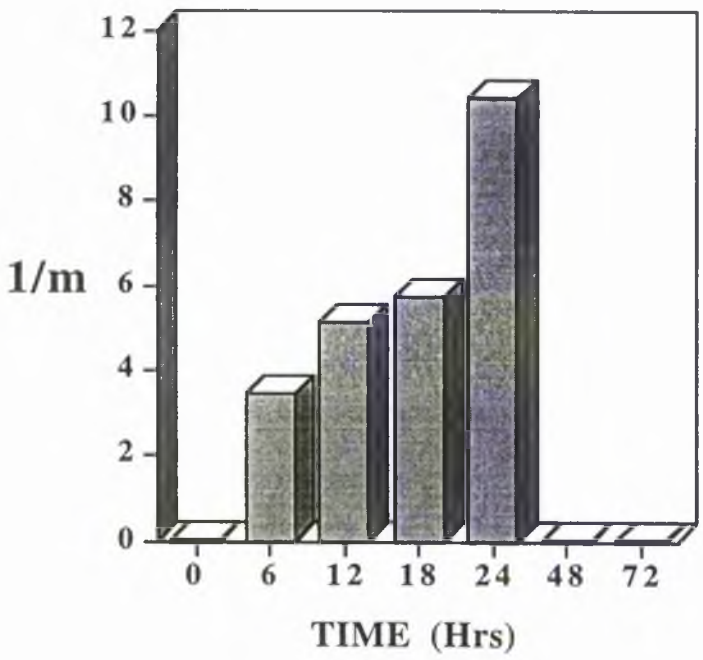


Fig. 3.21 *Shows the relative hyporesponsiveness increasing with time after LPS treatment.*

3.7 Discussion

3.7.1 Endotoxic Shock Model

Several studies have now reported on the effects of *Escherichia coli* LPS administration to rats *in vivo* (Wakabayashi *et al.*, 1987) and the resultant hyporesponiveness to the pressor effects of catecholamines *in vitro* and *ex vivo* (Fleming *et al.*, 1990; Julou-Schaeffer *et al.*, 1990, see discussion table 3.3).

REF	STIMULANT	HOURS (Post LPS)	PREPARATION	INHIBITORS	EFFECTS	MEDIATOR/ CONCLUSIONS
1	LPS (026:B6, *E. coli*) 10mg/kg i.p.	6	Aortic rings (Wistar)	-	↓ M.A.P. - *in vivo* ↓ contractility - *ex vivo*	Disorder of Ca^{2+} utilisation
2	LPS (*S. typhosa*) 100ng/ml i.v.	8	Aortic rings	Dexamethasone, Cycloheximide, L-NMMA, L-NIO, Polymyxin B	↓ contractility - *ex vivo*	Induction of NOS (VSM)
3	LPS (055:B5, *E. coli*) 20mg/kg i.p. (infused i.v. 5mg/kg/h)	4	Aortic rings (Wistar)	L-NMMA, MEB	↓ M.A.P. - *in vivo* ↓ contractility - ex vivo	↑ NO production (VSM)
4	LPS (*S. typhimurium*) 4mg/kg i.p.	6	Lung, liver and aorta tissue extracts (Wistar)	Dexamethasone	Induction of NOS *in vivo*	Similar to iNOS in macrophages *in vitro*
5	LPS (055:B5) 10mg/kg i.v. (*E. coli*).	4	Aortic rings (Sprague-Dawley)	L-Canavanine, N^G-nitro-L-arg	↓ contractility - *ex vivo*	↑ NO production (VSM)
6	LPS (0127:B8) 2mg/kg i.v.	3	Aortic rings (Wistar)	TNF_{ab} (16 hrs prior to LPS), L-NAME	↓ M.A.P.- *in vivo* ↓ contractility - *ex vivo*	TNF contributes to induction of NOS *in vivo*
7	LPS (0111:B4) 3mg/kg i.v. (*E. coli*)	5	Jejunal & colonic tissue extracts (Wistar)	Dexamethasone (2 hrs prior to LPS), L-NMMA	↑ in vascular permeability lag 3hr	↑ NO production by iNOS
8	LPS (*S. Enteritidis*) 3mg/kg i.p.	4	Brain, lung, liver, spleen, kidney & skeletal muscle extracts (Wistar)	Dexamethasone (40 min prior to LPS)	mRNA iNOS expression	Induces a widespread tissue expression of iNOS mRNA *in vivo*
9	LPS (0111:B4, *E. coli*).	5	Aortic rings (Wistar)	N^G-nitro-L-arg, L-NMMA, aminoguanidine bicarbonate	↓ contractility - *ex vivo*	selective inhibition of iNOS by aminoguanidine
10	LPS (055:B5) 4mg/kg i.p. (*E. coli*).	6 - 72	Perfused tail artery (Wistar)	L-NAME, L-NMMA, aminoguanidine hemisulfate	↓ M.A.P. - *in vivo* Hyporesponsive - *ex vivo*	iNOS expression in vascular tissue. Hypotension correlates with hyporesponsiveness - maximal at 24 hrs

Abbreviations; N-iminoethyl-L-ornithine (L-NIO), Tumour necrosis factor (TNF-α), TNF-α-antibody (TNF_{ab}), methylene blue (MEB)
References; [1]Wakabayashi I. *et al.*, 1987, [2]Rees D.D. *et al.*, 1990, [3]Julou-Schaeffer G. *et al.*, 1990, [4]Knowles R.G. *et al.*, 1990, [5]Umans J.G. & Samsel R.W., 1992, [6]Thiemermann C. *et al.*, 1993, [7]Broughton-Smith N.K., *et al.*, 1993, [8]Liu S. *et al.*, 1993, [9]Joly G.A. *et al.*, 1994, [10]Bisland S.K. *et al.*, unpublished.

Discussion table 3.3 *Showing some recent studies on the effects of LPS treatment in the rat.*

Reversal of the hyporeactivity to PE by stereospecific inhibitors of NO production clearly implicates NO as the major mediator of this effect. Some studies have been made comparing the effects of LPS *in vivo* and *ex vivo*, yet in the majority of instances the properties of the isolated vessel *ex vivo* is not consistent with what occurs *in vivo*. This is perhaps not surprising when one considers that many different homeostatic mechanisms will be operating *in vivo* at any given time. Furthermore, it is becoming clear that different signalling mechanisms exist for the induction of iNOS mRNA in culture and *in vitro*

(Sirsjo *et al.*, 1994). Thus it is unlikely that the situation *in vivo* will ever be fully emulated outside the body.

The model of endotoxic shock described here is unusual in that the *ex vivo* experiments employed isolated, endothelium-intact vessels which are constantly perfused. Most previous studies did not use intraluminal perfusion, which is surprising, since this more closely replicates the situation *in vivo*, where blood is constantly flowing through the vessels. Similarly, using lengths of artery and rather than rings contracting isometrically is more physiologically relevant. This model also emcompasses a pharmacological examination of LPS-induced hypotension and vascular hyporeactivity in combination with analysis of iNOS protein expression (ie. Western Blot). The dose and mode of administration of LPS was designed to ensure 100% survival of the animals.

3.7.2 Hyporesponsiveness of LPS-treated RTAs to PE. These experiments were carried out in a darkened laboratory to minimize the influence of artificial light on NO release during prolonged experiments (Megson *et al.*, 1995). The results from isolated segments of RTA *ex vivo* reveal that contractile responses to bolus injections of PE diminish with increasing time after LPS exposure. The effect is transient, lasting up to ~48hrs, and is maximal after ~18-24hrs. These results are completely consistent with the LPS-induced increase in blood plasma NO_x levels, indicative of NO levels, which increased steadily from t= 6hrs until 24hrs, by which time they were ~18 fold greater than controls, then subsequently returned to control levels over the next 48hrs. These results are also in agreement with a subsequent study by Tracey *et al.*, (1995), who showed that NO_x levels peak 20hrs after LPS.

L-arginine does not appear to be limiting the production of NO at t= 24hrs as added substrate did not alter responses to PE (data not shown). Equally, the apparent disparity at 6hrs, with high iNOS expression, but relatively low levels of circulating NO_x and the lack of hyporesponsiveness to PE, may be due to

insufficient substrate availability, limiting NO production. L-arginine has been shown to limit the diminished response to noradrenaline in endothelium-denuded aortic rings exposed to LPS *in vitro* (Schott *et al.*, 1993) and as the only physiological substrate for the NOS reaction and the high NO production associated with iNOS activation, arginine is likely to be in high demand following LPS treatment. Studies have shown that LPS upregulates L-arginine transport into activated smooth muscle cells (Stoclet *et al.*, 1994) as well as inducing the necessary enzymes involved in the regeneration of arginine from citrulline (Hattori *et al.*, 1993 &1995). However, neither of these mechanisms are operating by 6hrs-post LPS and only appear after a lag of ~8hrs, peaking by 24hrs and receding thereafter (Hattori *et al.*, 1994). With sufficient substrate to sustain iNOS-derived NO production it is perhaps not surprising that after 12hrs vessels begin to show a reduced sensitivity to PE. The increase in substrate availability may also partly explain why after 24hrs NO production remains high while iNOS expression is declining. It is perhaps necessary for future studies to more fully consider the turnover of L-arginine during high level NO production in vessels, in order to better appreciate its time-course in relation to iNOS expression.

3.7.3 Diminished sensitivity to SNAP It is well documented that vessels isolated from LPS-treated animals are hyporesponsive to endothelium-dependent vasodilators (Julou-Schaeffer *et al.*, 1990) but not to certain endothelium-independent agents like sodium nitroprusside (SNP; Parker & Adams, 1993). SNP undergoes tissue-catalysed reduction yielding NO. The tissue-dependent nature of SNP is thought to reflect the presence or absence of a membrane bound protein which is required to actively 'strip' NO off NP (Kowaluk *et al.*, 1992). Furthermore, there is controversy as to whether NO is actually responsible for the vasodilator properties of SNP, as responses are not always attenuated by inhibitors of guanylate cyclase (Diamond & Chu, 1983). In this respect SNP is generally not considered to be a 'typical' NO donor drug.

This study, using the more conventional NO donor drug, S-Nitroso-N-acetyl-DL-penicillamine (SNAP), showed that vasodilator responses were significantly reduced in LPS-treated vessels as compared to controls (these experiments were carried out in the dark to prevent photolysis). This result might be explained by the fact that LPS treated vessels are producing high levels of NO prior to injection with SNAP, so guanylate cyclase will be activated and may therefore be less sensitive to the additional NO release by SNAP.

3.7.4 iNOS Expression iNOS is maximally expressed in LPS-treated RTAs within 4-6hrs, some 6-8hrs before NO_x levels are significantly increased and before vessels become hyporesponsive to PE. Even more conspicuous is the lag between iNOS expression in RTAs and the resultant *in vivo* hypotension which is maximal after ~24hrs-post LPS. This was accompanied by a slight bradycardia which is consistent with myocardial depression observed in conditions of shock (Hom *et al.*, 1995). By 24hrs the expression of iNOS protein is considerably reduced and falls below the limit of detection by 72hrs.

It is important when comparing the *in vivo* response to LPS with that of the *ex vivo* response produced by the isolated RTA to bear in mind that the latter is only representative of one vessel, and with so many other vessels contributing to the cardiovascular system, it is inevitable that one will express iNOS after a lag period which differs from another. One must therefore appreciate that the same lag time separating iNOS expression and hypotension in this case may not be applicable had another vessel been used. This is exemplified by lung tissue which is shown using immunofluorescence to contain large numbers of activated macrophages expressing high levels of iNOS at t= 24hrs.

Other immunohistochemical techniques reveal that iNOS is also expressed in the vascular smooth muscle of LPS treated RTAs (t= 6hrs) but not controls, and is often seen in large (macrophage-like) cells attached to the endothelial layer of LPS RTAs.

3.7.5 eNOS Expression eNOS is strongly expressed both at t= 6hrs, 24 and 72hrs post-LPS with no apparent decrease due to increased iNOS expression. The evidence concerning whether or not constitutive NOS activity in endothelial cells (ie. eNOS) is reduced upon activation of iNOS remains ambiguous (Walter et al., 1994; Yen et al., 1993). Diminshed eNOS activity has been suggested by studies using endothelium-dependent agonists (Parker & Adams, 1993). It is conceivable that eNOS is indeed unaffected by LPS but that the transduction pathway involved in agonist-mediated NO synthesis in endothelial cells is disrupted. This hypothesis is borne out by the results of this study showing L-NMMA augmenting responses in t= 18hr LPS RTAs beyond that of AG. Assuming that AG inhibits iNOS and not eNOS activity, it is likely that the effect of L-NMMA is due to inhibition of eNOS activity.

3.7.6 Conclusion From this study it is clear that exposure to a single bolus injection of LPS (serotype 055:B5; 4mg/Kg, i.p.) is sufficient to induce endotoxic shock in the rat. The criteria chosen to reflect endotoxaemia are consistent with each other and clearly identify the time-course of LPS-induced NO production over 72hrs. NO_X levels, hyporesponsiveness to PE and the severe hypotension which occur *in vivo* all follow a similar time-course, with maximal effects seen at t= 24hrs and a subsequent return to control values over the next 48hrs. This establishes the validity of the model for studying the properties of blood vessels from animals in endotoxic shock.

Chapter 4

Effects of NOS inhibitors and L-arginine on responses of control and LPS-treated RTAs to PE

4.1 Introduction

It will be seen later (see Chapter 9) that the physiological basis for the hyporesponsiveness to PE of vessels isolated from LPS-treated rats (RTAs) and those which formerly supplied a solid tumour (TEAs) may not be identical. However, they appear sufficiently similar in some aspects to make it worthwhile trying to identify inhibitors which can most effecively reverse the condition in *ex vivo* experiments using RTAs from LPS-treated animals. The outcome of such experiments could ultimately provide a rational basis for predicting which compounds are most likely to selectively constrict tumour supply vessels *in vivo*, and thus compromise the delivery of oxygen and essential nutrients to the growing tumour.

This chapter deals with the effects of several NOS inhibitors on the senitivity to PE of RTAs taken *post mortem* from LPS-treated rats: two relatively non-specific NOS inhibitors, L-NMMA and L-NAME (Hibbs *et al.*, 1987) and one which reportedly (Misko *et al.*, 1993; see Section 1.13.3) displays some selectivity towards the inducible isoform of NOS, aminoguanidine (AG). The results will show that L-NMMA was most effective at reversing the hyporesponsiveness associated with LPS-treated RTAs, although AG selectively reversed the hyporesponsiveness without altering control RTA responses to PE.

4.2 Materials & Methods

4.2.1 Methods

Segments of RTAs from control and LPS-treated rats were isolated and perfused internally and externally with Krebs solution as described earlier (chapter 3). The following protocol was used:

1. An initial series of injections of PE was administered in the presence of Krebs solution *only*.

2. NOS inhibitors (L-NMMA, L-NAME or AG; all at [80μM]) were added to the internal perfusate for 30 mins before making a second series of injections.

3. The inhibitors were washed out with Krebs solution (10 mins) and then L-arginine (1mM) was added to the internal perfusate. Vessels were perfused with Krebs solution + L-arginine for 45mins and a third series of injections was made.

Peak perfusion pressures were measured and used to construct log dose-response (D.R.) curves (1^{st}, 2^{nd} and 3^{rd}, respectively). Statistical significance of differences between D.R. curves was evaluated by two way analysis of variance (ANOVA). Student's paired or unpaired t-tests determined the significance of any difference between data recorded at a given [PE] with a probability (P) value of 0.05 considered as statistically significant ($p \leq 0.05$*; $p \leq 0.01$**, $p \leq 0.001$***).

4.2.2 Materials

L-NMMA, L-NAME, AG and L-arginine were all obtained from Sigma Ltd.

4.3 Results

4.3.1 Comparing PE Responses in Proximal and Distal Segments of RTA.

In order to ensure that segments of RTA produced similar responses to PE irrespective of their position along the length of the tail, D.R. curves for proximal segments (1-4cm; n= 29) were compared with those more distal (< 4cm, n= 27).

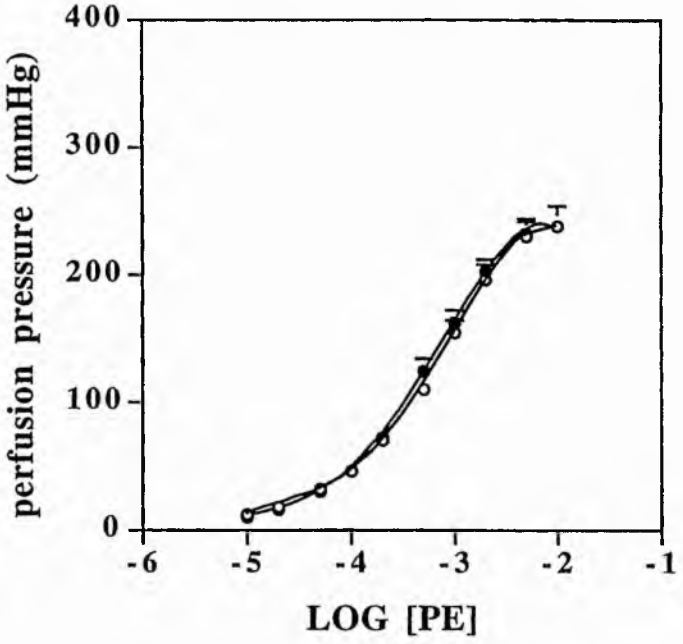


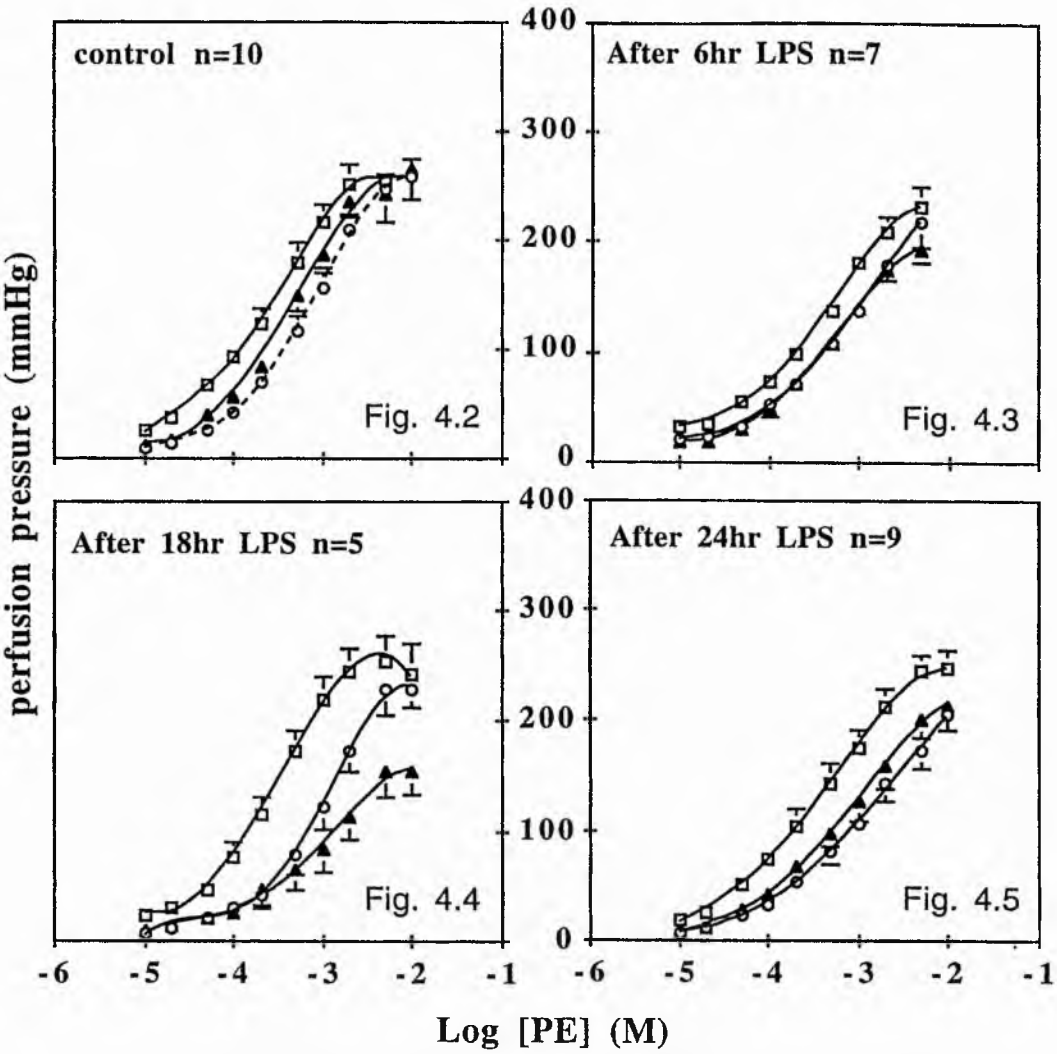
Fig. 4.1 *Comparing initial D.R.s to PE in RTAs from proximal (open circles, n= 29) and distal (closed circles, n= 27) portions of the tail.*

The results are shown in fig. 4.1. Contractile responses from distal and proximal segments were not significantly different (student's unpaired t-test; see Appendix table 4.6) and therefore results for all of these preparations were pooled.

4.3.2 Effects of L-NMMA on responses to PE of RTAs from control and LPS-treated rats: reversal by L-arginine. The effects of L-NMMA and the ability of L-arginine to reverse its action were studied in both control (t= 0) and LPS-treated RTAs (t= 6, 18, 24).

The results are shown in figs. 4.2-4.5. L-NMMA enhanced responses to PE in both control and LPS-treated RTAs, producing a leftward and upward displacement of the D.R. curve. The effect was significant (Student's paired t-test) for all groups, with the exception of vessels from LPS-treated animals sacrificed at t= 6hrs.

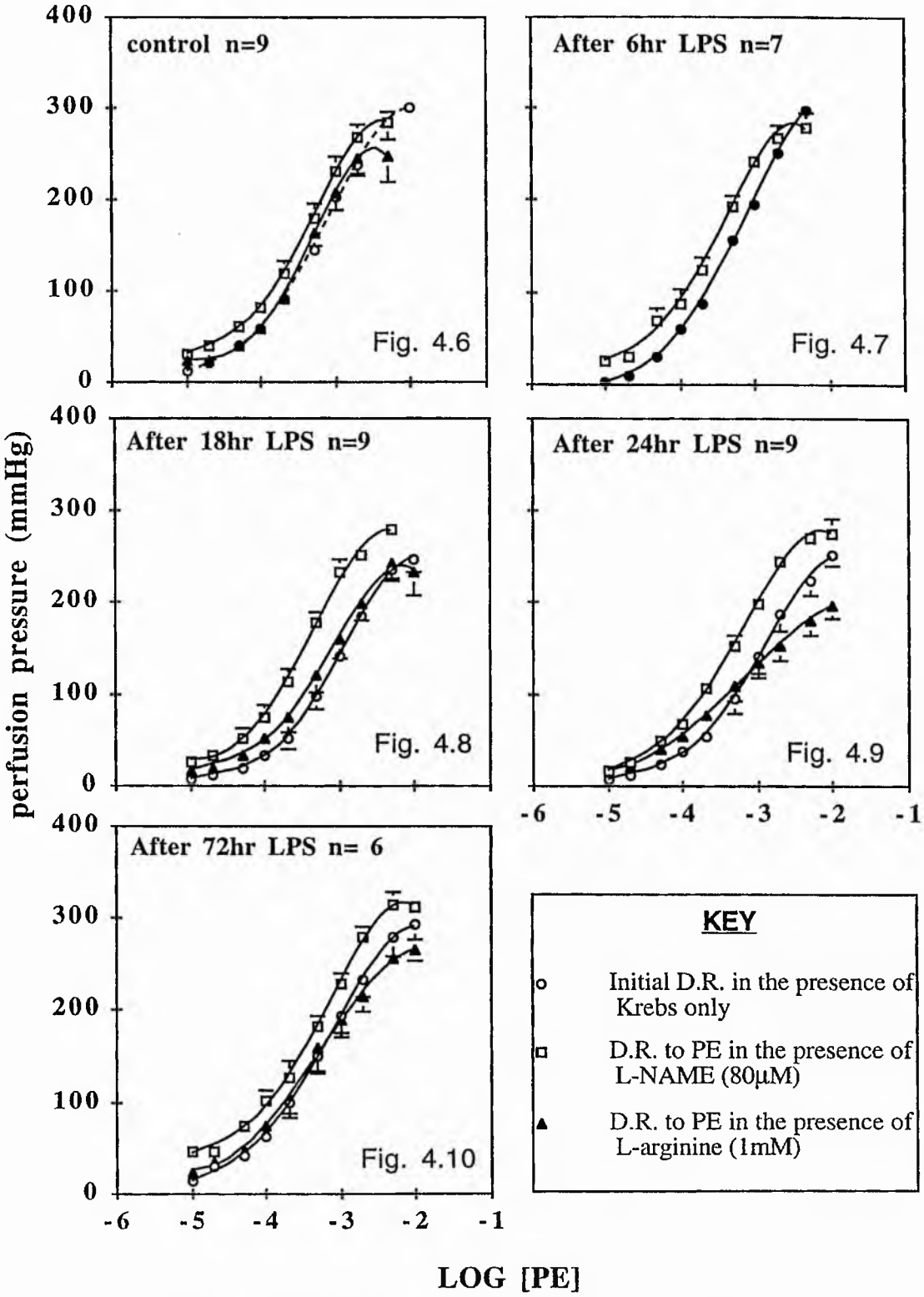
L-arginine completely blocked the effects of L-NMMA at all time points, but did so to differing extents. Interestingly, L-arginine more than reversed the effect of L-NMMA on t= 18hr RTAs, displacing the curve downwards and to the right, *beyond* the initial D.R. curve recorded in the presence of Krebs solution alone (fig. 4.4, filled triangles). This particular effect of L-arginine is considered again later (see 4.5, Discussion). Statistical analyses for the data presented in figs. 4.2-4.5 appear in Appendix tables 4.1 .



Figs. 4.2-4.5 *The D.R.s for control (fig. 4.2) and LPS-treated (t= 6, 18 and 24; figs. 4.3-4.5 respectively) RTAs before (open circles) and after treatment with L-NMMA (open squares; 80μM) and L-arginine (1mM; closed triangles).*

4.3.3 Effects of L-NAME on responses to PE of RTAs from control and LPS-treated rats: reversal by L-arginine. The effects of L-NAME and of subsequent treatment with L-arginine were studied in both control (t=0hrs) and LPS-treated RTAs (t= 6, 18, 24 and 72hr). The results are shown in figs. 4.6-4.10.

As with L-NMMA, L-NAME also enhanced responses to PE in both control and LPS-treated RTAs displacing the D.R curve upwards and to the left. The effect was statistically significant for t= 18 and 24hr LPS-treated arteries but not for the control or vessels sacrificed at t= 6 and 72hrs.



Figs. 4.6-4.10 *Showing the D.R.s for control (fig. 4.6) and LPS RTAs at t= 6 (fig. 4.7), 18 (fig. 4.8), 24 (fig. 4.9) and 72hrs (fig. 4.10) before and after treatment with L-NAME and L-arginine.*

Treatment with L-arginine completely reversed the effect of L-NAME and in this respect resembled results obtained with L-NMMA. Statistical analyses for these data appear in Appendix table 4.2.

4.3.4 Effects of aminoguanidine alone and of aminoguanidine followed by L-NAME on responses to PE of RTAs from control and LPS-treated (t= 18hr) rats. Experiments were made to test the effect of aminoguanidine (AG), a selective iNOS inhibitor, and of aminoguanidine followed by L-NAME, a relatively non-selective NOS inhibitor, on PE responses of both control RTAs and RTAs from LPS-treated (t= 18hr) animals. The protocol was as follows. First, isolated segments of RTA from control and LPS-treated animals were injected with PE in the presence of Krebs solution only. Second, vessels were perfused with Krebs solution containing AG (1mM) for 1hr before a second sequence of PE injections was given. Finally, following a 10 min washout with Krebs solution, L-NAME (80μM) was added to the internal perfusate before performing a third sequence of PE injections.

Fig. 4.11 shows the effect on control arteries. Contrary to what was seen with L-NMMA and L-NAME, AG produced a small *rightward* shift in the D.R. curve, but this was not statistically significant (2-way ANOVA, F= 0.58, df= 9, 118_e, p= 0.810). This effect was fully reversed by replacing AG with L-NAME. However, neither of the curves obtained with AG or with L-NAME was statistically different from that obtained in the presence of Krebs solution only.

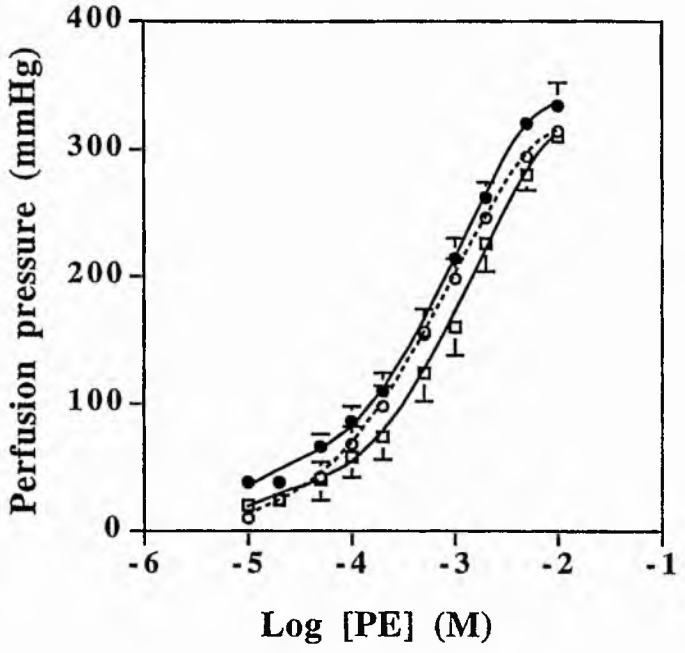


Fig. 4.11 *A graph showing the D.R.s for controls (n=5-8) initially perfused with Krebs (dotted line, open circles) then after 1hr AG (1mM; open squares) and 30mins L-NAME (80μM; closed circles).*

Fig. 4.12 shows the results obtained from a similar series of experiments, this time using RTAs from animals sacrificed at t= 18 hrs after LPS treatment. RTAs from LPS-treated rats (open circles) were significantly less responsive to PE compared to control vessels (dotted line), as determined by 2-way ANOVA (F= 2.51, df= 8, 153_e, p= 0.014). This difference was completely abolished by AG, which displaced the curve for LPS-treated vessels to the left and upward, so that it *almost exactly* coincided with that obtained for control (ie. non LPS-treated) vessels (2-way ANOVA, F= 3.04, df= 9, 191_e, p=0.002). Removal of AG followed by replacement with L-NAME further shifted the D.R. curve upward and to the left (2-way ANOVA, F= 2.10, df= $8,143_e$, p= 0.041). This difference is attributed (see Discussion) to the differential effect of the two NOS inhibitors on the constitutive and inducible NOS isoenzymes. Statistical (Student's t-test) analyses for these data appear in Appendix table 4.3.

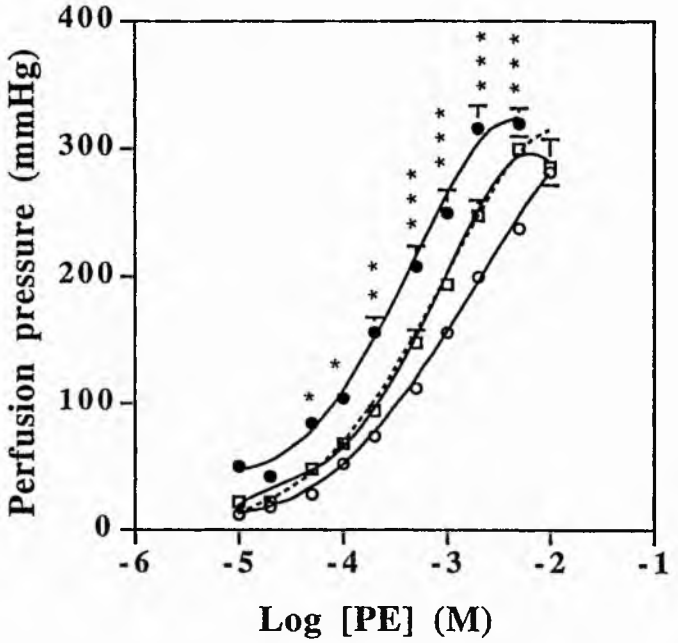


Fig. 4.12 *D.R.s for LPS treated RTAs (t= 18hrs, n= 7-11) perfused with Krebs (open circles), AG (1mM; open squares) and L-NAME (80μM; closed circles). The 1st D.R. curve for controls is included for comparison (dotted line). Asterisks indicate significant difference between the Krebs D.R. and the AG D.R. using t-test analysis.*

Finally, experiments were also made using vessels taken from animals at t= 72hrs after injection with LPS (data not shown). Results presented earlier (Chapter 3, section), clearly showed that such vessels do not display a hyporesponsiveness to PE and this was confirmed in this series of experiments. The results were comparable to those obtained for control (ie. non-LPS-treated) vessels ie. the log D.R. curve for vessels perfused with Krebs solution alone was not significantly different from that obtained with Krebs + AG (2-way ANOVA, f= 1.01, df= 9, 50_e, p= 0.445). Although, the log D.R. curve for Krebs + L-NAME was significantly shifted to the left (2-way ANOVA, F= 2.57, df= 9, 47_e, $p < 0.01$).

4.4 Quantitative analysis of the effect of NOS inhibitors on PE-induced responses. For clarity and some measure of quantification, experiments involving drugs which altered PE-induced contractile responses (ie. NOS inhibitors) are analysed with reference to 2 parameters (refer to fig.4.13):

a) the contractile response to a PE injection of $5x10^{-3}M$ (P),

and

b) the [PE] required to produce an active perfusion pressure of 150mmHg (ED_{150}).

The largest effect on both parameters when tested on the most hyporesponsive vessels (t= 24hrs) was seen with L-NMMA. For this reason the effects of L-NAME and AG are expressed as a % of the changes seen with L-NMMA (ie. % ΔED_{150} and % ΔP.This is shown diagrammatically in fig. 4.13 where:

ΔED_{150} for L-NMMA = A - B= 100%

and

ΔP for L-NMMA = B' - A' = 100%

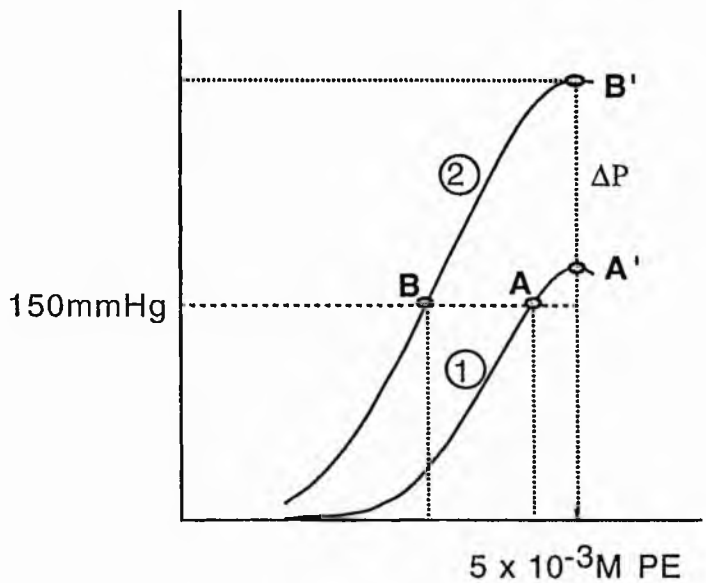


Fig. 4.13 *The relative effects of a NOS inhibitor are expressed as a % of the ΔED_{150} and ΔP between the D.R. curves for LPS-treated (t= 24hr) vessels in the presence of Krebs solution alone (1) and in the presence of L-NMMA (2).*

Fig 4.14- 4.16 show results for control and LPS-treated vessels exposed to L-NMMA (fig. 4.14), L-NAME (fig. 4.15) and AG (fig. 4.16). The effect of L-NMMA on both ΔP and ΔED_{150} increases progressively with time after LPS treatment, by definition reaching 100% at t= 24hrs. Responses to L-NAME, on the other hand rise to maximum at ~18hrs, increasing ΔP by ~50-60% and ΔED_{150} by ~40% of the maximum effect seen with L-NMMA.

The results obtained with AG refer only to control arteries and arteries from animals sacrificed t= 18hrs. Since AG caused a slight (not statistically significant) *rightward* and *downward* shift of the log D.R. curve when tested on control arteries (see fig. 4.11) the changes in the ED_{150} and P are negative. The effect of AG on the ED_{150} value for LPS-treated vessels (t= 18hrs) was ~20% of that produced by L-NMMA, but on the P value it produced an effect ~80% of that seen with L-NMMA. Generally, in terms of their potency, the three NOS inhibitors can be ranked as follows: L-NMMA > L-NAME > AG. The one exception to this is the effect of AG on P which is greater than that of L-NMMA and L-NAME at t= 18hrs.

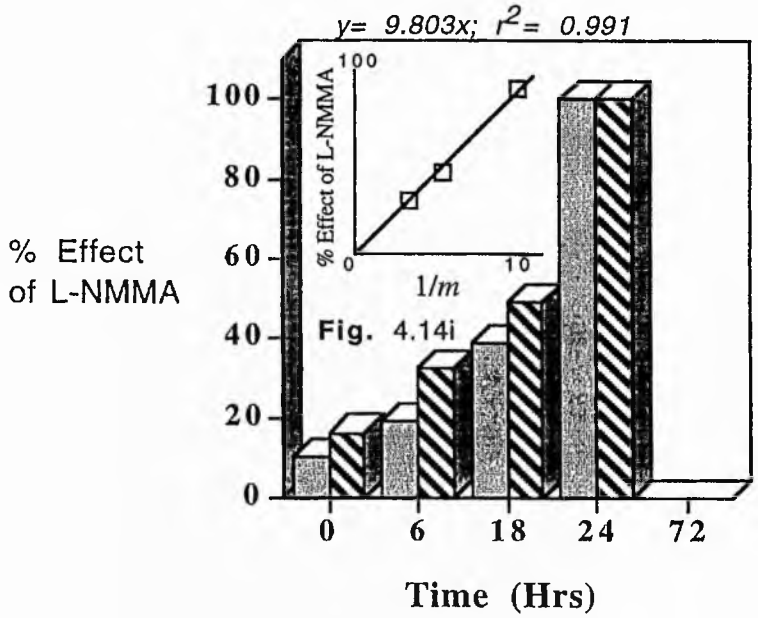


Fig. 4.14 *Shows the effect of L-NMMA on P (dark columns) and ED_{150} (striped columns). Results are expressed as % of LPS D.R. values (t= 24hrs).*

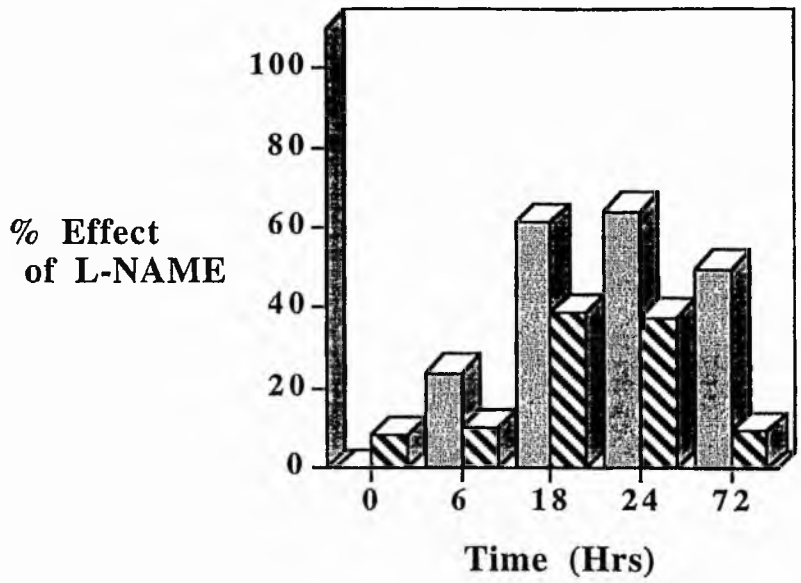
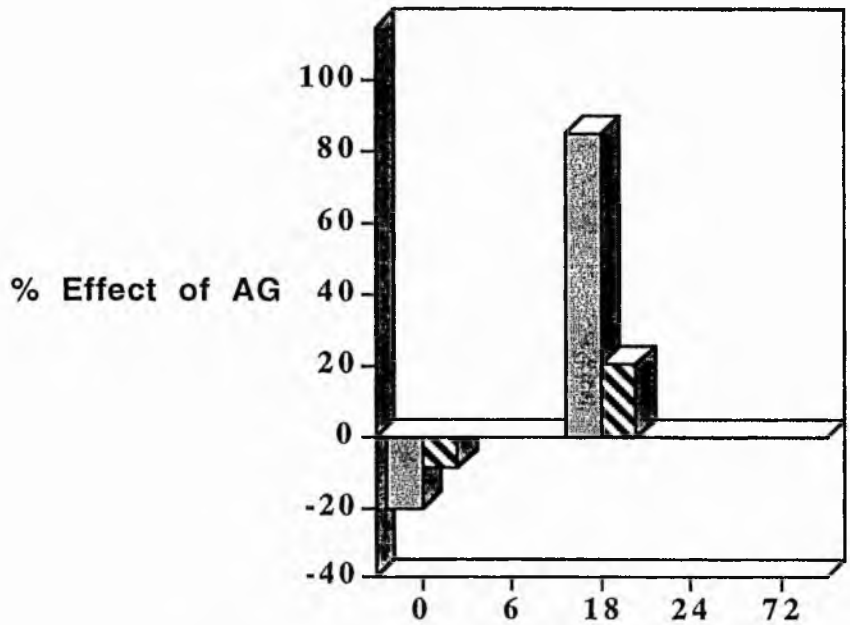


Fig. 4.15 A graph showing the effects of L-NAME on ED_{150} (striped columns) and P (dark columns) expressed as a percentage of LPS, t= 24hr L-NMMA treated data.



Figs. 4.16 A graph showing the effects of AG on ED_{150} (striped columns) and P (dark columns) expressed as a percentage of LPS, t= 24hr L-NMMA treated data.

The time-dependent increase in the effect of L-NMMA on ED_{150} from t= 6 - 24hrs is consistent with the increasing hyporesponsiveness and when the values for LPS RTAs (t= 6, 18, 24hrs) are plotted against $1/m$ a linear relation is obtained (see fig. 4.14i). This implies that L-NMMA is equally effective at

reversing the LPS-induced hyporesponsiveness at all time-points. A linear relation is not obtained if the same procedure is applied to L-NAME.

4.5 Discussion

The results presented in this chapter confirm the results of chapter 3 in showing that RTAs taken from LPS-treated rats are hyporesponsive to PE. Both L-NMMA and L-NAME increased the sensitivity to PE of control and LPS-treated vessels and thus abolished their hyporesponsiveness. Indeed, there was no difference in sensitivity in control and LPS-treated vessels in the presence of either NOS inhibitor. The effects of AG, widely acknowledged to be a more selective inhibitor preferentially blocking the inducible isoform (Joly *et al.*, 1994; Wu *et al.*, 1995; Griffiths *et al.*, 1993), were somewhat different. First, AG had no effect on responses of control arteries to PE. Second, AG fully restored the sensitivity of LPS-treated (t= 18hr) vessels to control values, but it did not further increase their sensitivity as did L-NMMA and L-NAME (see fig. 4.12). The differing effects of AG on the one hand and L-NMMA and L-NAME on the other can be explained if a) the hyporesponsiveness of LPS-treated vessels to PE is due to enhanced NO production via the action of the inducible isoenzyme. b) AG does not significantly effect NO production via the constitutive isoform. The increased sensitivity of vessels treated with L-NMMA and L-NAME, over and above control sensitivities, can equally be explained if both drugs inhibit the constitutive as well as the inducible NOS isoforms. The additional effects of L-NMMA and L-NAME over those seen with AG is presumably entirely due to blockade of cNOS. This is consistent with the fact that administering L-NAME to control and LPS-treated vessels after treatment with with AG produced an additional increase to PE.

The fact that a high dose of L-arginine (1mM), the natural substrate for all NOS isoforms, could fully reverse the effects of both L-NMMA and L-NAME confirms that both inhibitors increase the sensitivity to PE by inhibiting NO production.

Qualitatively similar results to those presented here were obtained when studying the effects of NOS inhibitors on arteries which previously supplied solid tumours and contralateral (control) arteries as will be seen in Chapter 7. Finally, the differential effects of non-selective and isoform selective NOS inhibitors might be usefully exploited in studies of the role of NO in solid tumour growth. This is considered in Chapter 8.

Although, L-NAME and L-NMMA are shown to reverse the LPS-induced hyporeactivity to PE, this does not rule out the possibility that other mediators other than NO are involved in this effect. A recent study suggests that NOS inhibitors can also inhibit cyclooxygenase activity (Salvemini et al., 1995). The implications of this become apparent in experiments shown in the next chapter.

Chapter 5

Effects of Indomethacin, Cycloheximide and L-NMMA on Contractility of Rat tail Artery.

5.1 Introduction

Kennovin *et al.*, (1994) showed that constant perfusion for 2hrs with the protein synthesis inhibitor cycloheximide (10μM) abolished the initial difference in sensitivity to PE between TEAs and CEAs. Furthermore, a time-dependent decrease in responsiveness of CEAs, occasioned by prolonged perfusion (up to 9hrs) with Krebs solution *only*, was also prevented by cycloheximide. A similar observation was reported by Rees *et al.*, (1990), using rat aortic rings, in which cycloheximide (10μM) abolished the gradual loss of tone of preparations incubated in Krebs for up to 8hrs. The time-course of the phenomenon and its prevention by both cycloheximide and inhibitors of NOS (Kennovin *et al.*, 1994; Rees *et al.*, 1990), led to the conclusion that this effect was mediated by increased NO synthesis by iNOS within the vascular smooth muscle of these preparations. It was also found that the expression of iNOS and the ensuing loss of tone began after a lag period of ~2hrs, and although LPS hastened and potentiated this effect, the time of onset remained the same.

Experiments were conducted to see whether control and LPS-treated RTAs behaved in a similar manner to CEAs and TEAs following treatment with cycloheximide. Indomethacin, the preferential inhibitor of the cyclooxygenase pathway involved in arachidonic acid metabolism, was also used to determine whether the hyporesponsiveness of LPS-treated arteries could be attributed to the inducible cyclooxygenase (iCOX), known to be co-induced with iNOS both *in vitro* and *in vivo* (Salvemini *et al.*, 1993). Cyclooxygenases are thought to be responsible, at least in part, for the symptoms associated with endotoxaemia (Salvemini *et al.*, 1995) and since they have the potential to synthesise both

vasoconstrictors (eg. thromboxane A_2) and vasodilators (eg. prostacyclin), this pathway is likely to be of importance when considering the properties of isolated blood vessels. Their possible role in the time-dependent desensitization was not addressed in the study by Rees *et al.*, (1990) since these authors used the synthetic glucocorticoid, dexamethasone, which inhibits both iNOS and iCOX. As a result, their experiments were unable to distinguish which enzyme system was responsible for the effect.

5.2 Materials & Methods

5.2.1 Methods

Isolated segments of RTA from control and LPS-treated (t= 18hrs) Wistar rats were perfused both internally and externally with pre-warmed Krebs solution as decribed earlier (chapter 3). The following protocol was adopted:

1. Control and LPS-treated RTAs were divided into two groups: after 45 mins. perfusion, indomethacin (final concentration of 10μM) was added to the internal perfusate of group 1 (n= 6-8 for LPS and control RTAs), while group 2 continued to be perfused with Krebs *only* (n= 5-6 for both). After a further 30mins, both groups were given an initial (0hrs) series of bolus injections of increasing [PE]s (10^{-5}-10^{-2}M).

2. Cycloheximide was added to the internal perfusate of both groups (final concentration of 10μM) and 1hr later another series of injections was performed and repeated hourly for 4hrs (1-4hrs).

3. Finally, L-NMMA (80μM) was added to the internal perfusate of both groups and after 30-45mins a final series of PE injections was given.

Peak perfusion pressures were measured and used to construct log dose-response (D.R.). Statistical analysis was similar to that described earlier in chapter 4.

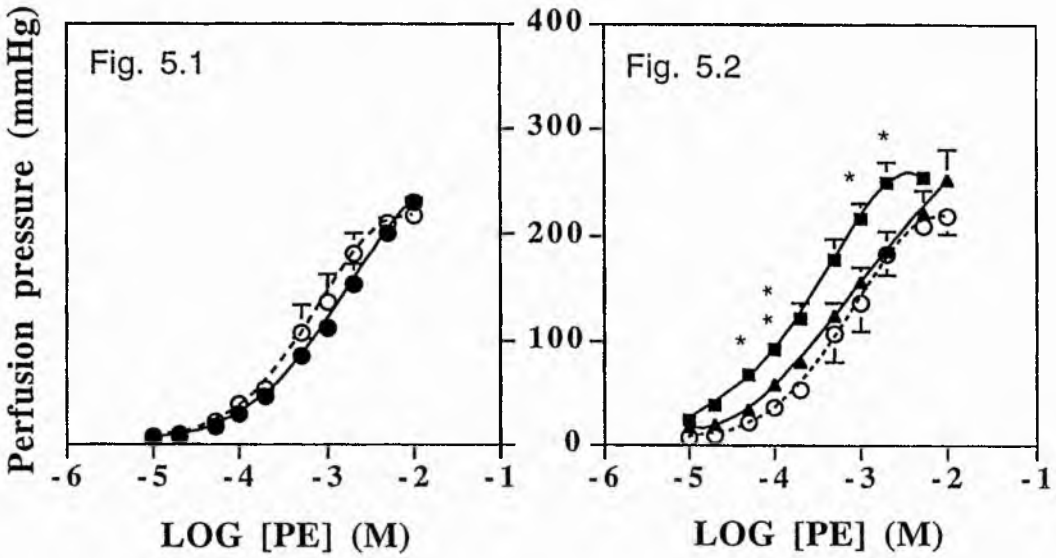
5.2.2 Materials

Cycloheximide, indomethacin and L-NMMA were all obtained from Sigma Ltd.

5.3 Results

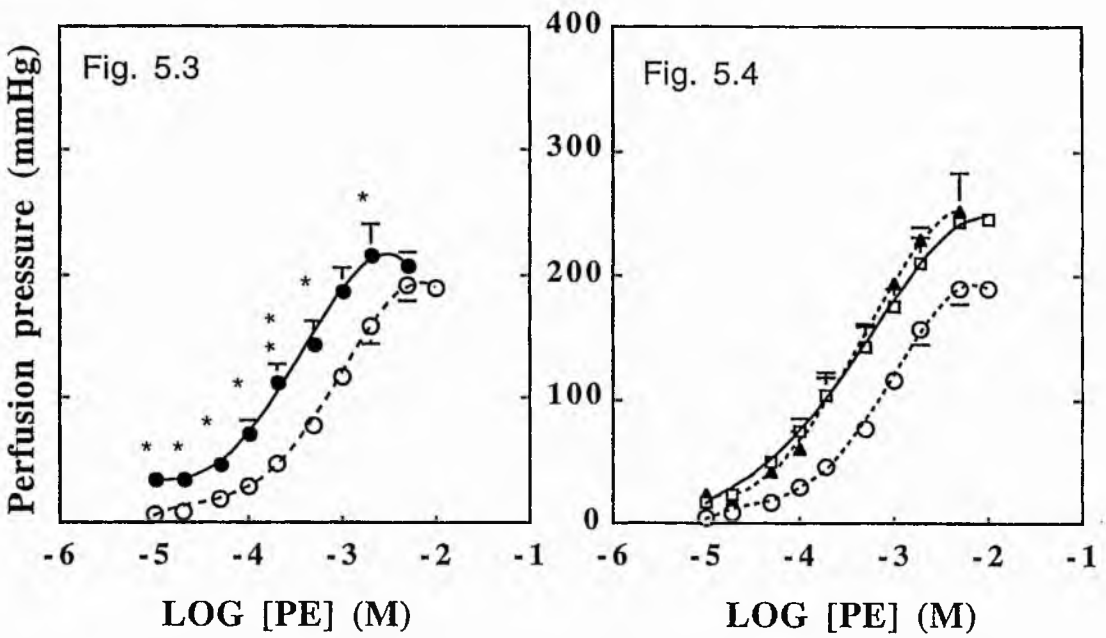
5.3.1 Effects of cycloheximide alone and cycloheximide + L-NMMA on PE-induced responses in control and LPS-treated RTAs. LPS-treated arteries were hyporesponsive to PE as compared to control arteries (as shown previously in Chapter 4).

Fig. 5.1 shows that the addition of cycloheximide alone to control vessels had no significant effect (2-way ANOVA, F= 0.38, df= 9, 85e, p= 0.94). Subsequent addition of L-NMMA to the internal perfusate (= Krebs + cycloheximide + L-NMMA) shifted the log D.R. curve to the left, but the effect was not as great as that produced by L-NMMA on control RTAs not previously exposed to cycloheximide (Fig. 5.2; Student's t-test analysis, see Appendix Table 5.5), i.e. cycloheximide significantly reduced the effect of L-NMMA.



Figs. 5.1 & 5.2 *Showing the D.R. curves for control RTAs perfused with Krebs (open circles, n= 5-6; Figs. 5.1 & 5.2), after 4hrs perfusion with cycloheximide alone (closed circles; Fig. 5.1) and following the addition of L-NMMA (closed triangles; Fig. 5.2). The D.R. curve for control RTAs not previously treated with cycloheximide is also shown in Fig. 5.2 (close squares, n=10) and the asterisks show a significant difference between the data represented by closed triangles and closed squares.*

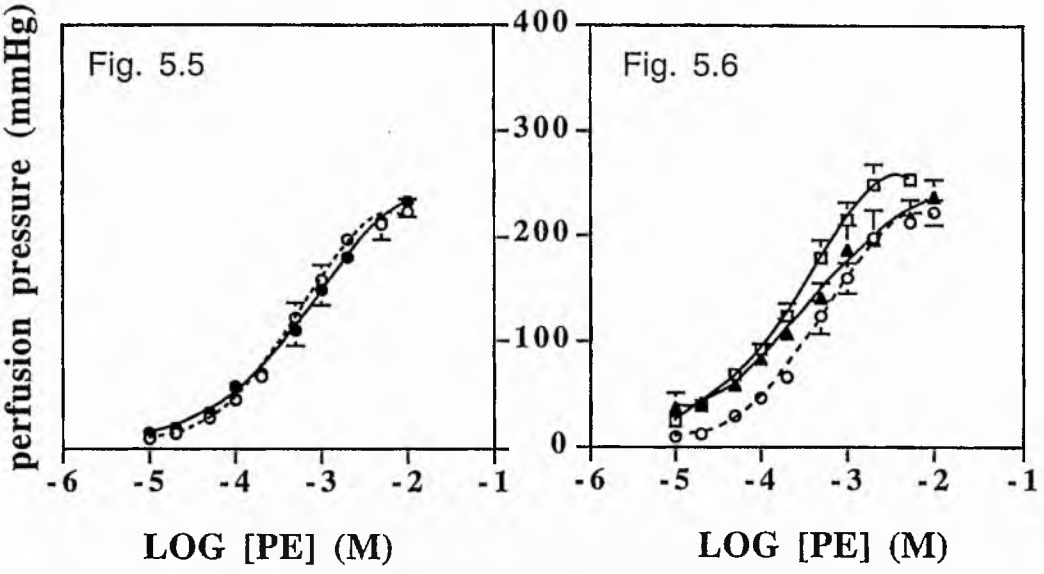
In contrast, responses to PE of LPS-treated arteries were significantly enhanced by cycloheximide (see Fig. 5.3; Asterisks represent p values < 0.05*, Student's paired t-test; see Appendix table 5.3). A similar effect has previously been shown for tumour-associated vessels (Kennovin et al., 1994). Indeed, the potentiation was as great as that seen after exposing LPS-treated vessels to L-NMMA *only* (i.e. without prior treatment with cycloheximide), so that responses were enhanced as compared to even *untreated* control vessels. Thus, the subsequent addition of L-NMMA (= Krebs + cycloheximide + L-NMMA) had no further potentiating action (see Fig. 5.4).



Figs. 5.3 & 5.4. *Showing the D.R. curves for LPS RTAs (t= 18hrs) perfused with Krebs (open circles, n= 5-6; Figs. 5.3 & 5.4), after 3hrs perfusion with cycloheximide alone (closed circles; Fig. 5.3) and following the addition of L-NMMA (closed triangles; Fig. 5.4). The D.R. curve for LPS RTAs not previously treated with cycloheximide is also shown in Fig. 5.4 (open squares, n=9).*

5.3.2 Effects of indomethacin and indomethacin + cycloheximide on PE-induced responses in control and LPS-treated RTAs. The addition of indomethacin to the internal perfusate of control RTAs (i.e. = Krebs + indomethacin) had no significant effect (not shown) and neither did the subsequent addition of cycloheximide (= Krebs + indomethacin +

cycloheximide; see Fig. 5.5 & Appendix table 5.4 for Student's t-test analysis). However, when L-NMMA was later added (= Krebs + indomethacin + cycloheximide + L-NMMA; Fig. 5.6, closed triangles), it produced a leftward shift of the curve approaching that seen in LPS-treated vessels not previously exposed to indomethacin or cycloheximide (Fig. 5.6; open squares).



Figs. 5.5 & 5.6 *Showing the D.R. curves for control RTAs perfused with Krebs (open circles, n= 6-8; Figs. 5.5 & 5.6), after 4hrs perfusion with* indomethacin + cyclo*heximide (closed circles; Fig. 5.5) and following the addition of L-NMMA (closed triangles; Fig. 5.6). The D.R. curve for control RTAs not previously treated with cycloheximide or indomethacin is also shown in Fig. 5.6 (open squares, n=10).*

Treatment of LPS-treated RTAs with indomethacin alone (= Krebs + indomethacin) had no effect on responses to PE (not shown), and the subsequent addition of cycloheximide to the internal perfusate also had no effect (= Krebs + indomethacin + cycloheximide, see Fig. 5.7; closed circles & Appendix table 5.4). This is marked contrast to the effect of cycloheximide alone (see section 5.3.1), which greatly potentiated responses of LPS-treated vessels, i.e. prior treatment with indomethacin abolished the potentiating effect of cycloheximide. When L-NMMA was later added (= Krebs + indomethacin + cycloheximide + L-NMMA; see Fig. 5.8, closed triangles), responses to PE were enhanced in the normal way i.e. although indomethacin inhibited the potentiating effect of

cycloheximide, it did not alter the effect of L-NMMA. See Appendix tables 5.5 for student's t-test analysis.

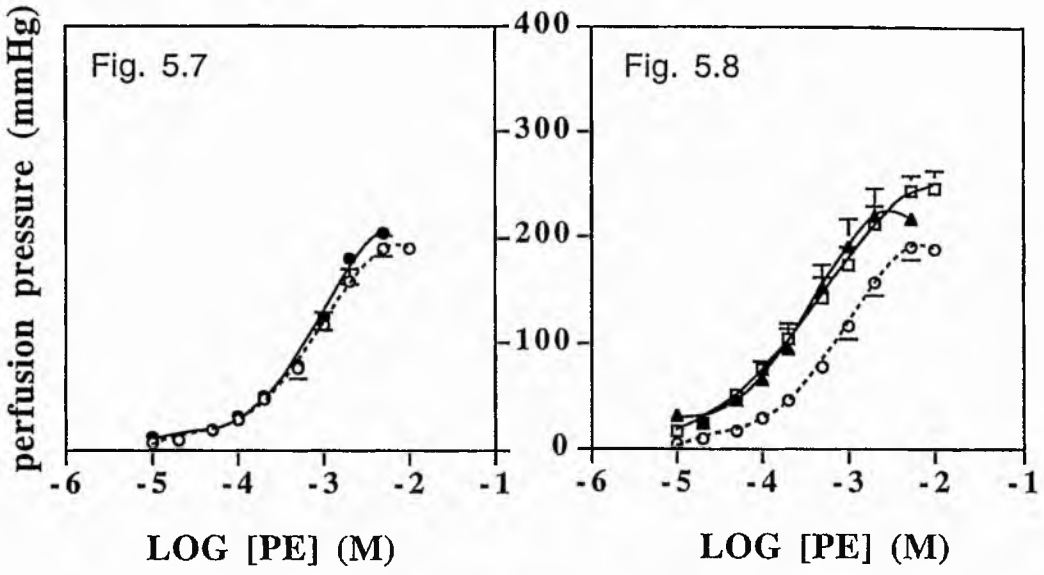


Fig. 5.7 & 5.8 *Showing the D.R. curves for LPS RTAs (t= 18hrs) perfused with Krebs (open circles, n= 6-8; Figs. 5.7 & 5.8), after 3hrs perfusion with indomethacin + cycloheximide closed circles; Fig. 5.7) and following the addition of L-NMMA (closed triangles; Fig. 5.8). The D.R. curve for LPS RTAs not previously treated with cycloheximide or indomethacin is also shown in Fig. 5.8 (open squares, n=9).*

5.4 Discussion

These experiments were designed to show whether a similar poteniating effect of cycloheximide as that described by Kennovin et al. (1994) in isolated tumour-supply vessels, was also seen in vessels taken from animals which had previously received an injection of LPS (t= 18hrs). The role of cyclooxygenase enzymes in this effect was also explored using the specific cyclooxygenase inhibitor, indomethacin.

5.4.1 Effects of cycloheximide and cycloheximide + L-NMMA on control and LPS-treated RTA responses to PE. A constant 4hr internal perfusion with the protein synthesis inhibitor cycloheximide (10μM) alone did not significantly alter control RTA responses to PE. Furthermore, when L-NMMA was subsequently added to the internal perfusate, the characteristic leftward shift caused by L-NMMA in untreated control RTAs (i.e. not previously exposed to cycloheximide) was not seen. Cycloheximide therefore abolished the potentiating effect of L-NMMA in control RTAs.

In contrast to control RTAs, the sensitivity to PE of LPS-treated (t= 18hrs) RTAs was significantly enhanced after 3hrs of perfusion with cycloheximide, such that the D.R. curve was shifted beyond that of untreated control RTAs, to lie almost exactly on the curve produced by L-NMMA in *untreated* LPS-treated vessels (i.e. vessels which had not previously been exposed to cycloheximide). Moreover, the subsequent addition of L-NMMA did not further enhance responses to PE.

From this evidence it would appear that the hyporesponsiveness to PE associated with LPS-treated RTAs requires *de novo* protein synthesis and is consequently abolished by cycloheximide. Furthermore, the diminished effect of L-NMMA after cycloheximide treatment in control RTAs suggests that *de novo* synthesis

may also be required for constitutive NO production by cNOS, although further work is needed to confirm this.

As a protein synthesis inhibitor, cycloheximide could potentially inhibit a number of enzymes thought to be stimulated by LPS, it is therefore unlikely that disruption of iNOS activity is solely responsible for the effects on LPS-treated vessels, if at all. Other factors which regulate iNOS activity could also be affected by cycloheximide, including interferon regulatory factor-1 (Kamijo *et al.*, 1994), nuclear factor-*K*B (Schreck *et al.*, 1992), transforming growth factor (Vodovitz *et al.*, 1993) and a number of other transcriptional factors (Gross *et al.*, 1994). However, the role of many of these factors in iNOS expression is dependent on whether cells are in culture or as part of an isolated vessel (Sirsjo *et al.*, 1994). Sirsjo *et al.*, (1994) found that in culture cycloheximide potentiated the induction of iNOS expression by LPS but in aortic tissue strips cycloheximide attentuated iNOS expression. This indicates that the induction of iNOS *ex vivo* requires *de novo* protein synthesis and strengthens the idea that inhibition of iNOS by cycloheximide explains the effect on LPS-treated vessels.

The production of NO from L-arginine requires several different cofactors, including tetrahydrobiopterin (BH_4), NADPH and flavins, all of which may be influenced by cycloheximide (see Section 1.12). *De novo* synthesis of BH_4 is an absolute requirement for full NOS activation (Baek *et al.*, 1993; Gross *et al.*, 1994) and may become limiting during cytokine-mediated activation of iNOS in VSM cells (Gross & Levi, 1992). It is clear therefore that cycloheximide could inhibit BH_4 synthesis and so prevent NO production without disrupting iNOS expression. Furthermore, most BH_4 (~90%) is produced by the endothelium and is then directed towards the VSM (Schoedon *et al.*, 1993). This stresses the importance of using endothelium-intact vessels for studying iNOS-derived NO production *ex vivo*.

In order that iNOS can produce high levels of NO there also has to be sufficent L-arginine substrate to satisfy demand. L-arginine is synthesised endogenously as part of the urea cycle (Meijer *et al.*, 1990), although it can also be recycled from L-citrulline by an LPS-inducible enzyme, argininosuccinate synthetase (iAS) within the vasculature (Morris *et al.*, 1994; see Section 1.13). Cycloheximide has been shown to attenuate the induction of iAS mRNA in cultured aortic smooth muscle cells (Hattori *et al.*, 1994). Whether the same also occurs in tissue preparations is not clear. If cycloheximide does inhibit the expression of iAS in isolated vessels then L-arginine may become limiting to NO production in LPS-treated RTAs, with the result that responses to PE are potentiated.

It is clear that cycloheximide could reduce NO production via a number of ways, many of which do not involve the NOS enzyme. Whatever the mechanism, it's potentiating effect was exclusive to vessels isolated from LPS-injected animals (t= 18hrs) and produced a leftward shift in their D.R. curve. This may imply that a) a vasodilator had been inhibited b) that a vasoconstrictor had been activated.

Further evidence that iNOS may be involved stems from two sources; a) Western Blot analysis shows that iNOS continues to be expressed beyond t= 24hrs and will therefore also be expressed at t= 18hrs. b) aminoguanidine was able to abolish the hyporesponsiveness to PE displayed by LPS-treated vessels (t= 18hrs). However, it is worth noting that unlike cycloheximide, aminoguanidine did not potentiate the responses beyond those of control RTAs.

5.4.2 Effects of indomethacin, indomethacin + cycloheximide and indomethacin + cycloheximide + L-NMMA on control and LPS-treated RTA responses to PE. Indomethacin did not have a significant effect on the 1st D.R. curves (= Krebs + indomethacin) for either control or LPS-treated vessels (data not shown). After 4hrs perfusion with indomethacin + cycloheximide (both at concentrations = 10μM) the control responses to PE were not significantly

altered. The addition of L-NMMA (= Krebs + indomethacin + cycloheximide + L-NMMA) produced a leftward shift comparable to that for *untreated,* control vessels.

As with control RTAs, indomethacin on its own did not influence responses to PE in LPS-treated vessels. However, the potentiating effect of cycloheximide seen when used on its own, was completely abolished when it was added after indomethacin (= Krebs + indomethacin + cycloheximide), the result of which was to reveal a large leftward shift when L-NMMA was later added (= Krebs + indomethacin + cycloheximide + L-NMMA). This shift was comparable to that produced by L-NMMA in *untreated* LPS-treated vessels.

These results suggest that LPS treatment induces the expression of cyclooxygenases in isolated RTA, and that the potentiating effect of cycloheximide in these vessels reflects the inhibition of vasoactive products synthesised by these enzymes. Furthermore, the effect of L-NMMA added after cycloheximide (= Krebs + indomethacin + cycloheximide + L-NMMA) appears to be enhanced in control RTAs as compared to its effect after cycloheximide treatment alone (= Krebs + cycloheximide; see Section 5.4.1), i.e. indomethacin prevents the inhibitory action of cycloheximide on the L-NMMA-mediated shift in control RTAs, and thereby exposes the synergism between NO and cyclooxygenase-derived products. This, together with the fact that in LPS-treated vessels L-NMMA (i.e. = Krebs + indomethacin + cycloheximide + L-NMMA) produced a shift similar to that in *untreated* LPS-treated vessels (i.e. not previously exposed to indomethacin or cycloheximide), implies that indomethacin does not inhibit cNOS-derived basal NO production in control RTAs or the upregulated NO production by iNOS in LPS-treated vessels.

Indomethacin inhibits the cyclooxygenase pathway and by doing so, may influence vascular tone by inhibiting vasoconstrictors (eg. thromboxane A_2) or vasodilators (eg. prostacyclin). Two forms of cyclooxygenase have been

identified, a constitutive isoform and an LPS/cytokine-inducible isoform (iCOX). iCOX is induced by endotoxin which can result in an upregulated thromboxane A_2 or prostacyclin synthesis (Salvemini *et al.*, 1993; Wu *et al.*, 1994). Therefore, the ability of indomethacin to abolish the potentiating effect of cycloheximide in LPS-treated RTA responses to PE suggests that vasoactive products such as these are synthesised and can modify responses to PE. These results also indicate that the effect of cycloheximide on its own reflects an inhibition of *de novo* synthesis of one or more of these products.

5.4.3 Conclusion

Cycloheximide selectively augments responses to PE in LPS-treated vessels. This effect was sensitive to indomethacin, indicating the involvement of vasoactive products of the cyclooxygenase pathway. The syngergistic relationship between the NOS and COX pathways has been thoroughly studied (Salvemini *et al.*, 1993, 1995; Vane *et al.*, 1994; Swierkosz *et al.*, 1994). Both enzymes can be induced by cytokines to produce high levels of vasodilator and in the case of COX, vasoconstrictor products, which influence the size of contraction produced in response to PE by an LPS/cytokine-activated vessel (Wu *et al.*, 1994). The fact that cycloheximide caused a leftward shift of LPS-treated RTA responses to PE suggests that production of an enzyme-derived vasodilator, synthesised *de novo* within the vessel wall, was inhibited. Further studies will have to be performed to determine the identity of these. The two most likely candidates are NO and/or prostacyclin. Prostacyclin is the main product of arachidonic acid metabolism in isolated vascular tissues (Honn *et al.*, 1983).

The fact that NOS inhibitors were shown to fully reverse the hyporesponsiveness to PE in LPS-treated vessels (see chapter 4) does not rule out the involvement of prostaglandins in this effect as a recent publication by Salvemini *et al.*, (1995), suggests that analogue inhibitors of NOS, including L-NAME, may also inhibit prostaglandin production. Whether this is due to a direct inhibition of the

enzymes synthesising the prostaglandins (ie. cyclooxygenases) or an indirect result of reduced NO production is not clear.

It could be argued that LPS-treated vessels did not show an altered respones to PE in the presence of indomethacin *only* since both vasodilator and vasoconstrictor products of the cyclooxygenase pathway are being produced and therefore counteract each other. The effect of cycloheximide may suggest that only the vasodilator product requires *de novo* synthesis. This explanation is speculative and further studies will have to be conducted to confirm or dismiss it.

Chapter 6

Ruthenium complexes as nitric oxide scavengers: therapeutic potential in nitric oxide-mediated disease.

6.1 Introduction

As already outlined in Part D of the Introduction and in Chapter 3, mounting evidence supports the view that iNOS-generated NO is an important causal factor in septic shock syndrome. The ability of NOS inhibitors to alleviate some of the haemodynamic manifestations of septic shock has focused attention on their therapeutic potential. The outcome thus far has been equivocal, with reports of adverse cardiovascular consequences, for example, pulmonary hypertension (Robertson *et al.*, 1994) and ultimately, increased mortality in animal models (Minnard *et al.*, 1994; Tracey *et al.*, 1995). It is becoming increasingly apparent that the capacity to discriminate between iNOS and cNOS is crucially important in this context and increasing effort is now devoted to finding more isoenzyme-selective inhibitors of NOS. To date, the most promising compound displaying a greater specificity for iNOS than for cNOS is aminoguanidine (Griffiths *et al.*, 1993) which has been shown to cause partial reversal of vascular hyporeactivity *ex vivo* (Joly *et al.*, 1994) and improved survival in endotoxic animals *in vivo* (Wu *et al.*, 1995). Certain isothioureas also show some selectivity for the inducible isoform (Southan *et al.*, 1995).

The present study addresses an alternative strategy to treating NO-mediated disease with the use of drugs which specifically scavenge and remove pathophysiological quantities of NO, rather than block its synthesis. The potential of a range of ruthenium compounds has been explored with this in mind and this study reports on the pharmacological properties of three ruthenium (III) complexes: penta-aminechloro-ruthenium-3-dichloride (or JM1006),

potassium chloro[hydrogen(ethylenedinitrilo)tetraacetato]ruthenate (or JM1226) and aqua[hydrogen(ethylenedinitrilo)tetraacetato]ruthenium (or JM6245).

6.1.1 Summary of findings.

Experiments conducted in collaboration with Dr. Simon Fricker (Johnson Matthey Technonology Centre, Reading, England), have established that:

1) Many ruthenium(III) complexes react readily with NO to form stable ruthenium(II) mononitrosyls. A range of Ru(III) complexes was synthesised and a study made of the ability of each to bind NO, both *in vitro* and *in vivo* and also in the model of endotoxic shock in the rat (see Chapter 3).

2) Binding of authenic NO gas by aqueous solutions of JM1226 was studied manometrically by Dr. Simon Fricker. Reaction with NO gave a product with an infra red (IR) spectrum having a peak absorbance at 1897 cm^{-1}, characteristic of an Ru(II)-NO adduct. Reaction of JM1226 with the NO donor drug *S*-nitroso-N-acetylpenicillamine (SNAP) yielded a compound with a similar IR spectrum.

3) Fricker et al., (*in press*), showed that the considerable increased accumulation of NO_2^- in growth media from cultures of LPS/interferon-gamma-treated RAW 264 macrophages was abolished in the presence of JM6245 or JM1226. Furthermore, the reduction in NO_2^- levels was comparable following treatment with L-NMMA and with all three compound, NO_2^- levels were similar to those obtained in media from *un*activated macrophages.

4) Addition of JM1226 or JM6245 (both 100μM) to the culture medium of stimulated RAW 264 macrophages significantly increased the cell viability of co-cultured P815 murine mastocytoma cells. The degree of protection afforded by JM compounds was the same as that seen after blockade of NO production by the NOS inhibitor, L-NMMA (Fricker *et al.*, *in press*).

5) Male Wistar rats were injected with bacterial LPS (4mg/Kg; i.p.) to induce endotoxic shock (see Chapter 3). JM1006, 1226 and 6245 (both 100μM) fully reversed the hyporesponsiveness to PE of internally-perfused RTAs isolated from animals injected with LPS 24hrs previously (ie. t= 24hrs). Furthermore, a single injection of JM1226 (100mg/Kg; i.p.) administered 20hr after an LPS injection (4mg/Kg; i.p.) reversed the hypotension associated with endotoxic shock within *ca* 9hrs.

6) These results show that JM1006, 1226 and 6245 are able to scavenge NO in biological systems and suggest a role for these ruthenium complexes in novel therapeutic strategies aimed at alleviating NO-mediated disease states.

I presented an account of some aspects of this work at the Fifth International Meeting on the Biology of Nitric Oxide held on Amelia Island, Florida (Fricker *et al.*, 1996).

6.1.2 Theory

Ruthenium forms more nitrosyl complexes than any other metal (Bottomley, 1978). Ru(II) reacts with NO to form six co-ordinate Ru(II) mononitrosyls containing a linear Ru-NO bond. The latter is very stable, able to resist a variety of redox and substitution reactions, and consequently the nitrosyl moiety is not easily displaced. Chelation of the metal with a suitable ligand can be employed to confer water solubility, facilitating rapid *in vivo* clearance and low toxicity, whilst providing an available binding site for NO. The polyaminocarboxylates, such as ethylenediaminetetraacetic acid (edta), satisfy these requirements as ligands. In all three complexes mentioned here edta is pentadentate so that one co-ordination position is available for NO binding.

Reaction of JM1226 with NO in aqueous solution (see fig. 6.1) involves formation of the aqua derivative, JM6245, followed by a rapid substitution of H_2O for NO. The ligand around ruthenium(III) is kinetically inert and formation

of the linear Ru-NO bond further stabilises the *trans* position in the resulting ruthenium(II) adduct (Fricker et al., *in press*).

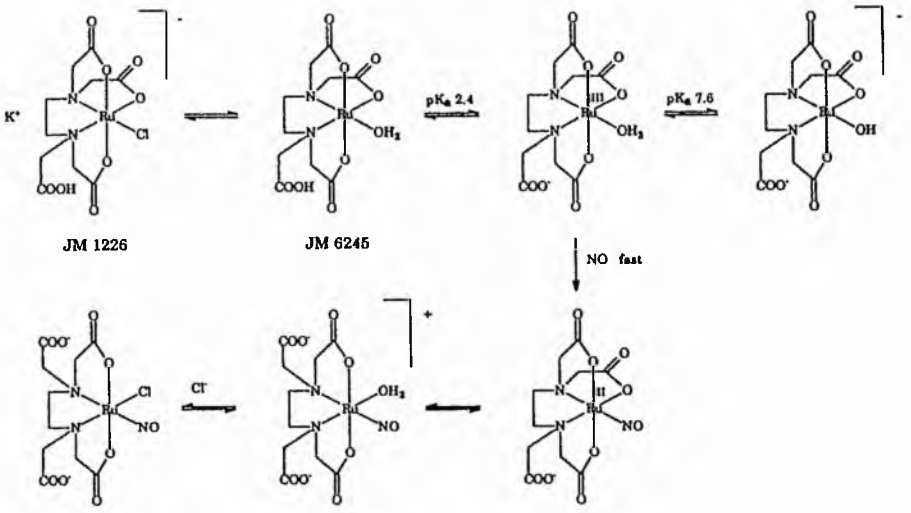


Fig. 6.1 *Equilibrium diagram of the reaction between JM1226 and NO in aqueous solution. Reaction between the aqua derivative, JM6245, and NO is rapid and proceeds via a seven co-ordinate intermediate. The resulting stable adduct contains a linear Ru(II)-NO bond (see fig. 6.2).*

6.2 Materials and Methods

6.2.1 Methods

Binding of NO by aqueous solutions of JM1226. Two experiments were conducted (Fricker *et al.*, 1995 and *in press*). First, a known volume of authenic NO gas ($3cm^3$; 1.3 x 10^{-4} moles) was introduced into the headspace above a stirred aqueous solution of JM1226 ($25cm^3$; 1.3 x 10^{-4} moles) in a closed apparatus under an argon atmosphere. Temperature was maintained at 22-24^oC. NO absorption was measured using a manometer. After complete absorption of NO (generally, *ca* 20 mins) the reaction mixture was freeze dried and the product examined by infra-red (IR) spectroscopy using a Perkin Elmer 1720X FT-IR spectrometer. Second, equimolar amounts (9 x 10^{-5}M) of JM1226 and the NO donor SNAP were dissolved in water and allowed to react for 24hrs. The resulting compound was freeze-dried and its IR spectrum recorded later.

Isolated rat tail artery preparation. Segments of of RTAs from control and LPS-treated (t= 24hrs) Wistar rats were isolated and perfused internally and externally with Krebs solution as described earlier (Chapter 3). The following protocol was used:

1. An initial series of bolus (10µl) injections of PE (10^{-5}-10^{-2}M) was administered in the presence of Krebs solution *only*.

2. JM1226, JM6245 and JM1006 (100µM) were added to the internal perfusate for 30 mins before making a second series of injections.

3. JM compounds were washed out for 2hrs, after which time a third series of injections of PE was administered.

4. Finally, L-NMMA (100µM) was added into the internal perfusate for 30 mins before a final series of PE injections was administered.

Evaluation of the ability of JM1226 to reverse the hypotension in LPS-treated rats. Using the well established model of endotoxic shock in male Wistar rats (see Chapter 3), two experiments were conducted to examine the ability of JM1226 to reverse the ensuing hypotension following LPS treatment. Blood pressure measurements were made in thirteen conscious rats (250 - 340g) at various times after a single injection of LPS (4mg/Kg; i.p.), followed later (at t= 20hrs) by a second injection of either (a) sterile saline (grp. 1) or (b) a sterile solution (100mg/Kg) of JM1226 (grp. 2). In the first experiment, blood pressures were measured at t= 0, 20 and 24hrs. In the second experiment recordings were made at t= 0, 20, 24, 29 and 32hrs. All blood pressure measurements were made using the tail cuff method (see Chapter 3, Section 3.5.1) and animals were familiarised to the procedure by a 'training' regimen (daily, for 1 or 4 weeks) before commencing an experiment so as to avoid undue stress. Recordings of systolic (S.b.p), mean (M.b.p.) and diastolic (Db.p.) arterial pressures were made at the following times relative to the time (t= 0hr) at which animals were injected with LPS: t= 0, 20, 24, 29 & 32hrs. Experiments 1& 2 followed exactly the same protocol and because blood pressure readings were made in the morning and afternoon a study was carried out on a group of rats (n= 8)to determine if this influenced the results. Blood pressure (b.p.) readings made in the morning were not significantly different from those made in the afternoon (results not shown).

6.2.2 Materials

L-NMMA and LPS (*E. coli* serotype 055:B5) were obtained from Sigma Ltd. JM1226, JM6245 and JM1006 were supplied by Johnson Matthey Technology Centre, Reading, England. Male Wistar rats (260-340gr. wt.) were bought from John Tuck Suppliers and the tail cuff apparatus (Model 179 Blood Pressure Analyzer) from IITC Life Science, Calif. USA.

6.3 Results

6.3.1 JM1226 binds NO to form a ruthenium(II) mononitrosyl. Fig. 6.2 shows FT-IR spectra for JM1226 alone (A) and for JM1226 after reacting it with authenic NO in aqueous solution (B; see methods). The sharp absorbance peak at 1897cm^{-1} in spectrum B (arrow) identifies a linear Ru(II)-NO bond in the resulting compound (Bottomley, 1978) and provides confirmation of the formation of a ruthenium(II) mononitrosyl. A similar result (spectrum not shown) was obtained after reacting JM1226 with the NO donor SNAP.

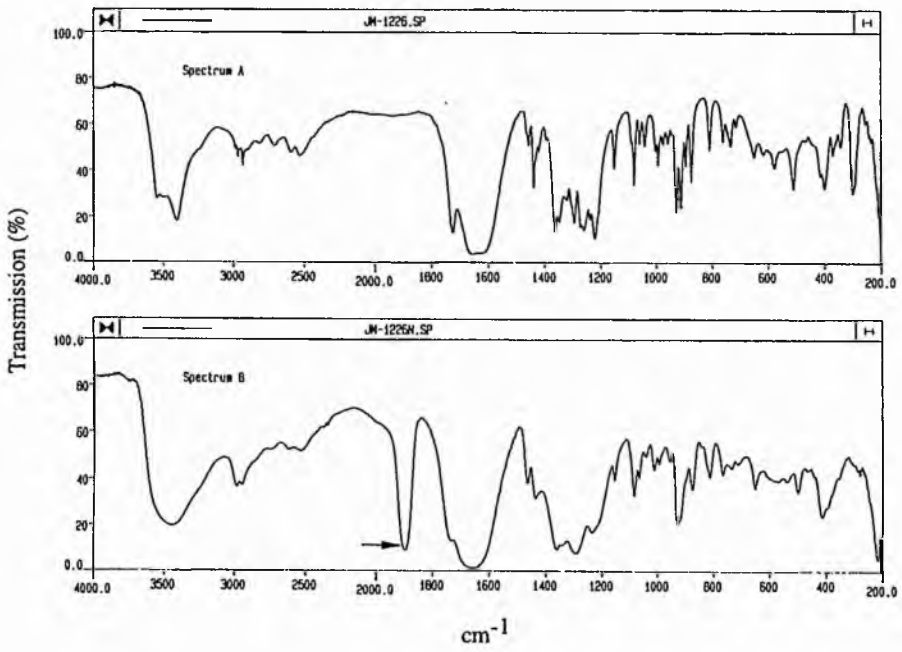


Fig. 6.2 Infra-red spectra of JM1226 (spectrum A) and JM1226 after reaction with NO (spectrum B). The product has a sharp absorbance peak at 1897cm^{-1} (arrow), characteristic of a linear Ru(II)-NO adduct.

6.3.2 Time course of LPS-induced endotoxaemia in an experimental rat model. LPS induced a marked hypotensive response (Table 1): at t= 20hrs, SAPs, MAPs and DAPs had fallen by 32%, 28.5% and 25.1% respectively. Systemic pressures spontaneously returned to pre-injection values over the ensuing 24-28hrs (t= 44-48hrs). LPS also induced a modest (*ca* 10%) but statistically significant tachycardia.

Time after injection	t= 0hr	t= 20hr	p value*
SAP	166.4 ±8.8	113.2 ±5.2	0.028
MAP	110.6 ±4.9	79.1 ±6.4	0.000
DAP	82.8 ±4.9	62.0 ±7.8	0.000
HR	376.6 ±9.4	411.1 ±11.7	0.017

Table 1 *Systemic blood pressure (mmHg) and heart rates (HR; beats/min) recorded with the rat tail cuff apparatus immediately before and 20hrs after injecting animals (n= 13) with bacterial LPS (4mg/Kg). Student's paired t-test (p values to the nearest 3rd decimal point).*

6.3.3 JM1006, JM1226 and JM6245 reverse the hyporeactivity to PE of RTAs from LPS-treated rats. Fig. 6.3a & 6.3b show that the addition of JM1006 (100μM) to the internal perfusate completely abolished the diminished sensitivity (2-way ANOVA, F= 6.78, df= 9, 136_e, p= < 0.001) to PE of isolated, internally-perfused RTAs from LPS-treated animals (t= 24hrs; closed circles; controls, open circles; n= 7) . Statistically, there was no significant difference between JM1006- (closed squares) and L-NMMA-treated (open triangles) arteries from LPS-treated rats (2-way ANOVA, F= 0.98, df= 9, 234_e, p= 0.362). Similarly, the difference in sensitivity to PE (2-way ANOVA, F= 7.39, df= 9, 155_e, p < 0.001) between vessels from control (open circles) and LPS-treated (t= 24hrs; closed circles, fig. 6.4a; n= 9) rats was reversed by JM1226 (100μM; closed squares, fig. 6.4b; 2-way ANOVA, F= 3.61, df= 9, 151_e, p < 0.001). The effect of JM1226 (and of JM6245; data not shown) on vessels from LPS-treated rats was closely comparable to that seen after blocking NO synthesis with L-NMMA (100μM; open triangles; fig. 6.4b). Statistically, there was no significant difference between JM1226- and L-NMMA-treated arteries from LPS-treated rats (2-way ANOVA, F= 0.46, df= 9, 189_e, p= 0.901). Meaned results are shown in Appendix tables 6.1 & 6.2.

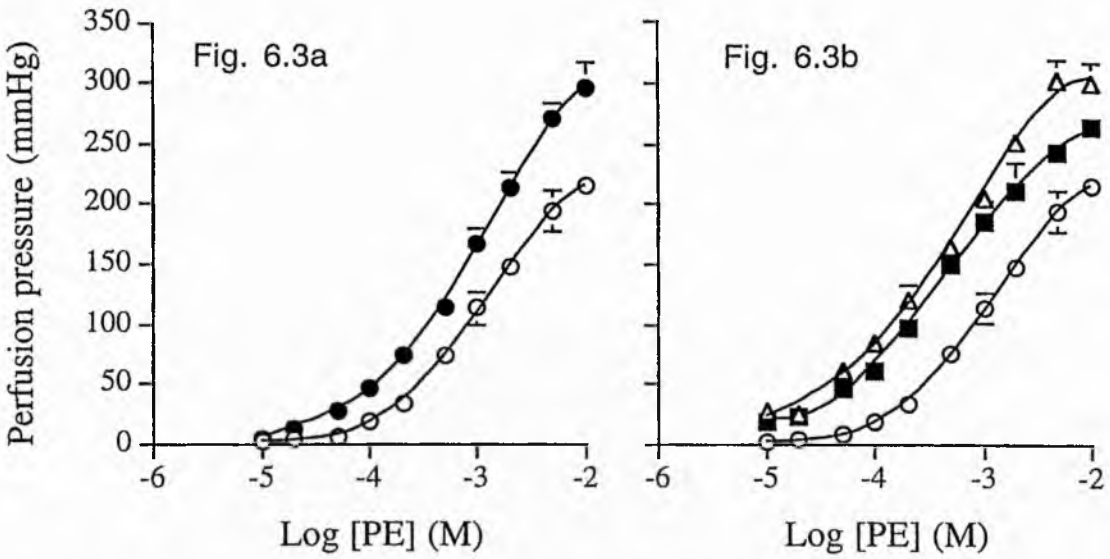


Fig. 6.3 a *Log D.R. curves showing the vasoconstrictor effect of bolus injections of PE on arteries isolated from animals previuosly injected with LPS 24hrs prior to sacrifice (open circles) compared to vessels from animals injected with sterile saline only (controls; closed circles). Mean ±S.E.M. shown.* ***b*** *Log D.R. curves to PE of vessels from LPS-treated rats (t= 24hrs) before (open circles; same data as fig. 6a) and after the addition of either JM1006 (closed squares) or L-NMMA (open triangles) to the internal perfusate. The sensitivity to PE of L-NMMA- and JM1006-treated vessels were not statistically different and both were significantly greater than that for LPS-treated arteries (see text).*

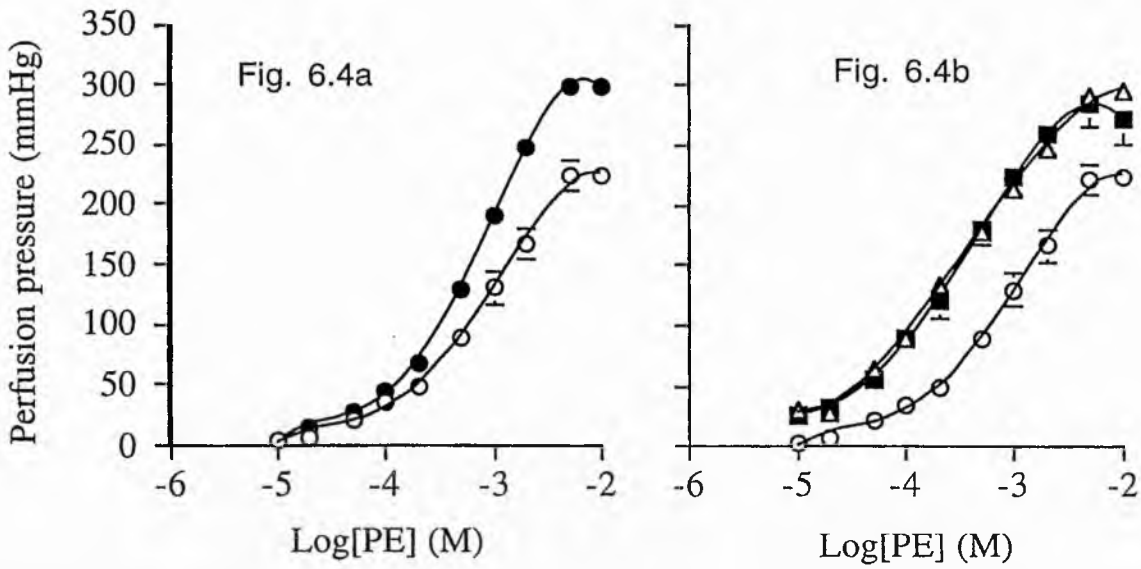


Fig. 6.4a *Log D.R. curves showing the vasoconstrictor effect of bolus injections of PE on arteries isolated from animals previuosly injected with LPS 24hrs prior to sacrifice (open circles) compared to vessels from animals injected with sterile saline only (controls; closed circles). Mean ±S.E.M. shown.* ***b*** *Log D.R. curves to PE of vessels from LPS-treated rats (t= 24hrs) before (open circles; same data as fig. 6a) and after the addition of either JM1226 (closed squares) or L-NMMA (open triangles) to the internal perfusate. The sensitivity to PE of L-NMMA- and JM1226-treated vessels were not statistically different and both were significantly greater than that for LPS-treated arteries (see text).*

6.3.4 JM1226 accelerates recovery of arterial blood pressures in LPS-treated (hypotensive) rats. Fig. 6.5 summarises the results in the form of a histogram in which the data from experiments 1&2 are combined for time points t= 0, 20, 24hrs, but for time points t= 29 and 32hrs the data is from experiment 2 only. The are results also shown in Appendix table 6.3.

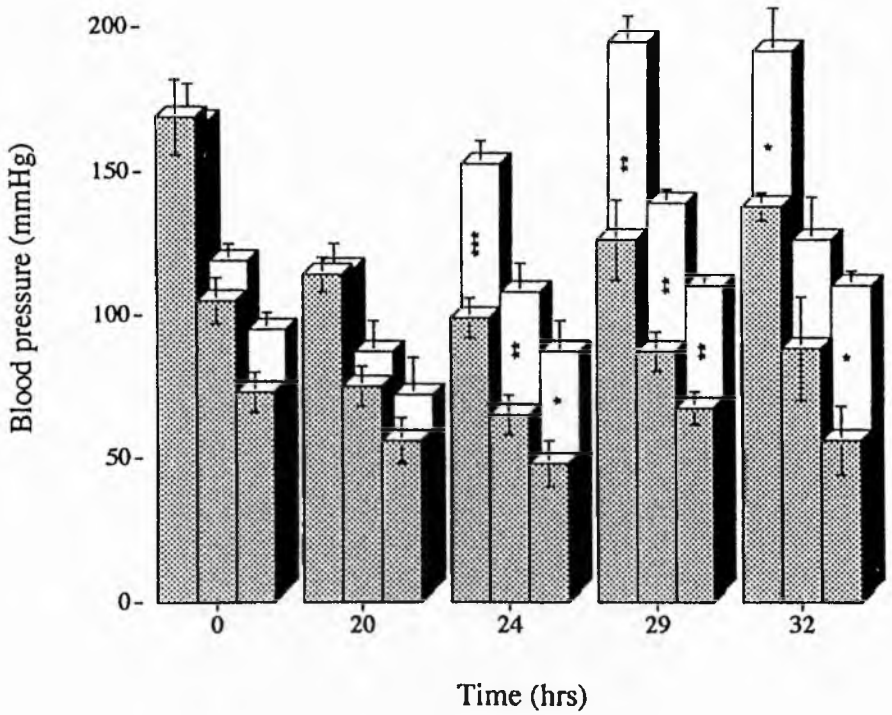


Fig. 6.5 *Blood pressure measurements for rats immediately before (t= ohr; first block) and 20hrs after (second block) a single i.p. injection (4mg/Kg) of bacterial LPS (data also in Table 1). Animals in group 1 (stippled columns) received an injection of saline only and those in group 2 (blank columns) received JM1226 (i.p.; 100mg/Kg) at t= 20hrs. Blood pressure recovered more rapidly in animals treated with JM1226 and was essentially complete after ca. 9hrs. See text for details.*

Each of the 5 grouped vertical columns in the histogram represents (from left to right) S.b.p.s, M.b.p.s, and D.b.p.s. Both groups of LPS-treated animals were markedly hypotensive by t= 20hrs (see Table 1 which shows combined data ie.

experiments 1&2, n=13 animals) and shows some recovery thereafter. However, the recovery of saline-injected animals (stipplied columns) was incomplete even after 12hrs (t= 32hrs), whereas the rate of recovery of animals that received JM1226 (open columns) was enhanced and essentially complete within *ca.* 9hrs (t= 29hrs).

6.4 Discussion

From these results it is clear that ruthenium-based scavengers could offer an alternative therapeutic strategy to the use of NOS inhibitors for alleviating the symptoms of septic shock, and possibly other mediated diseases too. Two chemically-related polyaminocarboxylate-ruthenium(III) complexes - JM1226 and its aqua derivative JM6245 - were found to react with either authentic NO or with an NO donor (SNAP) in aqueous solution to yield the corresponding Ru(II) mononitrosyl adduct (Fig. 2; Fricker *et al.*, 1995 and *in press*). This has yet to be confirmed for JM1006. Their ability to react with NO in several biological systems of increasing complexity (from cultured cells to whole animals) was therefore explored, following induction of the iNOS-driven, 'high-ouput' pathway for NO synthesis, using either LPS alone (whole animals) or combined LPS + interferon-gamma treatment (cell cultures; Fricker *et al.*, 1995). It has been shown that ruthenium complexes reduced NO_2^- accumulation by cultured macrophages and afforded a significant degree of protection against the tumouricidal action of these macrophages (Fricker *et al.*, *in press*).

The results of *in vivo* experiments using the rat model of endotoxic shock are clearly more relevant when evaluating the therapeutic potential of these compounds. The effects of LPS treatment (4mg/Kg; i.p.) have been defined according to five separate parameters all of which are consistent with the principal physiological manifestations of septic shock (Glauser *et al.*, 1991) and therefore, establish the LPS-treated rat as a valid paradigm for testing the ability of JM compounds to scavenge pathophysiological quantities of NO under both *ex vivo* (isolated RTAs) and *in vivo* conditions.

The hyporesponsiveness to PE displayed by internally-perfused RTAs isolated from LPS-treated animals was maximal at t= 24hrs (see Chapter 3, fig. 3.7). The addition of JM1006, JM1226 and JM6245 (results not shown) to the internal

perfusate of such vessels enhanced their sensitivity to PE: indeed, the effect was indistinguishable from that seen after blockade of NO synthesis at source using the NOS inhibitor, L-NMMA.

Treatment with LPS produced a profound but *transient* hypotensive response in laboratory animals. As it will be seen in Chapter 8, S.b.p.s, M.b.p.s amd M.b.p.s fall to a minimum around t= 20 - 24hrs and gradually recover over the ensuing 24- 28hrs (t= 44 - 48hrs). Fig. 6.5 shows that some spontaneous recovery of blood pressures was seen in the saline-injected (control) group of LPS-treated rats but this was clearly incomplete after 12hrs (t= 32hrs). In contrast, the recovery of the LPS-treated group that received a single i.p. injection of JM1226 given at t= 20hr was accelerated and complete after a further *ca.* 9hrs (t= 29hrs).

These results and those of Fricker et al., (1995) show that JM1226, 6245 and 1006 are effective scavengers of NO in a variety of biological systems and they establish these complexes as potentially useful alternatives to NOS inhibitors for alleviating some of the symptoms of endotoxic shock. It has previously been shown, and is further confirmed in this study (see Chapter 7, Section 7.4.1), that tumour-supply vessels are remarkably unresponsive to vasoactive agents (Hirst & Wood, 1989; Kennovin *et al.*, 1993) and in this respect they strongly resemble RTAs from LPS-treated rats. It is also shown in Chapter 7 (Section 7.4.2) that the sensitivity to PE of isolated, internally perfused epigastric arteries which previously supplied an inguinal tumour implant, can be fully restored using the non-selective NOS inhibitors L-NMMA or L-NAME. Moreover, chronic oral administration of L-NAME, via the drinking water, significantly retarded solid tumour growth (Chapter 8, Section 8.3.5). Based on the data presented here it is likely that JM1226, 6245 and/or 1006, could have similarly benefical effects to L-NAME.

Further studies will have to be conducted detailing the toxicological and pharmacokinetic properties of these JM compounds. However, preliminary

toxicity tests in vivo have shown that Wistar rats can tolerate maximum doses of JM1226 some 2-4 times greater than those used here (200 - 400mg/Kg; i.p.) and pilot pharmacokinetic experiments indicate rapid plasma clearance times (Dr. Simon Fricker; *personal commun.*). Studies will also have to be conducted to optimize the dosing schedule of these drugs.

6.4.1 Conclusions. In conclusion, the results of this study demonstrate that by efficiently scavenging NO, ruthenium(III) complexes JM1006, 1226 and its aqua derivative, 6245, might be usefully exploited in new therapeutic strategies aimed at combating NO mediated disease.

Chapter 7

Vasoconstrictor Properties of Tumour-Supply Arteries

7.1 Introduction

Experiments were carried out to establish whether isolated arteries which had previously supplied a solid tumour (ie. TEAs) displayed altered vasocontractile responses to PE when compared to non-tumour associated arteries (ie. CEAs), and if so, whether their responsiveness could be restored using specific inhibitors of NOS. Kennovin *et al.*, (1994) first showed that TEAs were hyporesponsive to PE as compared to CEAs and the results of their study suggested that the reason for the difference was an upregulated NO production in TEAs. This hypothesis was borne out by their subsequent findings that inhibitors of NOS could abolish their their diminished hyporesponsiveness to PE. These inhibitors are specific for NOS but not selective for one or other of the different NOS isoforms. This study follows on from that of of Kennovin *et al.* and uses the selective iNOS inhibitor, aminoguanidine (AG), in addition to the non-selective NOS inhibitors, L-NMMA and L-NAME, to ascertain whether indeed increased iNOS-derived NO production is responsible for the hyporeactivity of TEAs to PE.

Materials & Methods

7.2 Methods

7.2.1 Preparation of Isolated Tumour Supply Artery

Preparing the Tumour Tissue (step 1). Pieces of a spontaneous P22 carcinosarcoma tumour found in a BD9 rat were taken and stored in vials containing minimum essential medium (MEM). In preparation for implantation, pieces were incubated at 37°C in fresh MEM for 20 mins. and minced using sterile scissors. To obtain sufficient tissue, tumours were initially grown subdermally on the backs of isogeneic female BD9 rats until they were of suitable size (8-10g), after which they were removed, minced and re-implanted into isogeneic males. Third generation (T_3) tumour tissue was used for all experiments. Sterile technique was applied at all times when handling tumour samples.

Tumour Implantation (step 2). Small pieces of P22 carcinosarcoma tumour tissue (T_3) were finely chopped into a slurry and injected (0.05mls; using a sterile 19G1.5 needle) into the right inguinal fat pads of male isogeneic BD9 rats under general anaesthesia (90mg/Kg Ketamine: 10mg.Kg Xylazine; i.p.), close to the first bifurcation between the right inferior epigastric and femoral arteries. The developing tumour therefore derived its blood supply primarily through the right inferior epigastric artery (TEA). The left inferior epigastric artery (CEA) from the same animal did not supply a tumour and was used as a control (see fig. 7.1). This model is very similar to the isolated tumour model devised by Gullino & Grantham, (1961) and subsequently modified by Tozer *et al.*, (1995) and others.

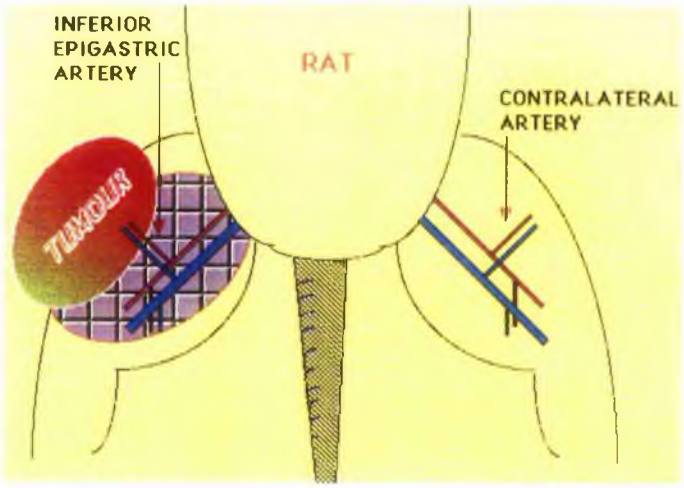


Fig. 7.1 *A diagram of the vascular supply to the implanted tumour in the rat hindlimb (refer to text for details).*

Isolation of TEA and CEA (step 3). The side branches along the femoral artery were ligated and a perfusion canula (Portex; with green mount) containing Krebs solution (see below) was fed into the femoral artery until the tip was adjacent to the junction with the epigastric artery (see fig. 7.2). Once the TEA and CEA had been removed (ie. a section of the femoral artery and the complete length of the epigastric artery), the animals were killed by anaesthetic overdose.

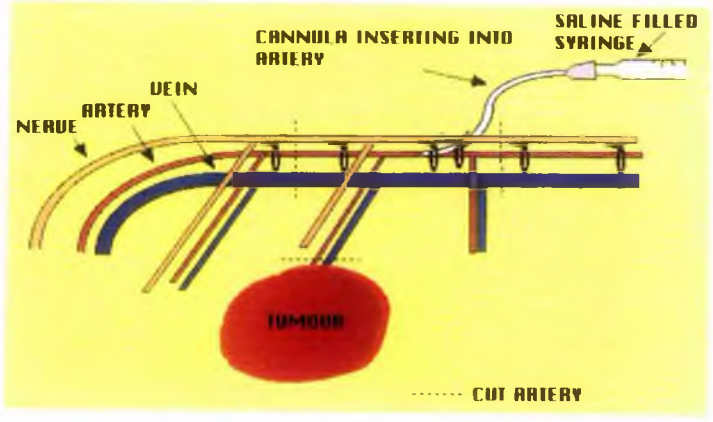


Fig. 7.2 *A diagram showing the right inferior epigastric artery feeding into the developed tumour.*

7.2.2 Experimental Protocol. Isolated segments of TEA and CEA were mounted in a perfusion apparatus (similar to the one described in chapter 3). Vessels were allowed to acclimatise for ~1hr during which time the internal perfusion rate was gradually increased from 0.05 to 0.6mls/min. TEAs and CEAs were subjected to a similar set of experiments as RTAs (outlined in chapter 4).

7.3 Materials

Isogeneic male BD9 rats and tumour tissue originated from the CRC Gray lab., Mount Vernon Hospital, England. Minimum essential medium was obtained from GibcoBRL Ltd.(cat. No. 11700). Arteries were perfused using a constant flow pump; Harvard Apparatus, South Natick., Massach. USA.

7.4 Results

7.4.1 The hyporesponsiveness of isolated TEAs. Responses to PE are expressed as mean (± S.E.M) perfusion pressure (mmHg) and plotted as a function of $\log_{10}$ phenylephrine (PE) concentration (M). In agreement with findings by Kennovin *et al.*, (1994), vasoconstrictor responses to increasing concentrations of PE were significantly attenuated in TEAs (2 way ANOVA; F= 7.80, df= 9, 445_e $p < 0.001$) compared to CEAs (see fig. 7.4 below and Appendix table 7.1).

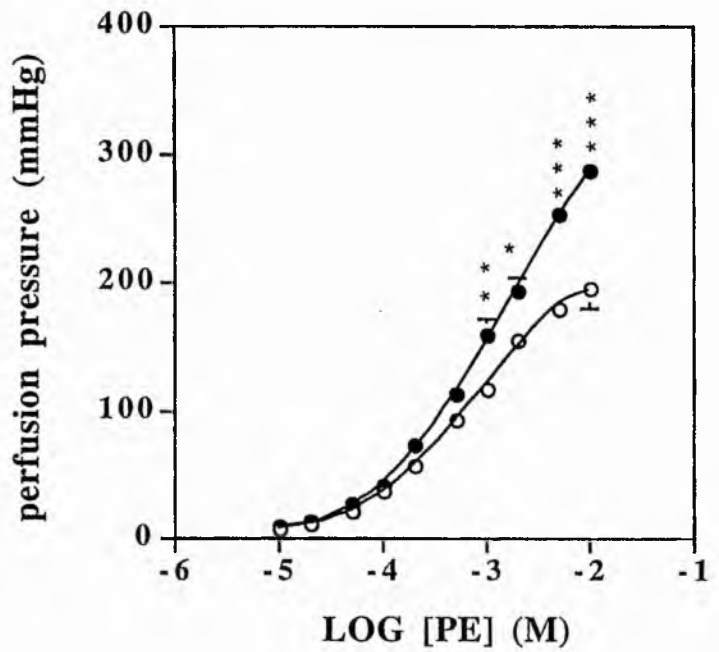
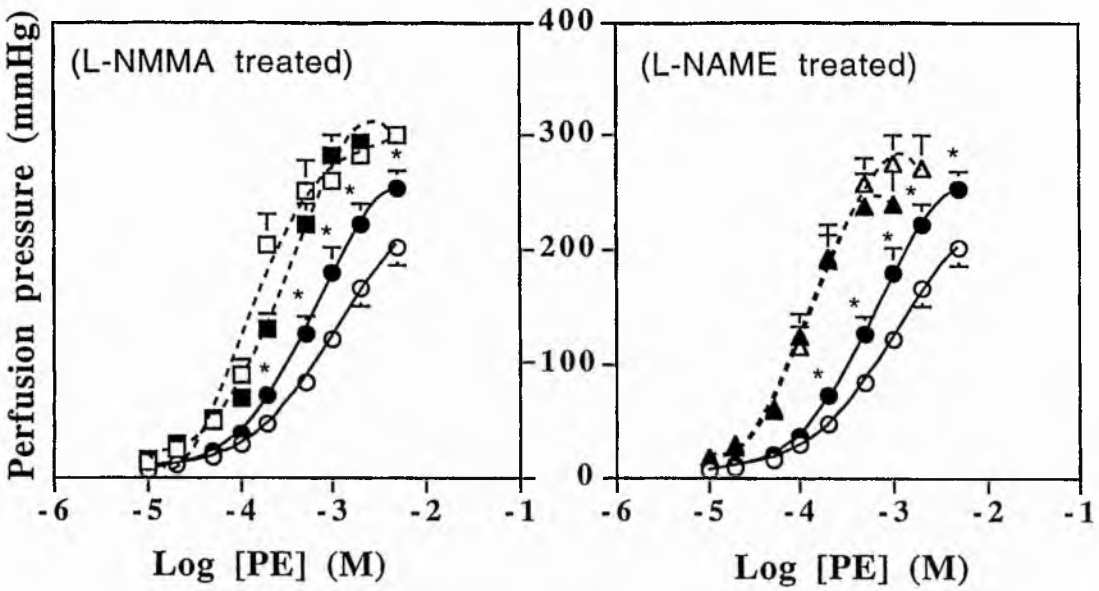


Fig. 7.4 *A graph comparing the initial D.R. to PE concentration (M) in TEAs (n=28; open circles) and CEAs (n=29; closed circles) perfused with Krebs only. Asterisks indicate a significant difference between using a student's unpaired t-test.*

7.4.2 The Effect of NOS Inhibitiors on PE-Induced Vasocontractile Responses: Effects of L-NMMA or L-NAME. The effect of a number of NOS inhibitors was tested, in order to determine whether the difference between TEAs and CEAs is due to upregulated NOS activity. Exposure to L-NMMA or L-NAME (80μM) for 20-30 mins did not affect passive perfusion pressures but augmented the responses to PE and completely abolished the difference between TEAs and CEAs (figs.7.5 & 7.6 respectively).

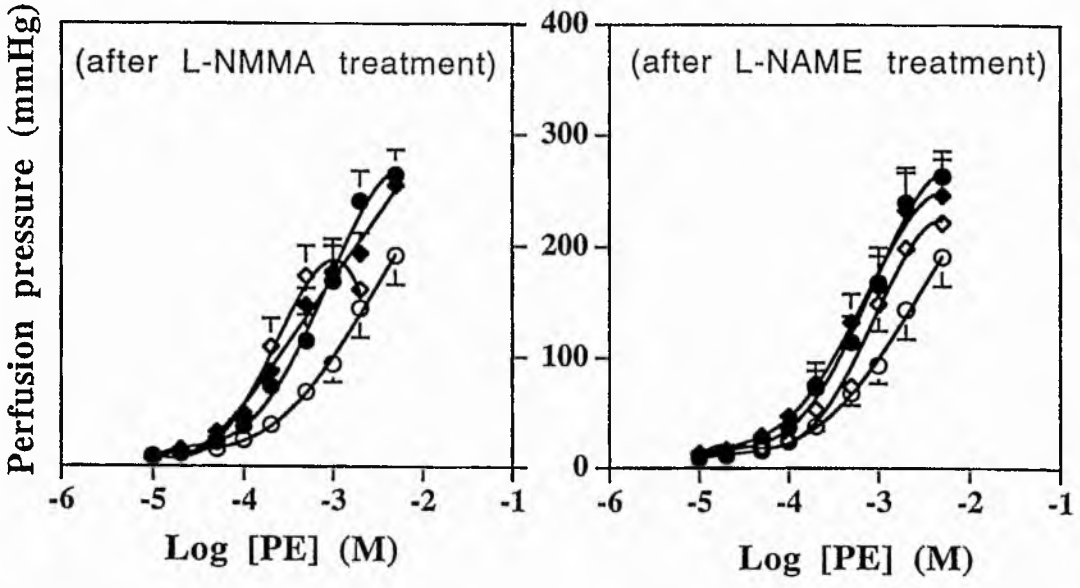


***Figs. 7.5 & 7.6** (LHS and RHS respectively). Graphs of log dose response curves for CEAs (n=14; closed symbols) and TEAs (n=15; open symbols) in Krebs solution (circles), in the presence of L-NMMA (80μM; squares; n= 6 &7, respectively, fig. 7.5), or L-NAME (80μM; triangles; n= 10; fig. 7.6). Asterisks represent a significant difference between CEAs and TEAs (unpaired student's t-test, $p< 0.05^{*}$).*

Initial responses to PE for CEAs and TEAs were statistically significantly different (2 way ANOVA; F= 2.85, df= $8,230_e$, p= 0.005^{**}). however, their responses to PE were not significantly different from each other after treatment with L-NMMA (2 way ANOVA; F= 0.96, df= $8,83_e$, p= 0.471) or L-NAME (2 way ANOVA; F= 0.32, df= $6,85_e$, p= 0.927) showing that both NOS inhibitors abolished the TEA- associated hyporesponsiveness to PE (see figs.7.5 & 7.6). Furthermore, student's *unpaired* t-test analysis did not reveal a statistically significant difference between TEA curves after treatment with L-NMMA or L-NAME. The same was also true for CEAs (see Appendix table 7.3).

The effects of L-arginine after 30-60 mins internal perfusion are shown in Figs. 7.7 & 7.8. L-arginine completely reversed the shift produced by L-NMMA or L-NAME in CEAs (see figs. 7.7 & 7.8 respectively), such that the initial D.R. curve in the presence of Krebs solution alone (ie. 1st D.R.) did not differ significantly from that after treatment with L-arginine (3rd D.R.; 2 way ANOVAs; F= 0.87, df= $8,95_e$, p= 0.546 & F= 0.15, df= $8,91_e$, p= 0.996, respectively). The

potentiating effect of L-NAME to PE-induced responses in TEAs was also reversed in the presence of L-arginine. This is shown by the fact that the 1st D.R. curve did not significantly differ from the 3rd D.R. curve (2 way ANOVA; F= 0.71, df= $8,124_e$, p= 0.681; see fig. 7.8). In contrast however, L-arginine was somewhat less effective at reversing the potentiating effect of L-NMMA in TEAs, such that the 1st and 3rd D.R. curves remained significantly different (2 way ANOVA; F= 3.94, df= $7,78_e$, p= 0.001^{***}; see fig.7.7).



Figs. 7.7 & 7.8 (LHS and RHS respectively). CEAs (closed symbols, n= 5) and TEAs (open symbols, n= 4-8) comparing the initial D.R. curves (Krebs only; circles) with those in the presence of L-arginine (diamonds) after L-NMMA (fig. 7.7) or L-NAME (fig. 7.8) treatment.

7.4.3 The Effect of a selective NOS Inhibitor on PE-Induced Vasocontractile Responses: Effects of Aminoguanidine. Aminoguanidine (AG) is a relatively selective inhibitor of inducible NOS activity. Joly *et al.*, (1994) found that AG had no effect on the responses evoked by PE in rat aortic rings. However exposure to LPS (5hrs incubation; 200ng/ml) induced a significant hyporeactivity to PE which could subsequently be reversed after 1hr treatment with AG (100μM). To test whether AG had any effect on the PE-induced responses of TEAs and CEAs, an a sequence of injections to PE was followed by a 1hr perfusion with Krebs + AG (1mM), before a second series of

PE injections was given. Experiments were carried out on CEAs (n=6) and TEAs (n=5) and the results plotted in figs. 7.9 & 7.10 respectively.

It is clear from fig. 7.9 that AG does not significantly alter CEAs responses to PE. This was confirmed by comparing the 1st D.R. curve (in the presence of Krebs) and the 2nd D.R. curve (Krebs + AG) using 2 way ANOVA (F= 0.91, df= 9, 89_e, p= 0.347) and a student's *paired* t-test (see Appendix table 7.4).

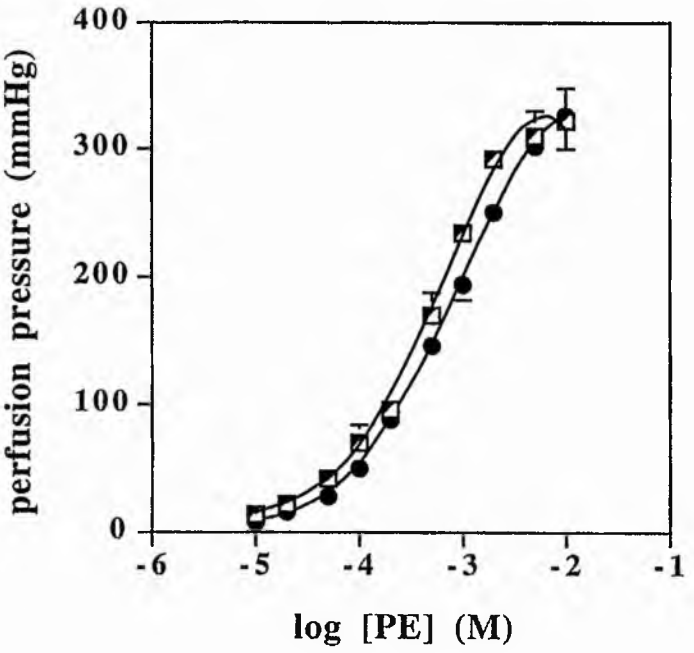


Fig. 7.9 *A graph of the D.R. curves to PE for CEAs (n=6) in the presence of Krebs only (closed circles) and Krebs + aminoguanidine (1mM; 1hr, semi-closed squares).*

Fig. 7.10 shows that TEA responses to PE were, in contrast to CEAs, significantly shifted to the left by AG (2 way ANOVA; F= 2.20, df= $9,66_e$, p= 0.033). Furthermore, a Student's *unpaired* t- test revealed that AG-treated TEA responses (Fig. 7.10, squares) were not significantly different from those of CEAs in the presence of Krebs only (Fig. 7.9, closed circles). In other words, AG completely abolished the TEA-associated hyporesponsiveness to PE.

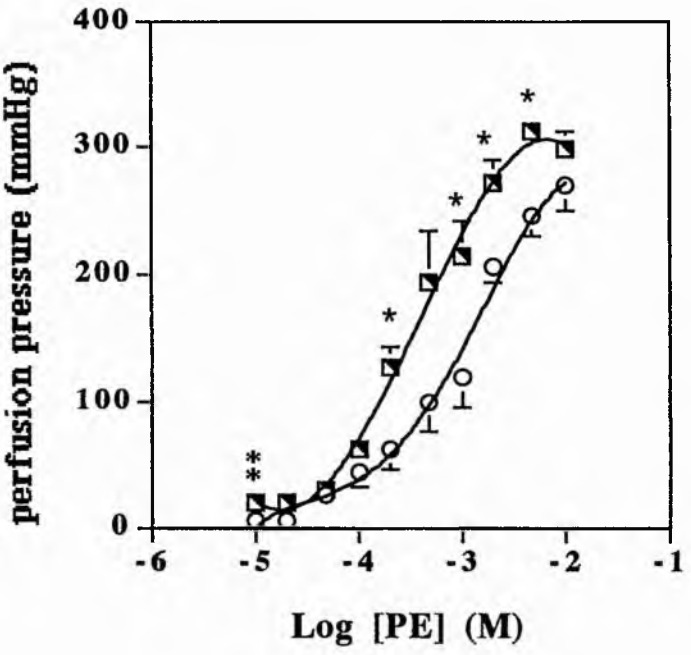


Fig. 7.10 *A graph comparing the D.R. curves to PE for TEAs (n=5) in the presence of Krebs only (open circles) and Krebs + aminoguanidine (1mM; 1hr, semi-closed squares).*

7.4.4 Immunohistochemistry of CEAs and TEAs. The above results suggest that the hyporesponsivness of TEAs to PE is attributable to iNOS-derived NO. Immunohistochemically staining with an antiserum specific to iNOS (see Material and Methods in chapter 3) revealed intense staining in the vascular smooth muscle layer of TEAs and little or no staining in CEAs (see figs. 7.11 and 7.12 respectively). It can also be seen in Fig. 7.11 that endothelial cells or cells attaching to the endothelial layer are also positive for iNOS.

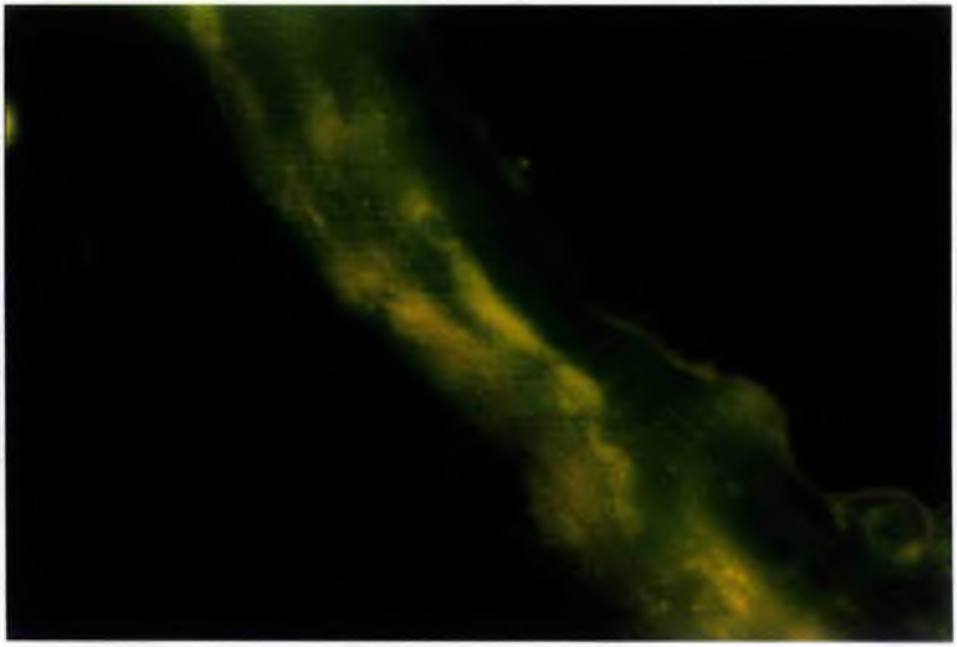


Fig. 7.11 *Cryostat section through a TEA immunostained with antiserum to iNOS (x1000).*

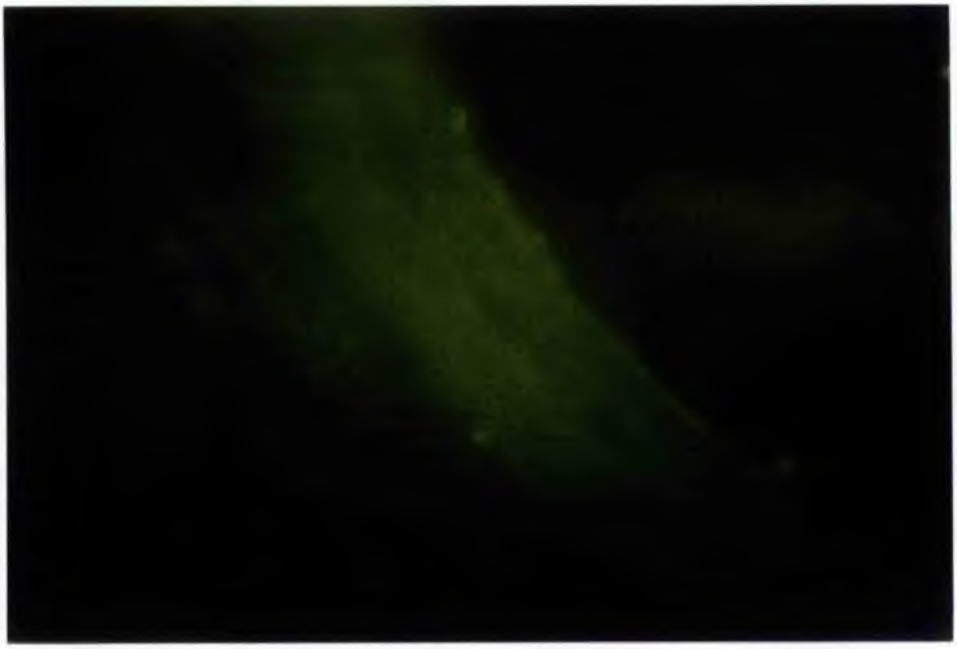


Fig 7.12 *Cryostat section of a CEA immunostained with antiserum to iNOS (x1000).*

7.4.5 The Effect of cyclooxygenase Inhibition on PE-Induced Vasocontractile Responses: Effects of Indomethacin. NO activates the vasomodulating family of cyclooxygenases (COX) and LPS has been shown to induce a novel isoform of COX *in vivo* and *in vitro* (iCOX; Wu *et al.*, 1993; Salvemini *et al.*, 1993). This experiment aimed to establish whether or not COXs were involved in regulating the responses to PE of TEAs and CEAs.

Indomethacin is a selective inhibitor of COX and has been shown to increase metastasis of certain cancers (Honn *et al.*, 1983) and suppress growth in prostaglandin-producing solid tumours (Milas *et al.*, 1991). It was of interest therefore, to see what role COXs may have in regulating tumour blood flow through arterial-supply vessels.

After an initial sequence of PE injections in the presence of Krebs only, indomethacin was dissolved and added into the internal perfusate to give a final concentration of 10μM. 30mins later a second series of PE injections was performed. Indomethacin significantly supressed the responses to PE of both CEAs and TEAs (2-way ANOVA, $p < 0.001$ for both groups) and although this effect appeared to be greater in TEAs as compared to CEAs, (with a reduction in contractile pressure at the highest [PE] of 64.17 ±5.23% for TEAs and 45.30 ±8.11% for CEAs), it was not statistically significant according to 2 way ANOVA analysis ($F= 1.69$, $df= 9, 90_e$, $p= 0.103$).

The diminished responsiveness in the presence of of indomethacin suggests that a COX-derived vasoconstrictor is being inhibited. Therefore, indomethacin may potentiate the TEA-associated hyporesponsiveness to PE.

7.4.6 Spontaneous Pressure Oscillations in Isolated TEAs

Spontaneous rythmic oscillations in perfusion pressure were seen in approx. 20% of all the TEAs isolated from solid, inguinal tumours. Kennovin *et al.*, (1994) reported similar oscillations in ~60% of TEAs. Oscillations were more commonly seen in TEAs isolated from large (>8g) tumours, however they were never

observed in CEAs. This unusual characteristic of tumour vasculature is thought to reflect the irregular, intermittent blood flow through these vessels, although the exact nature of the factors or mechanisms involved remains ill-defined (see p74 of Introduction for more details).

Oscillations were often observed in TEAs perfused with Krebs solution only. Their amplitude and frequency varied considerably. They were initially small and infrequent (9-18mmHg; every 20-40mins), but increased in both size (up to 60mmHg) and frequency (every 8-10mins) with longer term (>120mins) perfusions of Krebs (see fig. 7.13). They were also enhanced (amplitude and frequency) after vessels were injected with PE.

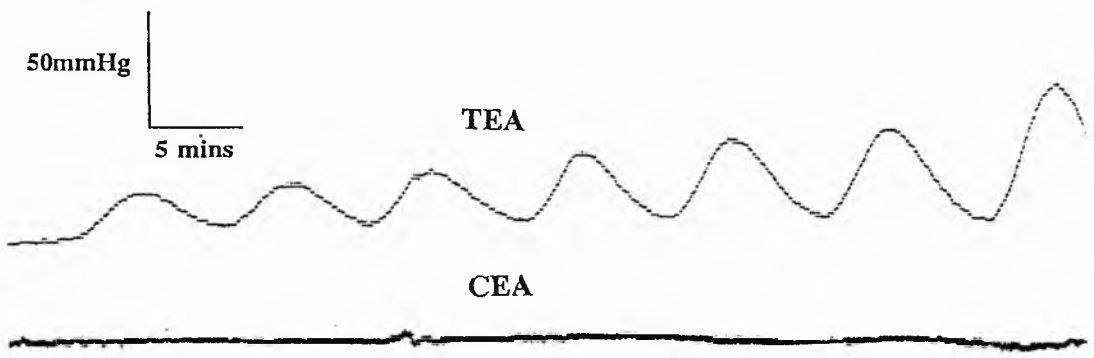


Fig. 7.13 *A trace showing spontaneous pulsatile pressures (18-60mmHg; every 8mins) in a TEA being perfused with Krebs (t > 200mins). The bottom trace shows the baseline pressure in the respective CEA also being perfused with Krebs. The BD9 rat was 397g in body weight with an inguinal solid tumour of 11.65g).*

Perfusion with AG (1mM) also potentiated their amplitude and frequency (40-136mmHg; every 15 mins; see figs. 7.14 & 7.15). The time of onset of this effect was rapid ~10mins after the initial addition of AG. In order to see if the effects of AG could be reversed or influenced by excess L-arginine (1mM), a TEA preparation which had been treated with AG were then perfused through with Krebs + L-arginine. L-arginine dramatically altered both the frequency and amplitude, such that they now resembled oscillations prior to AG treatment (see fig. 7.15). If L-arginine was then removed from the internal perfusate the oscillations again increased in size (see fig. 7.16).

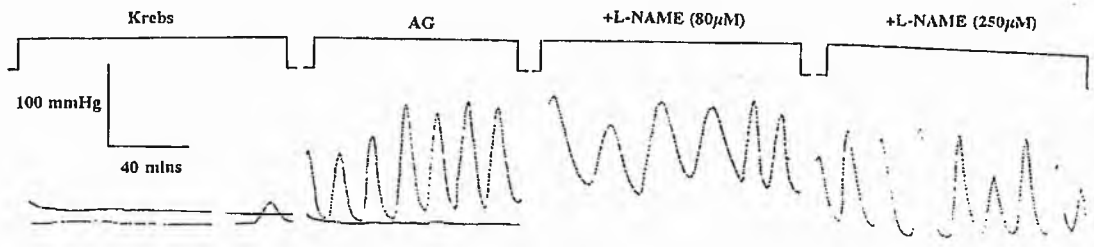


Fig. 7.14 *A trace showing the effects of aminoguanidine (AG; 1mM) and AG + L-NAME (80μM & 250μM) on the spontaneous pulsatile pressures in a TEA. AG potentiates the frequency and amplitude of the pulses. Oscillations in pressure in the presence of AG were not inhibited by L-NAME.*

Fig. 7.14 also shows that the addition of L-NAME to the Krebs + AG solution for an extended period of time (~2hrs) had no clear cut effect on the rythmic oscillations in TEAs.

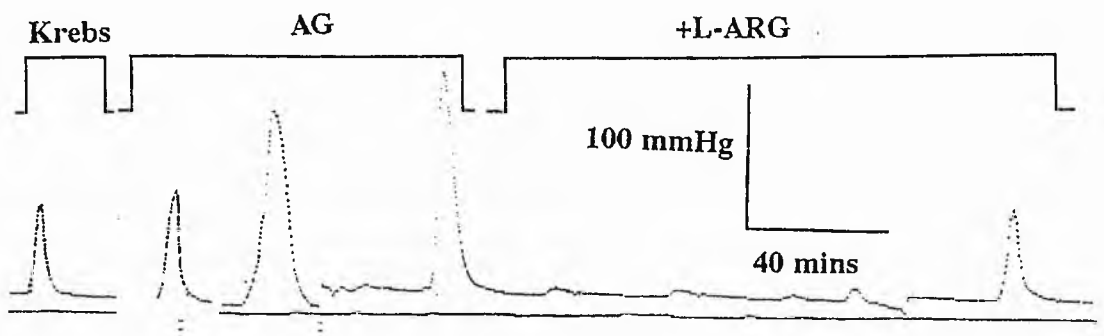


Fig. 7.15 *A trace showing the potentiating effect of AG (1mM) on rythmic oscillations in a TEA preparation and the subsequent reversal of this by excess L-arginine (1mM).*

L-arginine reduced the size of the oscillations from approx. 40mmHg (in the presence of AG) to approx. 10mmHg. The removal of L-arginine from the internal perfusate augmented the contractions back to approx. 30mmHg (see fig. 7.16).

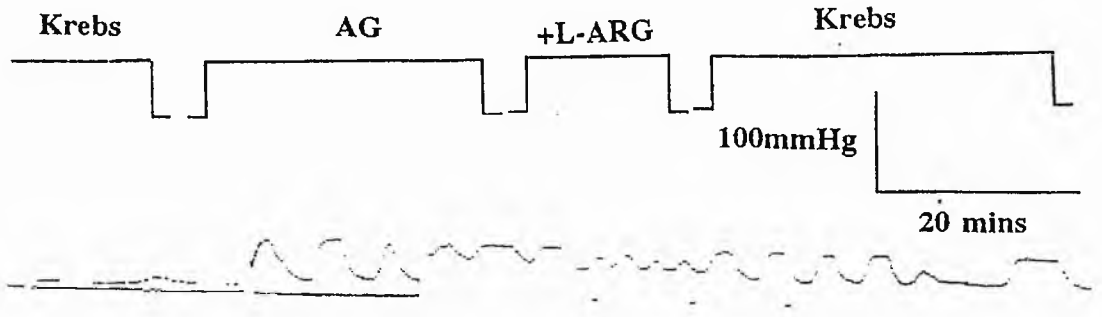


Fig. 7.16 *A trace of spontaneous oscillations in pressure in a TEA initially perfused with Krebs only, then AG (1mM) and AG + L-arginine (+L-arginine 1mM). On washout; when the excess L-arginine is removed from the internal perfusate, the inhibitory effect of L-arginine is apparently lost and oscillations again increase in size.*

7.5 Quantitative analysis concerning hyporesponsiveness of TEAs to PE and the effects of NOS inhibitors.

7.5.1 TEA-associated Hyporesponsiveness to PE. The hyporesponsiveness of TEAs was quantified according to the protocols outlined in chapter 3, section 3.6 and chapter 4, section 4.7 for the effects of a drug on PE-induced responses. Fig. 7.17 shows factor (*f*) plotted against [PE] and reveals a negative slope (*m*) = -0.048. It will be recalled that a *positive* slope tending towards a maximum value of *f*= 1.0 indicates that hyporesponsiveness of TEAs could in theory be overcome at higher [PE]s.

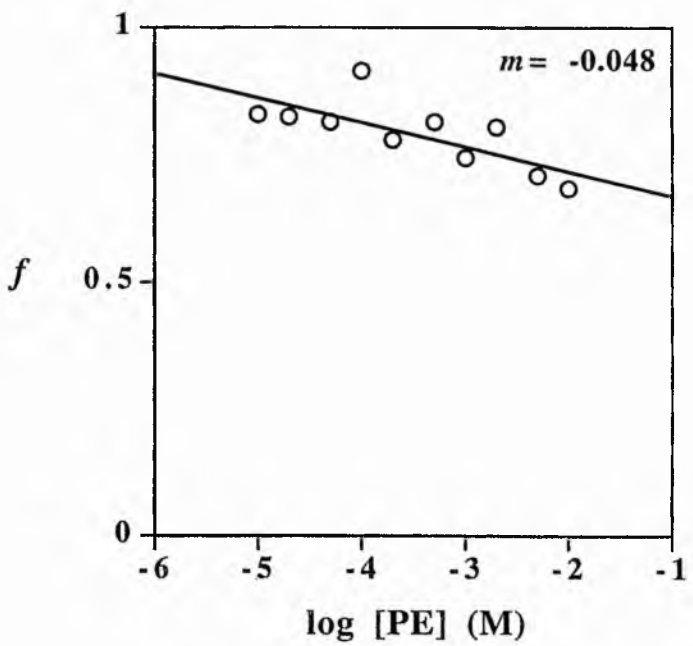


Fig. 7.17 *A graph showing TEA responses plotted as a fraction (f) of CEA responses, with the resultant linear curve fit revealing a negative gradient.*

It is clear from Fig. 7.17 that this not likely to be the case for TEAs and moreover suggests an underlying difference in the hyporesponsive nature of TEAs and RTAs.

7.5.2 Quantitative analysis (ED_{150}) of the effects of L-NMMA and L-NAME on PE-induced contractile responses are shown in tables 7.1 & 7.2 below. See Chapter 4, section 4.3.5 for details of quantiative analysis.

CURVE (A-C)	Krebs	L-NMMA (80μM)	L-Arg (1mM)	ΔED_{150} L-NMMA	ΔED_{150} L-ARG	% ΔED_{150}	% reversal
Group	A ($x10^{-4}M$)	B ($x10^{-4}M$)	C($x10^{-4}M$)	a(A-B)	b(B-C)	%(A-B)	%(b÷a)
CEAs	7.07	2.39	6.92	4.68	-4.53	20.18	96.79
TEAs	24.50	1.32	3.89	23.18	-2.57	100	11.08

***Table 7.1** Showing the ED_{150} values for TEAs and CEAs treated with L-NMMA*

CURVE (A-C)	Krebs	L-NAME (80μM)	L-Arg (1mM)	ΔED^{150} L-NAME	ΔED^{150} L-ARG	% ΔED_{150}	% reversal
Group	A ($x10^{-4}M$)	B ($x10^{-4}M$)	C($x10^{-4}M$)	a(A-B)	b(B-C)	%(A-B)	%(b÷a)
CEAs	6.67	1.26	6.43	5.50	-5.17	23.73	94.00
TEAs	14.16	1.28	10.23	12.88	-8.95	55.56	69.48

***Table 7.2** Showing the ED_{150} values for TEAs and CEAs treated with L-NAME*

Using a concentration that exceeds that required to inhibit cNOS and iNOS activity (Rees *et al.*, 1990) L-NMMA shifted the TEA D.R. curve ~5 times further to the left than the corresponding curve for CEAs (%ΔED_{150} = 100 and ~20% respectively; see table 7.1). The %ΔP by L-NMMA was similarly greater for TEAs (%ΔP in TEAs= ~57% & ~14% in CEAs; results not shown). A similar dose of L-NAME (ie. 80μM) caused a comparable leftward shift in the CEA D.R. curve to that caused by L-NMMA (%ΔED_{150}= ~24%). However, the %ΔED_{150} in L-NAME-treated TEAs, although more than twice that for CEAs (%ΔED_{150} = 55.56%), was nearly half that caused by L-NMMA (~44% less). If we assume that the ΔED_{150} caused by L-NMMA in TEAs represents the inhibition of all the NO being produced within these vessels (derived from iNOS and eNOS), and the %ΔED_{150} caused by L-NMMA and L-NAME in CEAs (average = ~22%) represents basal NO production (derived from eNOS) it can be concluded that nearly 80% (100% - 22%) of the total ΔED_{150} is due to the production of NO from iNOS (see tables 7.1 & 7.2).

The ΔED_{150} by L-NMMA was reversed by ~11% in TEAs and almost fully reversed in CEAs (~97%; see table 7.1). The % reversal by L-arginine of the leftward shift caused by L-NAME of the TEA D.R. curve was considerably greater than that after L-NMMA treatment (~70%). Similarly, L-arginine almost fully reversed the effects of L-NAME (% reversal= 94%). ΔP tables are not shown as most vessels were not viable at $5x10^{-3}M$ [PE] after treatment with an inhibitor.

This data shows that the potentiating effect of L-NMMA on the TEA D.R. curve (ie. $\%\Delta ED_{150}$) was considerably greater than that by L-NAME, whereas the effect of L-NMMA on the CEA D.R. curve was similar to that by L-NAME, with only a marginally greater $\%\Delta ED_{150}$ caused by L-NAME. Similarly in CEAs, the % reversal by L-arginine (in terms of ED_{150}) was nearing 100% after treatment with L-NMMA or L-NAME. In contrast, the % reversal by L-arginine in TEAs was almost a 60% ($\%\Delta ED_{150s}$ of 69-11%) greater after treatment with L-NAME. The results in tables 7.1 & 7.2 are shown graphically in fig. 7.18.

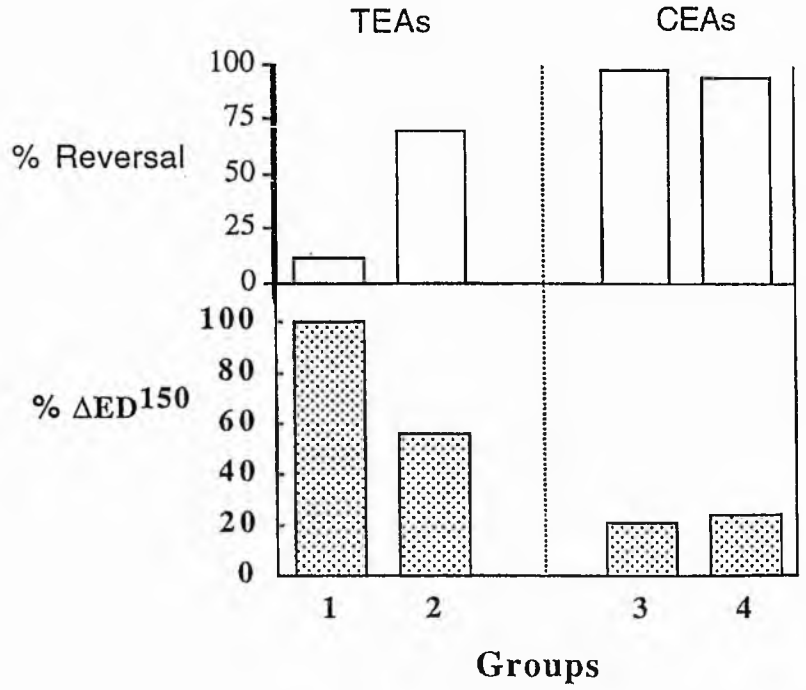


Fig. 7.18 *Showing the $\%\Delta ED_{150}$ produced by L-NMMA and L-NAME (stippled columns) and the % reversal by L-arginine (open columns) in TEAs (columns 1 & 2 respectively) and CEAs (columns 3 & 4 respectively).*

The results of this analysis indicate that L-NMMA has a greater effect on the ED_{150} TEA responses as compared to those of CEAs. Furthermore, with the ability of L-arginine to reverse the effects of L-NMMA in CEAs but, only partially reverse the effects in TEAs, there is perhaps therapeutic potential for using L-NMMA in combination with L-arginine to facilitate prolonged vasoconstriction in tumour supply vessels without unduly compromising the function of normal host vessels.

7.5.3 The effects of Aminoguandine. AG shifted the TEA D.R. curve to PE significantly to the left, ($\%\Delta ED_{150}$ = ~35%; 2 way ANOVA; F= 2.20, df= $9,66_e$, p= 0.033; see appendix table 7.3 for Student's t-test analysis & table 7.3 below) although, the maximal contraction at 10mM [PE] was only increased from 268 ±18.7mmHg to 298.2 ±14.1mmHg. On the contrary, CEAs responses to PE were not significantly enhanced by AG ($\%\Delta ED_{150}$ = 7.67%; see fig. 7.19). AG therefore offers some measure of selectivity for reversing the TEA-associated hyporesponsiveness to PE and provides further evidence that iNOS-derived NO production is responsible for this anomaly.

CURVE (A-B)	**Krebs ED^{150}**	**AG ED^{150}**	**Krebs Pmax**	**AG Pmax**	**ΔPmax**	**ΔED_{150}**	**% ΔED^{150}**
Group	A ($x10^{-4}M$)	B ($x10^{-4}M$)	A(mmHg)	B(mmHg)	(mmHg)	[PE]$x10^{-4}M$	%
CEA	5.62	3.84	301.00	310.00	9	1.78	7.67
TEA	11.2	3.23	245.00	312.00	67	7.99	34.47

Table 7.3 *Showing the Δpp and ΔED_{150} values for TEAs and CEAs treated with AG.*

For a comparison of the $\%\Delta ED_{150}$ caused by L-NMMA, L-NAME or AG, the results have been plotted together and are shown in Fig.7.19.

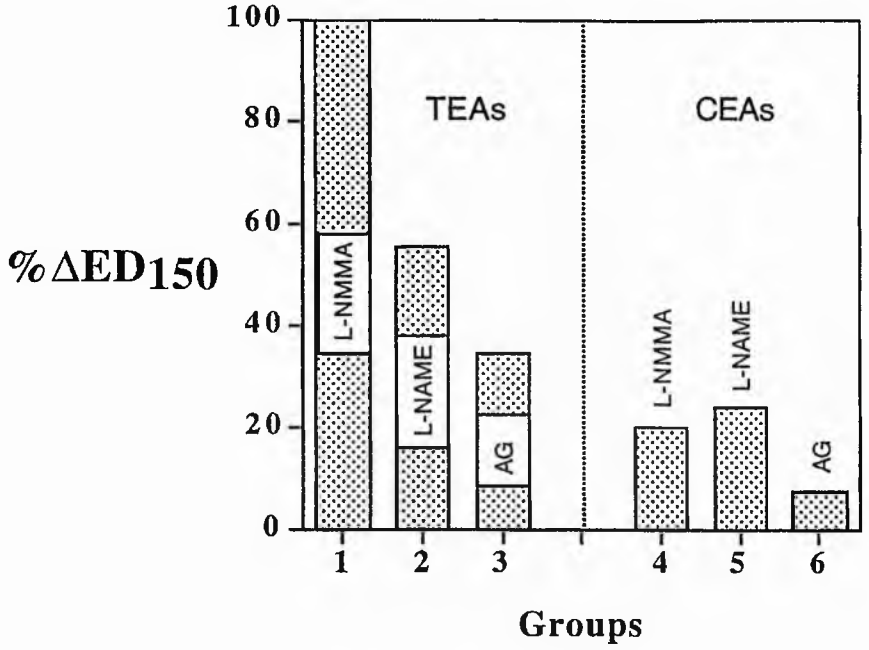


Fig. 7.19 *Compares the %ΔED_{150} in TEA and CEA D.R. curves caused by L-NMMA (columns 1 & 4 respectively), L-NAME (columns 2 & 5 respectively) or AG (columns 3 & 6 respectively).*

It is also clear from Fig. 7.19 that AG is not as effective as L-NMMA or L-NAME at shifting the TEA curve to the left with ~65% greater %ΔED_{150} after L-NMMA compared to that by AG. The rank order of potency for the effect of NOS inhibitors on ED_{150} is therefore L-NMMA > L-NAME > AG for TEA responses to PE and L-NAME > L-NMMA > AG for CEA responses to PE.

7.5 Discussion

7.5.1 TEA-associated Hyporesponsiveness These results establish a difference in PE-induced responses for TEAs and CEAs and provide evidence that iNOS-derived NO production in TEAs may be partly responsible for this. However, the effects of indomethacin indicate that COX-derived vasoconstrictors are also active in TEAs and CEAs. A similar suppressor effect by indomethacin has been reported in aortic rings isolated from LPS-treated animals (Wu *et al.*, 1994). Quantitative analysis of TEA-associated hyporesponsiveness reveals an inverse relation between f and [PE], indicating that the extent of hyporesponsiveness is greater at higher [PE]s and is unlikely to be overcome at [PE]s greater than 10mM. This contrasts with the RTA-associated hyporesponsiveness, point which is further discussed in chapter 9.

7.5.2 The effects of NOS inhibitors and cycloheximide on PE-induced responses. Competitive inhibitors of the constitutive and inducible NOS isoforms, L-NAME and L-NMMA, potentiated responses to PE in CEAs and TEAs and completely abolished the TEA-associated hyporesponsiveness to PE. Quantitative analysis indicates that L-NMMA had ~2 fold greater effect on the ED_{150} for TEA responses as compared to L-NAME, while the effects of L-NMMA and L-NAME were similar for CEAs, with only a marginally greater ΔED_{150} with L-NAME. The ΔED_{150} caused by L-NMMA and L-NAME was ~5 fold and ~2 fold greater in TEAs than CEAs respectively, indicating enhanced NOS-derived NO production in TEAs. Moreover, this also shows that L-NMMA is more potent at inhibiting iNOS-derived NO production than L-NAME. This agrees with previous findings (Moncada *et al.*, 1991).

L-arginine reversed the effects of both inhibitors in CEAs and the effect of L-NAME on TEA responses. However, the effects of L-NMMA in TEAs were not fully reversed by L-arginine and the 1st D.R. curve differed significantly from the 3rd D.R. curve after 30-45 mins. perfusion. The inability of L-arginine to fully

reverse the effects of L-NMMA in TEAs may prove to be of considerable therapeutic value. The ability to reduce blood flow into solid tumours without compromising host vessel function is clearly desirable. If arterial-supply vessels *in vivo* show a similar response to L-NMMA and L-arginine treatment it may be possible to reduce blood flowing into a solid tumour using L-NMMA (Andrade *et al.*, 1992) and then selectively reverse the NOS inhibition in non-tumour associated host vessels and therefore reduce the risk of detrimental side effects eg. hypertension.

A time-dependent decrease in CEA responses has been reported (Kennovin *et al.*, 1994), such that after 400mins perfusion with Krebs there was no longer any significant difference between CEA and TEA responses. The time-dependent desensitisation of CEA was reversed by L-NMMA (40μM) and prevented by cycloheximide (10μM), implicating the expression of iNOS in this effect. They also showed that the TEA-associated hyporesponsiveness could be reversed after ~2hrs incubation with cycloheximide but were unable to clarify the involvement of iNOS. Experiments were conducted to resolve whether iNOS is expressed in TEAs and if so, whether it is responsible for their diminished senitivity to PE. The selective iNOS inhibitor, AG was found to significantly augment TEA responses to PE with the result that they came to resemble those of CEAs. This was not due to the inhibition of eNOS-derived NO as AG did *not* alter CEA responses to PE. Furthermore, immunohistochemical staining with antiserum to iNOS revealed positive staining in TEAs but not in CEAs. These results confirm that TEAs express iNOS and CEAs do not. Moreover, the effects of AG indicate that iNOS-derived NO production is responsible for hyporesponsiveness to PE exhibited by TEAs.

7.5.3 Spontaneous Vasomotion Spontaneous rythmic oscillations in perfusion pressure were seen in a number of TEAs (~20%) isolated from large tumours, but never in CEAs. Kennovin *et al.*, (1991), reported an even larger proportion, as much as 60%. This characteristic of tumour associated vasculature

is thought to reflect spontaneous vasoconstriction within these vessels and may be responsible for the intermittent blood flow through them (Peters *et al.*, 1980; Intaglietta *et al.*, 1977). It is probable that this phenomenon is exacerbated in larger tumours, due to increased interstitial pressures elevating resistance to flow through the tumour vasculature. Oscillations were potentiated following exposure to phenylephrine, which suggests that irregular Ca^{2+} flux is involved, although nicotinamide which has been shown to reduce the amplitude and frequency of these oscillations (Hirst *et al.*, 1995) has not been reported to affect $[Ca^{2+}]_i$ levels and may act via an alternative, as yet unidentified mechanism. This study addresses the possibility that NO is involved: a transient down-regulation of the high output NO production in TEAs may result in vasoconstriction.

Perfusion with the selective iNOS inhibitor AG potentiated spontaneous contractions to more than twice their original amplitude and frequency, an effect which was not further enhanced when L-NAME was added in combination with AG. This suggests that L-arginine-derived NO release acts to supress spontaneous vasomotion, with by far the greatest influence attributed to iNOS-derived NO production. AG is an analogue-inhibitor and as such is reversible in the presence of excess L-arginine (Griffiths *et al.*, 1993). Consistent with this, the addition of L-arginine dramatically reduced both the amplitude and frequency of contractions, which then resembled those prior to treatment with AG. Furthermore, contractions increased in size when L-arginine was subsequently removed from the internal perfusate. This evidence establishes NO as a mediator of spontaneous pulsatile vasoactivity in TEAs and points to a mechanism involving L-arginine substrate.

It is possible that with increased NO production by iNOS, L-arginine eventually becomes limiting. Thereafter, NO production is regulated by L-arginine and may fluctuate depending on its availability, increasing momentarily as more L-arginine becomes available, and subsequently decreasing as it is used up. This might explain the pulsatile nature of these contractions. Moreover, the inhibitory effect of nicotinamide on spontaneous vasoactivity may also reflect increased NO

production, as nicotinamide is required for the production of NADPH which in turn is necessary for the formation of NO from L-arginine and molecular oxygen (Sagar *et al.*, 1995). However, nicotinamide may also cause vasodilatation independently of NO, since its actions are not blocked by the NOS inhibitor, L-NAME (Hirst *et al.*, 1994).

Spontaneous vasomotion is not seen in LPS-treated RTAs which implies that it probably relates to the presence of the tumour *per se* and not merely to iNOS activity and associated NO production within the vasculature.

7.5.4 Conclusion The main findings of this chapter are that TEAs express high levels of iNOS and are significantly hyporesponsive to bolus injections of PE. The hyporesponsiveness is abolished by specific NOS inhibitors and quantitative analysis reveals a rank order of potency as L-NMMA > L-NAME > AG. However, if these inhibitors are to be used to treat clinical disease they must be not compromise the constitutive NO production (ie. eNOS in blood vessels) which is essential for normal physiological function. Analysis of the ΔED_{150} reveals a rank order of potency in CEAs as L-NAME > L-NMMA > AG suggesting that L-NAME inhibits eNOS the most, while AG inhibits it the least. This confirms AG as a relatively selective inhibitor for iNOS and therefore of greater clinical value than non-selective inhibitors. However, it was also shown that the effects of L-NMMA in CEAs were reversed completely in the presence of excess L-arginine, while its effects in TEAs were more resistant to reversal. This suggests that L-NMMA may be of clinical use for treating NO-mediated disease when used in combination with L-arginine.

The potentiating effect of AG on spontaneous oscillations in TEAs indicates that iNOS-derived NO production normally suppresses this phenomenon and that AG may consequently reduce blood flow into a solid tumour *in vivo* by enhancing spontaneous vasomotion of supplying vessels. The effect of AG on the growth of solid tumours is addressed in chapter 8.

Chapter 8

NO and Solid Tumour Growth: effect of LPS and NOS inhibitors

8.1 Introduction

Kennovin *et al.*, (1993) showed that the relatively non-selective inhibitors of NOS, L-NMMA and L-NAME, enhance the sensitivity to a vasoconstrictor agent of arteries which previously supplied a solid tumour. This is confirmed in the present study (Chapter 7). Another vasoconstrictor, serotonin, has also been shown to have a greater effect on arteries supplying subcutaneously grown tumours in mice as compared to non-tumour associated arteries (Stücker *et al.*, 1991). Furthermore, Stücker et al., (1991) found that serotonin significantly suppressed the rate of growth of these tumours.

These findings raise the possibility that NOS inhibitors could also delay tumour growth *in vivo*. This was confirmed by Kennovin *et al.*, (1994), who first showed that chronic administration of L-NAME significantly retarded growth of solid tumours in BD-9 rats. To date, the effects of more isoform-selective NOS inhibitors, such as aminoguanidine (AG), have not been studied and this and related questions are addressed in the present chapter.

8.1.1 Enhanced NO production and tumour growth: effects of bacterial lipopolysaccharide. It has been known for more than a hundred years that tumour growth is delayed by bacterial infection (Coley, 1893). The mechanism for this effect is not understood, but presumably involves the immune response. More recent experiments have used purified components of the bacterial cell wall (lipopolysaccharide; LPS) to mimick the effects of bacterial infection. This chapter also deals with the effects on tumour growth of LPS treatment alone and in combination with AG or L-NAME. LPS is known to induce NOS activity, resulting in potentially harmful levels of NO within the vascular system (Stoclet *et al.*, 1993, Hom *et al.*, 1995). The role of NO in relation to the growth of solid

tumours remains unclear. Reports that it helps to improve tumour blood supply, and so promote growth, is contested by others showing that it is involved in killing tumour cells and would therefore act to inhibit tumour growth (see Part C of Introduction). By stimulating high levels of NO production with LPS administered on its own, or in combination with the NOS inhibitors, L-NAME or AG, the following experiments were designed to see whether LPS promotes or inhibits solid tumour growth and if so to what extent NO is involved in this effect. Having characterised the time-course of iNOS expression and enhanced NO production associated with LPS exposure in the rat (see Chapter 3), it was hoped to correlate the LPS-induced NO production with any effects on tumour growth.

8.1.2 Effects of NOS inhibitors on tumour growth. L-NAME and AG, were also administered on their own to determine the role of NO in tumour bearing animals which had not been exposed to LPS. L-NAME is a non-selective inhibitor of NOS and as such inhibits both cNOS and iNOS (Rees *et al.*, 1990). Vasoconstrictor agents are generally associated with decreased blood flow in experimental tumours (Peterson 1991). L-NAME has also been shown to cause a decrease in solid tumour blood flow in the rat (Anrade *et al.*, 1992; Wood *et al.*, 1993). However, the vasoconstrictor, angiotensin II reportedly increases blood flow in solid tumours (Chan *et al.*, 1984). It is clear that the effects of vasoconstrictor agents on tumour blood flow are not consistent (Suzuki *et al.*, 1981, Jirtle, 1978) and the mechanism is poorly understood.

8.1.3 Altered haemodynamics and tumour growth. It has long been known that vasodilators which ↓b.p. can reduce blood flow through tumours (Algire *et al.*, 1951). This effect is often referred to as the 'steal effect' and describes the decrease in blood flow in concert with the fall in *local* or systemic blood pressure (Hirst *et al.*, 1991). Tumour vasculature is widely regarded as being poorly differentiated, generally lacking smooth muscle cells (Chan *et al.*, 1984; Jain, 1988) and existing in a state of near maximal dilatation. For this reason it is

usually less sensitive to vasodilators than normal vasculature (Peterson *et al.*, 1991). However, since NOS inhibitors can effectively constrict these vessels it seems likely that NO or some other endothelium-derived vasodilator (Salvemini *et al.*, 1995) is mediating this effect. This is supported by the results of the present study, showing that LPS-treated vessels which release high levels of NO are also less sensitive to the vasodilator, SNAP (see chapter 3, Section 3.5.4).

It is possible then, that an altered haemodynamic state in the host, whether severe hypotension caused by vasodilators, or hypertension caused by vasoconstrictors, is a major determinant of tumour growth. A comparison of the effects of AG and L-NAME provides us with the means for testing whether inhibition of cNOS and the subsequent systemic hypertension contributes to their antitumour. AG does not inhibit cNOS activity and therefore does not result in hypertension.

8.1.4 Blood flow and pattern of solid tumour growth. Recent literature on subcutaneous solid tumours grown in rodents support the hypothesis that they conform to a distinct pattern of growth, governed by a number of fundamental processes ultimately determining the survival of tumour cells within the tumour mass (Thomas *et al.*, 1986; Kennovin *et al.*, 1994; Lepoivre *et al.*, 1989; Jain, 1988; Buttery *et al.*, 1993). From these studies a pattern of growth is proposed which may prove to be of value when trying to interpret the effects of drugs on the tumour. Three separate stages have been defined:

stage 1, Angiogenic phase:- ~Days 1-7. An initial slow period of growth during which time there is intensive angiogenesis (Jain, 1988). iNOS activity is not expressed in the tumour vasculature at this stage (Buttery *et al.*, 1993).

stage 2, Growth phase:- ~Days 7-14. The vascular supply is firmly established and tumour growth increases exponentially. iNOS is detectable in the tumour neovasculature (Buttery *et al.*, 1993).

stage 3, Necrotic phase:- ~Days > 14. There is an increasing incidence of central necrosis as elevated interstitial pressures within the tumour compress thin walled vessels in the centre and the tumour begins to outgrow its vasculature. This stage also sees a pronounced expression of iNOS in the tumour vasculature (Buttery *et al.*, 1993) and it has been suggested that this is the best time to deliver drugs which inhibit iNOS activity (Kennovin *et al.*, 1994). This is indeed a critical stage when the tumour can no longer sustain exponential growth and growth rate plateaus due to inadequate nutritional supply. This may also prove to be the most likely period for enhanced metastases, with new cells starved of blood migrating away from the primary tumour to reach more suitable environments elsewhere.

8.1.5 Scope of the present study: This chapter addresses the following questions.

1) Is iNOS expressed in well established (23-27 day old) subcutaneously grown solid tumours ?

2) Does a single bolus injection of LPS significantly influence solid tumour growth and if so is the effect due to upregulated NO production?

3) Are the effects of LPS on solid tumour growth dependent on when LPS treatment is administered relative to the time of implantation?

4) Does AG influence solid tumour growth ?

The results will show:

First, LPS administration delays tumour growth. The effect is maximal on day 11 post-implantation ie. the greatest inhibition is seen close to the critical transition between the rapid phase of growth and the necrotic phase. Furthermore, the resulting delay in growth (4-6 days) compares favourably with the time-course of enhanced NO production initiated by LPS.

Second, chronic administration of L-NAME alone (via the drinking water) also suppresses tumour growth and its effects are potentiated when LPS is administered one day after treatment with L-NAME commences. AG on its own also delays tumour growth, but to a lesser extent than L-NAME. However, in contrast to L-NAME, the administration of LPS one day after AG, completely abolishes the growth retarding effect of the latter.

Third, iNOS expression in solid tumours is confirmed by immunohistochemical and Western blot analysis.

Finally, LPS treatment enhances the expression of iNOS in vessels and activates macrophage-like cells resident within the tumour. Furthermore, LPS also increases the incidence of lung metastases in tumour bearing rats.

8.2 Materials & Methods

8.2.1 Methods

Isogeneic male BD9 rats were anaesthetised and shaved along their backs. Tumour slurry (from third generation tumour tissue; T_3) was injected (0.05mls) subdermally at four specific sites on the back of each animal (see fig. 8.1 below).

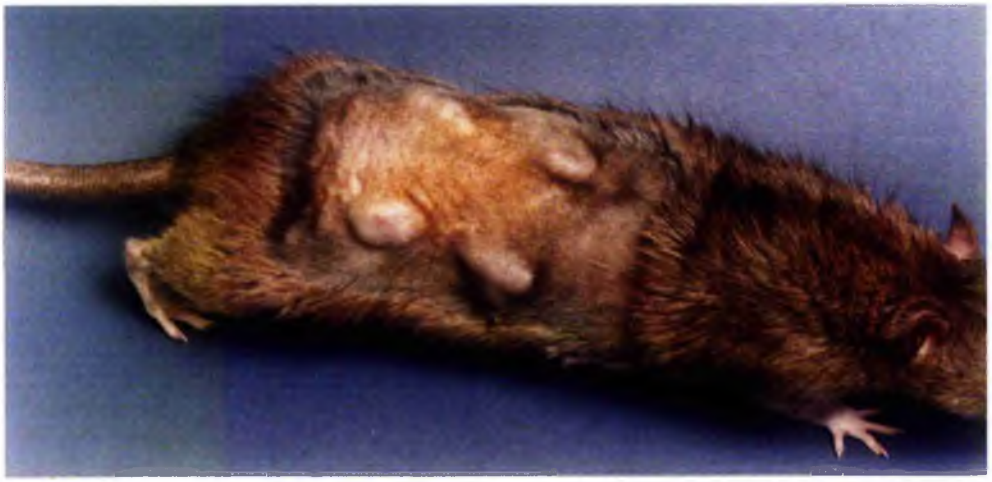


Fig. 8.1 Photograph showing a rat with four tumours on its back.

The surgical protocol for tumour implantation was outlined earlier in Chapter 7. Tumours were measured throughout the course of their growth (3-4 weeks) using hand-held, skin-fold calipers. The long (Y) and short (X) axis measurements were used to calculate tumour volume using the hemiellipsoid formula (Heitjan, *et al.*, 1993; see below):

$$\textbf{Tumour volume} = \prod (Y \times X) \div 6.$$

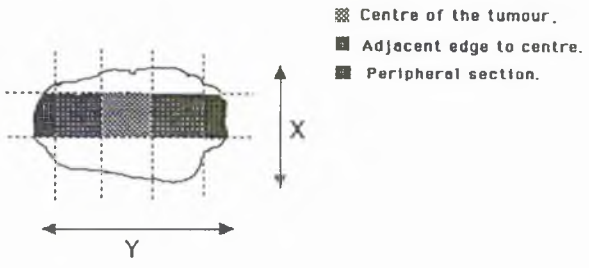


Fig. 8.2 A diagram showing the different areas within a tumour.

During the study, animals were divided into 12 groups, each fed *ad libitum*, with the mean daily ingested dose (mg/Kg/day) of a particular drug defined as:

$$\textbf{Dose}\ (\text{mg/Kg/day}) = \frac{(\text{water drunk (ml)/day}) \times [\text{drug}]:(1\text{mg/ml})}{\text{weight of rat (Kg)}}$$

Four separate experiments were conducted to determine the effect of L-NAME and AG on tumour growth, either administered singly or in combination with LPS treatment. A similar protocol was adopted for each experiment. AG or L-NAME was added to the drinking water at a concentration of 1mg/ml, along with 10mg/ml of sucrose to disguise the taste of the drug. According to a previous study, 1mg/ml of AG or L-NAME provides an ingested dose of ~100mg/Kg/day (Kennovin *et al.* 1994). Animals not treated with a drug also received 10mg/ml sucrose in their drinking water. LPS was administered via single bolus injection (4mg/Kg; i.p.) with control rats receiving an equivalent volume (v/v) of 0.9% sterile saline (i.p.).

1) **Protocol for LPS or LPS + AG treatment.** In two separate experiments (1& 2) rats were grouped and LPS administered at 24hrs prior to tumour implantation (exp. 2, grp. 2a; n=4 rats), 5 days post-implantation (exp. 1, grp. 2b; n= 3 rats) and 11 days post-implantation (exp. 1, grp. 2c; n= 4 rats). Control animals for each experiment (grp 1a, exp.1; n= 3 rats & grp. 1b, exp. 2; n= 4 rats) received equivalent saline (0.9%) injections 5 days post- and 24 hrs prior to implantation respectively. AG was also continuously fed from day 10 to rats which had not been injected with LPS (grp. 3a, exp. 2; n= 4) and from day 4 to rats which were subsequently injected with LPS on day 5 (exp. 3b, exp. 1; n=5).

Sucrose was added to the drinking water (all groups) on day 4.

2) **Protocol for LPS or LPS + L-NAME treatment.** In a further two experiments (3 & 4) the separate effects of LPS (grp. 4a, exp. 4; n=5 rats) and L-NAME (grp. 4b, exp.3, n= 3 rats) treatment were compared with those seen

when both were administered together (grp. 4c, exp. 4; n= 5). The blood pressures of rats used in exp. 4 were recorded every 1-2 days throughout the growth study (from days 5-21 post-implantation) using the tail cuff method, as outlined in Chapter 3, Section 3.3.1.

Grp 4a animals were injected with LPS on day 11 post-implantation while Grp 4c animals were fed L-NAME from day 10 post-implantation and subsequently injected with LPS on day 11 (sucrose was added to the drinking water on day 4). Grp. 4b received L-NAME from day 0 (sucrose was added to the drinking water on day 0). Control grps 1c & 1d were included for exps. 3 & 4 respectively, with grp. 1d receiving a saline injection on day 11.

Histochemical and immunohistochemical analysis was used to detect iNOS expression and the extent of necrosis within the tumour tissue (see Appendices 3.1 & 3.2). Sections of lung and liver from tumour bearing animals were also examined for evidence of metastases. Tumours were cut into blocks in preparation for sectioning, each block representing a different area in the tumour, as shown in Fig. 8.2.

Mean tumour volumes (mm^3) were used to construct graphs of tumour growth against time (days post-implantantion). Student's paired or unpaired t-tests determined the statistical differences between data on a given day post-implantation.

For clarity, the experiments performed in this chapter are shown schematically in Fig. 8A over the page. Steps in a line indicates a single injection of LPS (4mg.Kg; i.p.; solid lines) or when the NOS inhibitors were added to the drinking water (1mg/ml; broken lines). The lines are drawn to correlate with the scale bar labelled 'Real days'. N.B. Although Grps 2a, 2b and 2c are drawn on the same line, rat received a single injection of LPS on either day -1, or day 5, or day 11.

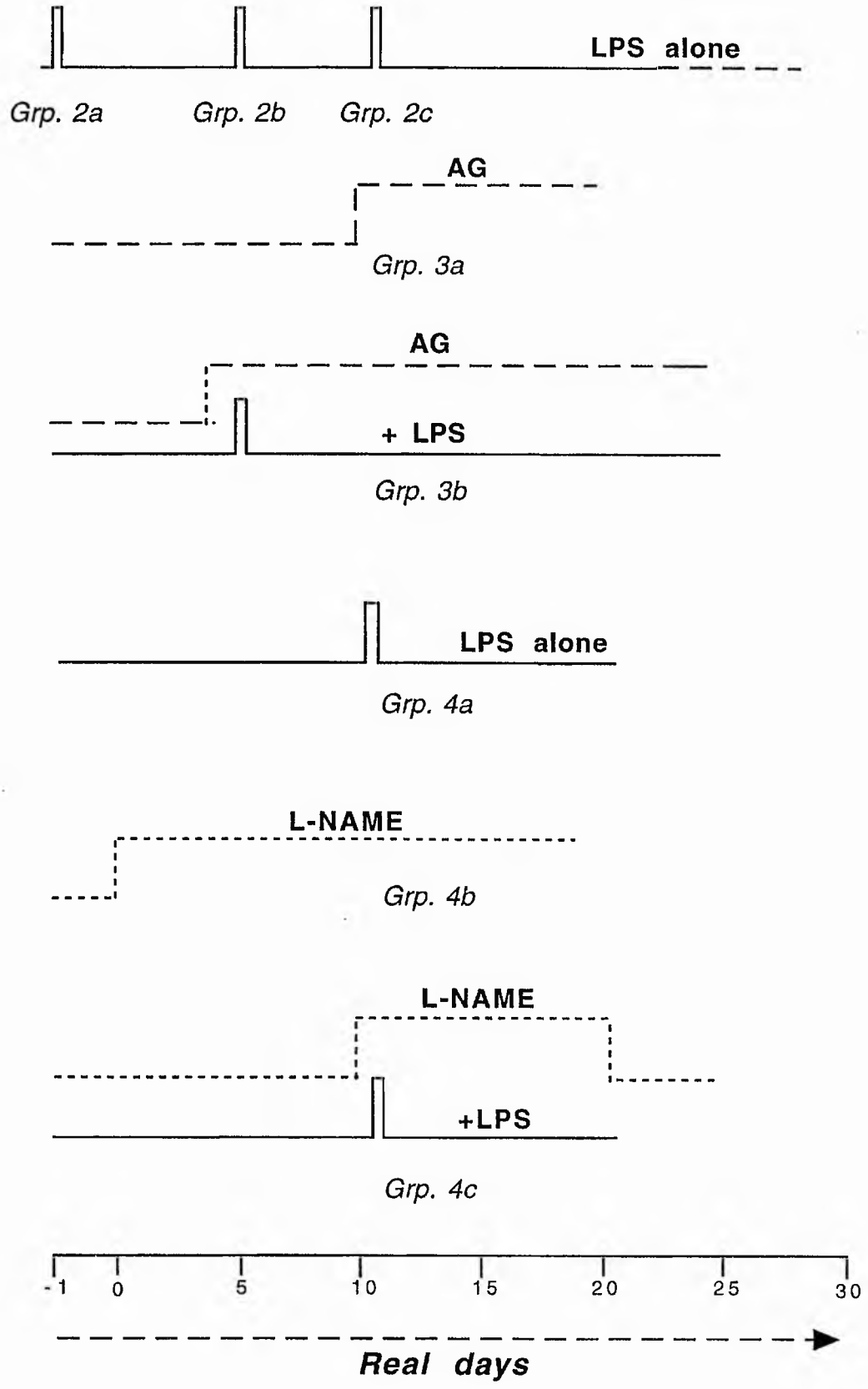

LPS alone
Grp. 2a
Grp. 2b
Grp. 2c
AG
Grp. 3a
AG
+ LPS
Grp. 3b
LPS alone
Grp. 4a
L-NAME
Grp. 4b
L-NAME
+LPS
Grp. 4c
-1
0
5
10
15
20
25
30
Real days

***Fig. 8A** Schematic representation of the protocols and groups used in this study.*

8.2.2 Materials

Male isogeneic BD9 rats (300-450g) and P22 carcinosarcoma (T_1) were obtained from the CRC Gray Lab., Mount Vernon Hospital, Middlesex. LPS (serotype 055:B5). L-NAME and aminoguanidine (hemisulfate) were bought from Sigma Ltd. Sucrose and sodium chloride were obtained from BDH (AnalaR grade).

8.3 Results

8.3.1 Histomorphology Microscopic examination of haematoxylin & eosin (H&E)-stained tumour sections from control animals at day 27 post-implantation revealed distinct layering from the centre to the periphery of the tumours. The border between concentric layers was often dramatic, with more intense staining in peripheral layers and a distinct lack of staining in the centre. H&E staining exposed the chaotic arrangement of tumour cells within the tumour mass. Cells were often seen as streams or chords radiating in a disorderly fashion throughout the stroma matrix, as is characteristic of carcinosarcomas (see fig. 8.3).

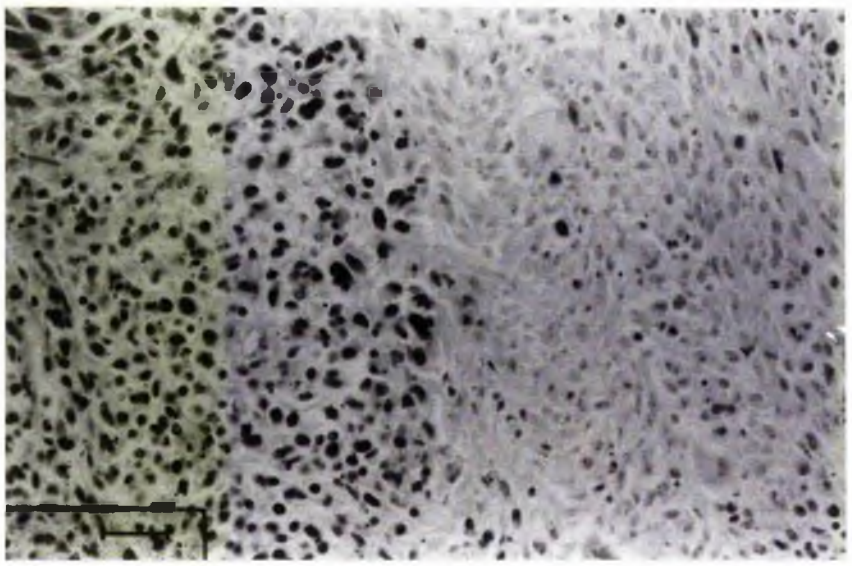


Fig. 8.3 *A frozen section (~7μm thickness) through the centre of a solid tumour stained with H&E illustrates the chaotic arrangement of tumour cells within the tumour and the distinct areas of less intensely stained cells indicating necrotic foci. (obj. mag. x40).*

8.3.2 iNOS expression in solid tumours. Immunofluorescence was used to detect iNOS in sections of tumour removed (day 27) from tumour-bearing control animals (grps. 1a - 1d). Intense staining was seen in ~75% of tumours and appeared as discretely dispersed areas within the tumour mass (see fig. 8.4).

The presence of iNOS within solid tumours was confirmed using dot blot and Western blot analysis (see figs. 8.5a & 8.5b). Staining by the ABC method revealed darkly stained macrophage-like cells in the centre of 17-day old tumours removed from animals which had been injected with LPS on day 11 (grp. 4a animals; see fig. 8.6). Similarly, capillaries at the periphery of the tumour showed intense staining for iNOS following LPS treatment (see fig. 8.7).

Fig. 8.4 *Cryostat section through the centre of a 27-day old tumour immunostained with antiserum to iNOS and labelled with TRITC. Staining was confined to discrete areas within the tumour mass. (obj. mag. x100).*

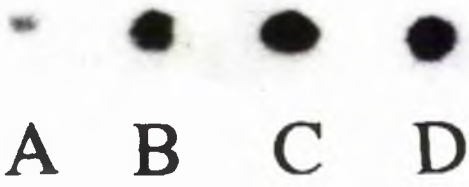


Fig. 8.5a *Dot blot of homogenised tumours (1μl; [protein = 5mg/ml]) from four individual rats (A-D) immunostained with antiserum to iNOS and detected by the ABC method.*

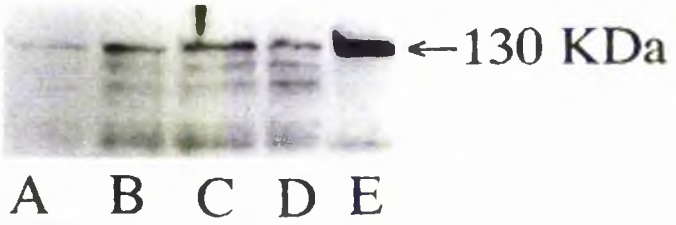


Fig. 8.5b *Western blot of tumour extracts (A-D) with lung extract from an LPS-treated rat at t= 24hrs as a positive control (E). This clearly shows that 27-day old tumours express iNOS, although levels of expression vary.*

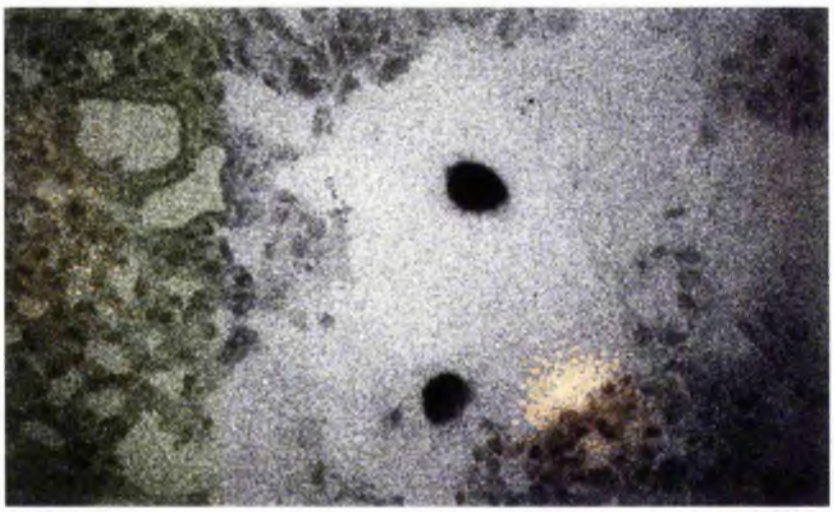
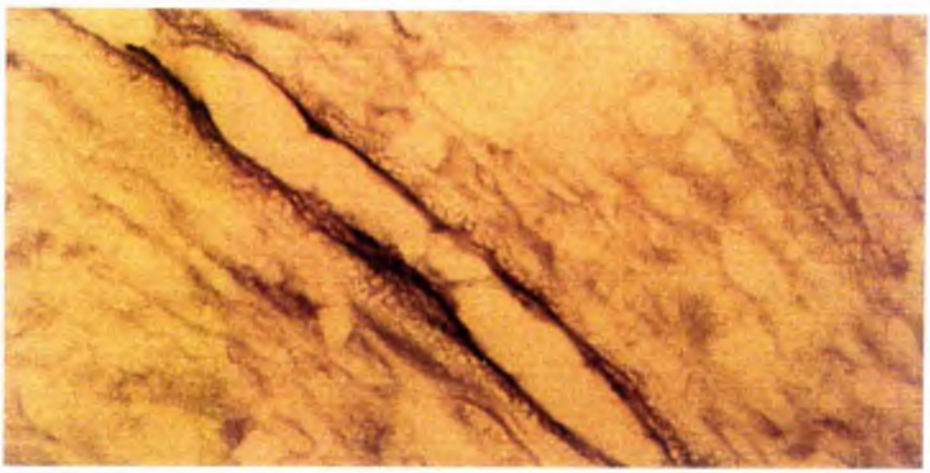


Fig. 8.6 *Cryostat section through a 17-day old tumour removed from an LPS-treated rat immunostained with antiserum to iNOS and detected using the ABC method. Large macrophage-like cells were seen within the necrotic foci and displayed intense staining for iNOS. (obj. mag. x100).*



Figs. 8.7 *Cryostat sections through 17-day old tumours removed from LPS-treated rats immunostained with antiserum to iNOS and detected using the ABC method. Staining was evident in capillaries around the periphery of the tumour removed six days after LPS treatment.*

8.3.3 Evidence of metastases. Cryostat sections of lung tissue removed on day 27 post-implantation from tumour-bearing control animals and tumour-bearing

animals which had previously been treated with LPS on days 5 and 11 post-implantation were stained with H&E. Staining revealed that ~30% of all the lung samples examined from LPS-treated animals (3 out of 10) showed considerable secondary tumour invasion. Metastases were not seen in lung tissue from tumour-bearing control animals. Fig. 8.8 shows a cryostat section of lung tissue removed from a tumour-bearing animal which had been injected with LPS on day 11 post-implantantion. Staining with H&E clearly shows the interface between the densely packed area of the invading tumour and the less densely packed area of the surrounding lung tissue.

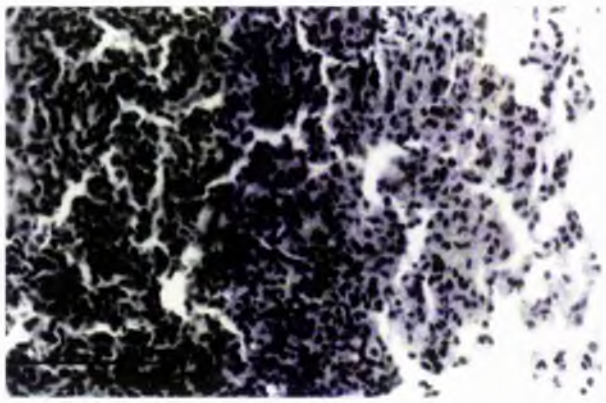


Fig. 8.8 *H&E stained cryostat section of lung tissue removed from a tumour-bearing animal 16 days after a single LPS injection. The lung tissue is seen as the paler, less densely packed area with the adjacent, darker staining tumour cells tightly packed together to form a compact solid mass. (obj. mag. x40).*

Large, densely packed balls of tumour cells within the lung tissue were also observed (see fig. 8.9). These balls of cells, often referred to as 'canon-ball tumours', were situated within the visceral matrix of the lung and could undoubtedly have the potential to form solid tumours depending on their ability to establish an adequate vascular supply.

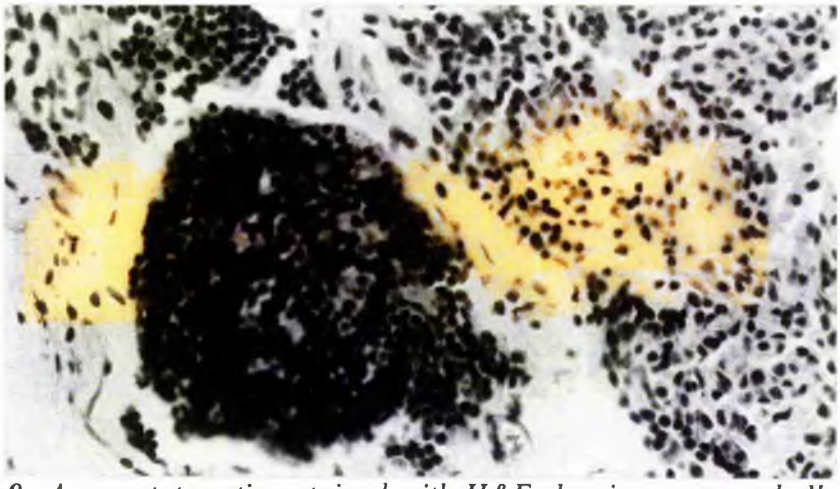


Fig. 8.9 A cryostat section stained with H&E showing a canon-ball tumour within lung tissue taken from a rat 22 days after being injected with LPS. (obj. mag. x40).

8.3.4 Time-dependent suppression of solid tumour growth by LPS. LPS suppressed the growth of solid tumours to differing degrees depending on when it was administered. Administering LPS 24hrs prior to tumour implantantion (ie. grp. 2a) had a no effect on tumour growth (see fig. 8.10). This represents one stage of the growth pattern. Delaying the LPS treatment until day 5 post-implantantion (ie. grp. 2b) resulted in a significant, albeit transient, effect on tumour growth (see fig. 8.11 & Appendix tables 8.1 - 8.2).

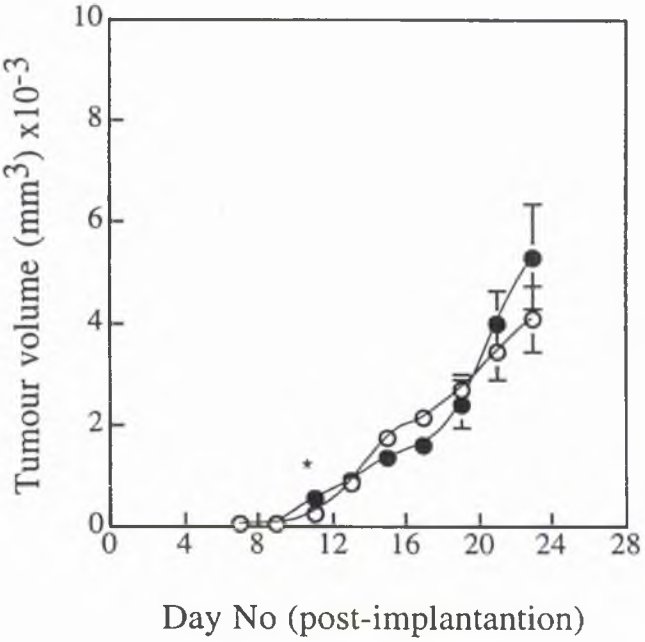


Fig. 8.10 Showing that a single LPS injection had no effect when given 24hrs prior to tumour implantation (grp. 1b; closed circles, n= 16) as compared to controls (grp. 2a; open cirlces, n= 16).

This effect on tumour growth lasted for up to 10 - 11 days after treatment (growth delay of ~4 days). The initial effect was evident after 24hrs with maximal suppression occurring 6 - 10 days post-LPS injection. According to the proposed pattern of growth, tumours more than 7 days old are in the second stage of growth, a period of prolific cell division.

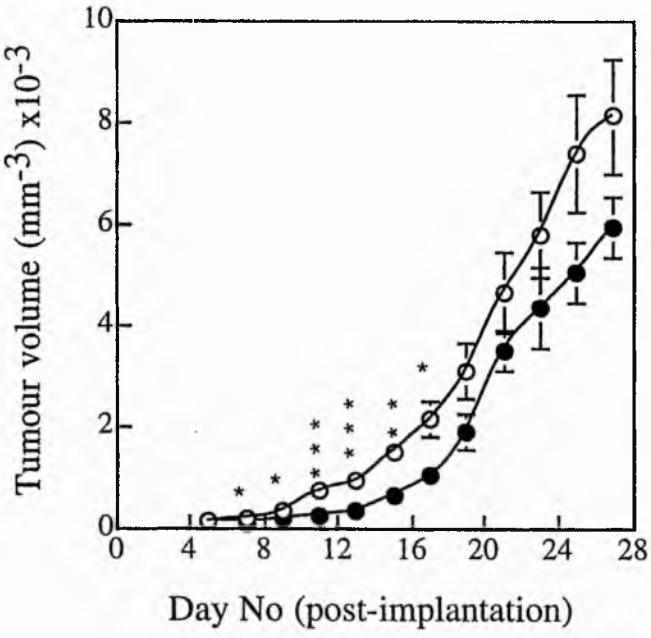


Fig. 8.11 *Comparing the growth curves for control (grp. 1a; open circles, n= 12) and day 5 LPS-treated animals (grp. 2b; closed circles, n= 12).*

The greatest inhibition of tumour growth was seen when LPS was injected 11 days post-implantation (see fig. 8.12). In this experiment the growth of tumours on LPS-treated animals was significantly suppressed within 12hrs of treatment (2-way ANOVA, F= 2.44, df= $7,183_e$, p= 0.02). The effect was less transient than that produced when LPS was injected on day 5 post-implantation, lasting up to 3-4 days longer, until ~day 14 post-LPS treatment.

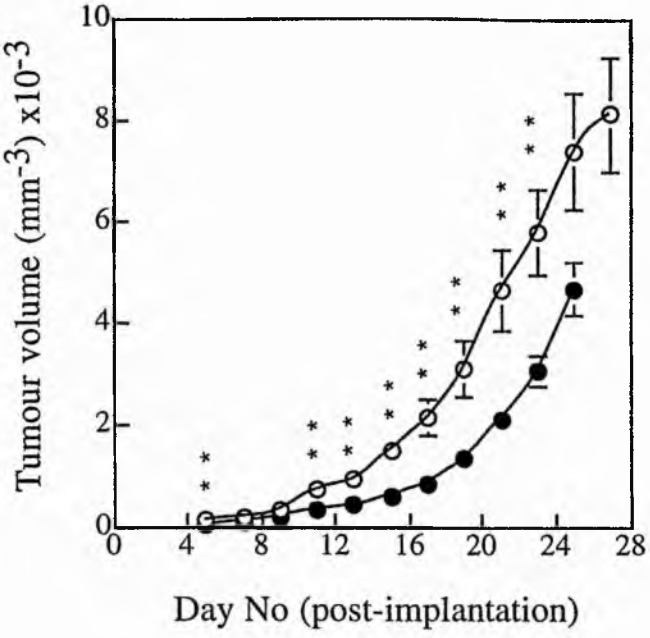


Fig. 8.12 *Comparing the growth curves for control (grp. 1a; open circles, n= 12) and day 11 LPS-treated animals (grp. 2c; closed circles, n= 14).*

This period extends into the last stage of growth, 'the necrotic stage', when tumours begin to outstrip their vascular supply and the rate of growth decreases. Fig. 8.12 shows that the growth rate of tumours in control animals began to decline at ~day 20-23 post-implantation, while the tumours in LPS-treated rats continued to increase exponentially.

Figs. 8.13-8.15 are photographs of tumour-bearing rats on day 15 post-implantation, representing controls (grp. 1a), day 5 LPS-treated (grp. 2b) and day 11 LPS-treated animals (grp. 2c) respectively. These picture show visually the difference in tumour size in animals treated with on day 5 and day 11 post-implantation. It is clear that tumours in the latter group are considerably smaller than those of the former.

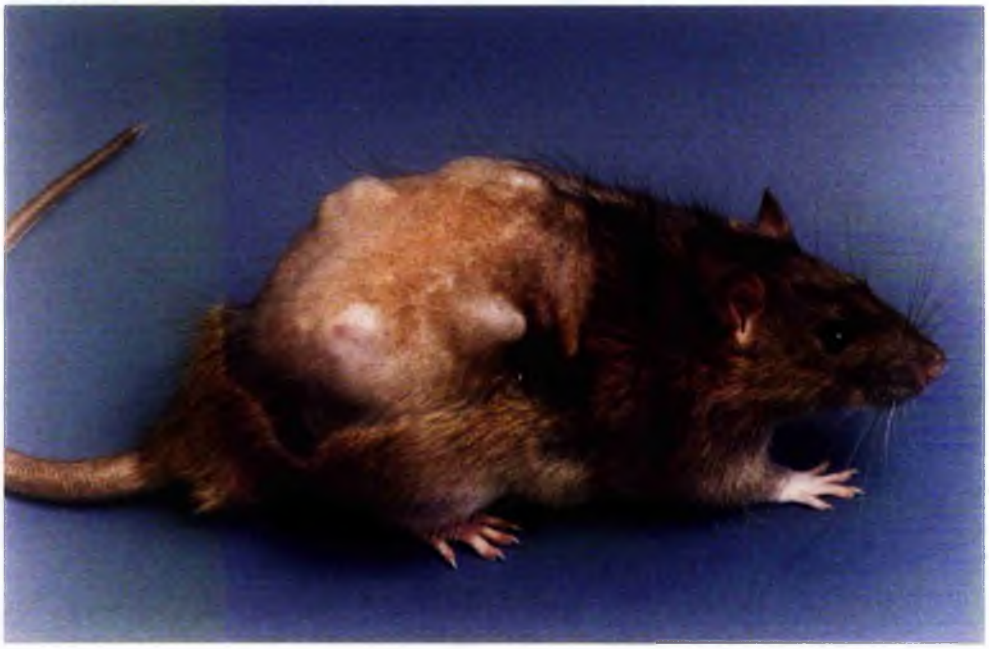


Fig. 8.13 *A photograph of a tumour-bearing rat from control grp. 1a 15 days post-tumour implantation.*

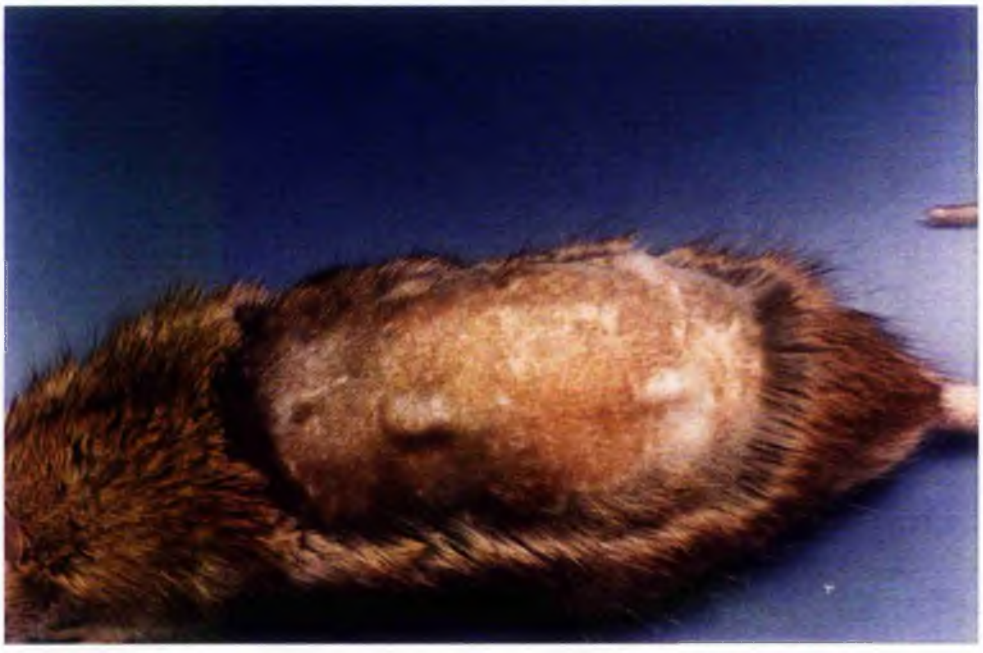


Fig. 8.14 *A photograph of a tumour-bearing rat from LPS-treated grp. 2b 15 days post-tumour implantation.*

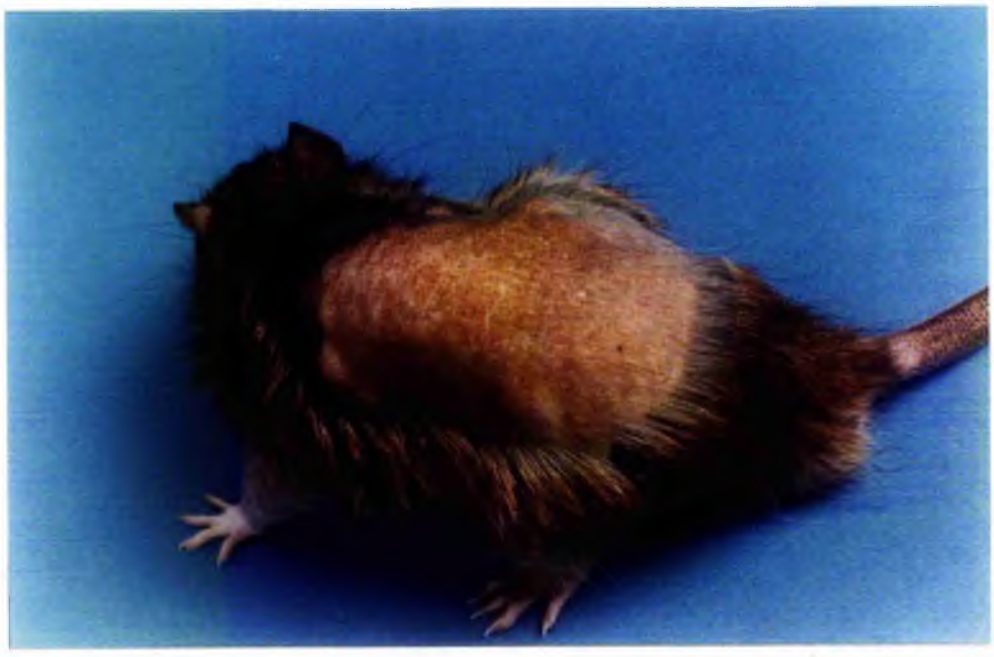


Fig. 8.15 *A photograph of a tumour-bearing rat from control grp. 2c 15 days post-tumour implantation.*

8.3.5 The effect of NOS inhibitors on the growth of solid tumours. Kennovin *et al.*, (1994), showed that feeding L-NAME (1mg/ml) caused a marked reduction in tumour growth rate and that this effect was near maximal and not significantly enhanced with a six fold increase in [L-NAME].

In the present study, L-NAME (1mg/ml) significantly inhibited tumour growth when added to the drinking water of grp. 4b rats on the same day as tumour implantation (day 0; 2-way ANOVA, F= 7.73, df= 4, 130_e, $p < 0.001$). The same concentration of AG on its own (grp. 3a rats) also suppressed tumour growth although the effect was less than that of L-NAME (still revealed that its effect was highly statistically significant, 2-way ANOVA, F= 15.26, df= $8,252_e$, p= < 0.001). The reduction in growth rate only became statistically significant 5 days after AG was added to the drinking water (see Appendix tables 8.2 & 8.3 for meaned data). Assuming that the growth profile for each group can be drawn

linearly, the difference in growth rates (mm^3/day) of tumours for each group can be estimated. According to this criteria, tumours in L-NAME-treated animals grew at a rate of ~0.1mm^3/day and those in AG-treated animals at ~0.19mm^3/day.

8.3.6 The combined effects of LPS + L-NAME and LPS + AG on the growth of solid tumours. The effects of LPS injection on day 11 post-implantation (ie. grp. 4a) were compared with the combined effect of LPS (injected on day 11) + L-NAME (ie. grp. 4c) and the effects of LPS injection on day 5 (ie. grp. 2b) were compared with the combined effect of AG + LPS (on days 4 & 5 respectively; grp. 3b). The results are shown in Appendix tables 8.1 & 8.4. As with exp. 1, grp. 2c animals, LPS significantly suppressed tumour growth (2-way ANOVA, F-= 3.15, df= 8,2914$_e$, p=0.002). Moreover, the inhibitory effect of L-NAME in combination with LPS was statistically significantly greater than that by LPS alone (2-way ANOVA, F= 2.30, df= 8,318$_e$, p= 0.0021; see fig. 8.16a).

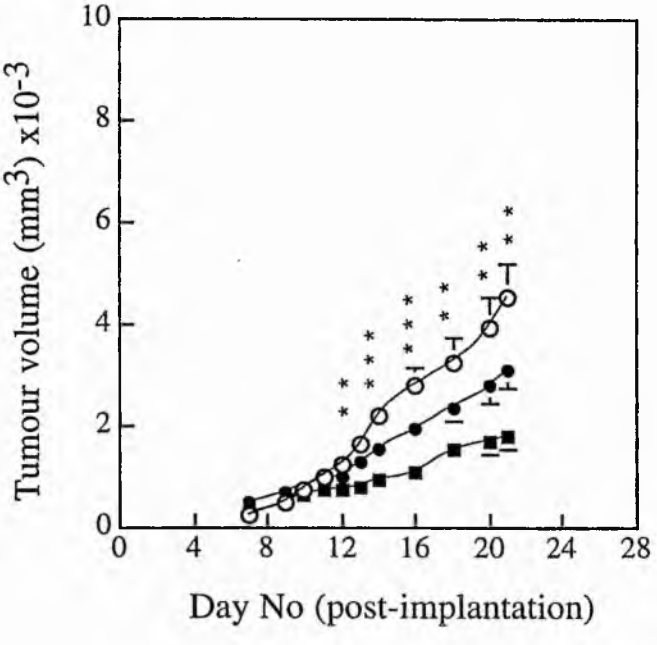


Fig. 8.16a *Comparing the growth of tumours in control (grp. 1d; open circles, n= 16), LPS-treated (grp. 4a; closed circles, n=16-20) and LPS + L-NAME-treated animals (grp. 4c; closed squares, n= 16-20). Asterisks indicate a significant difference between control and LPS-treated animals.*

the growth rate of tumours in control animals (~0.44mm^3/day) was ~1.6x that of the LPS-treated group (~0.27mm^3/day) and ~3.3x that of the LPS + L-NAME-treated group (0.13mm^3/day). In other words, the rate of tumour growth in the LPS + L-NAME-treated group was reduced by ~$^1/_2$ compared to that of the LPS-treated group. Students' t-test analysis is shown in Appendix table 8.5.

Fig. 8.16b shows that in contrast to the enhanced suppression seen with LPS + L-NAME, treatment of LPS to animals being fed AG, completely abolished the retarding effects of AG (2-way ANOVA F= 0.76, df= 10, 245$_e$, p= 0.851; see fig. 8.11).

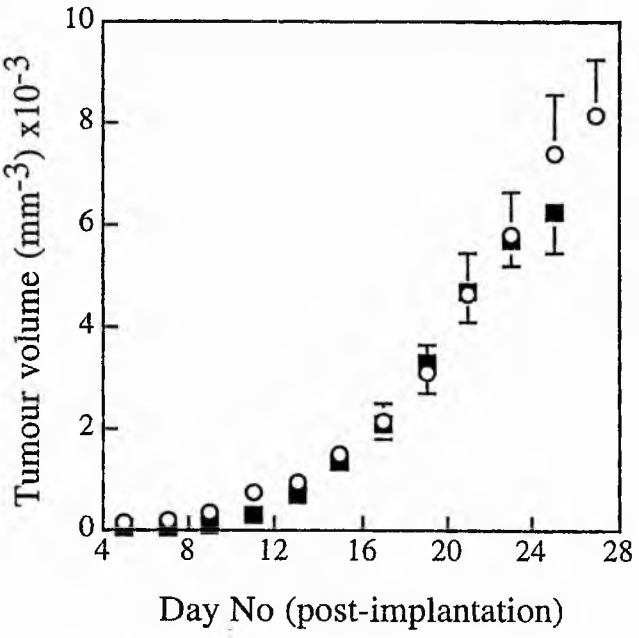


Fig. 8.16b *Comparing the growth of tumours in control (grp. 1a; open circles) and AG + LPS-treated (on days 4 & 5 respectively) animals (grp. 3b; closed squares). LPS abolishes the inhibitory effects of AG alone on tumour growth.*

To confirm that the inhibitory action of L-NAME is reversible (Kennovin *et al.*, 1994), L-NAME was removed from the drinking water of grp. 4c rats on day 21 and tumour volume was measure over the subsequent 4 days. The removal of L-NAME resulted in a dramatic increase in growth rate (> 6 fold, from ~0.078 to ~0.52mm^3/day) which exceeded that of controls (see Table 8.1)

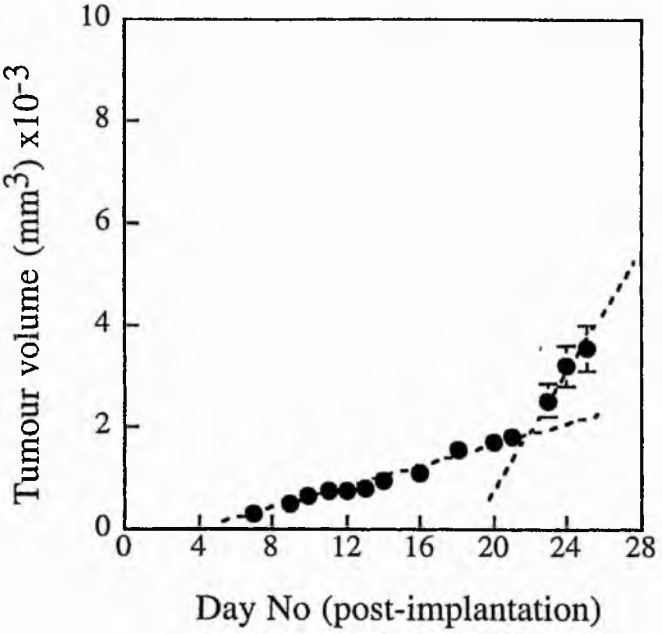


Fig. 8.17 *The inhibitory effect of L-NAME on grp. 4c tumours was reversed when L-NAME was removed from the drinking water on day 21. The rate of tumour growth increased over the subsequent 4 days to that of controls.*

Table 8.1 shows the changes in tumour growth rate (mm^3/day) for groups 1d, 4a and 4c. The rate of tumour growth was initially similar for all groups (days 7 -10). It increased in control animals (grp. 1d) by ~2.5 fold from the early growth phase (days 7-10) to the late growth phase (days 11-14), then over the subsequent 4-5 days (during the necrotic phase; days 16-21), the growth rate decreased by ~12%. The relative increase in growth rate between the early and late growth phases was less for tumours in LPS-treated rats compared to those of control rats, however, the growth rate *increased* by ~20% during the necrotic phase. In contrast to tumours in either the control group or the LPS-treated group, those in L-NAME + LPS-treated animals showed a significant *decrease* (~46%) in growth rate from the early to the late growth phase.

Growth rate (mm^3/day) Group	Days 7-10 (early growth phase)	Days 11-14 (late growth phase)	Days 16-21 (necrotic phase)	Days 23-25 (necrotic phase) - L-NAME
Control (1d)	0.16	0.40	0.35	-
LPS treated(4a)	0.10	0.20**	0.24	-
LPS+L-NAME (4c)	0.13	0.07***	0.14**	0.52

Table 8.1 *Showing the tumour growth rates for each experimental group. Asterisks indicate a significant difference compared to the control group.*

8.3.7 Haemodynamic effects of LPS alone and LPS + L-NAME treatment.

In order to determine whether the growth of tumours influenced haemodynamic parameters and assess the effects of LPS and LPS + L-NAME, systolic, mean and diastolic blood pressures of two rats from each group were recorded using the tail-cuff method. Control tumour bearing rats did not show any significant decrease in blood pressure throughout the experiment (see fig. 8.18).

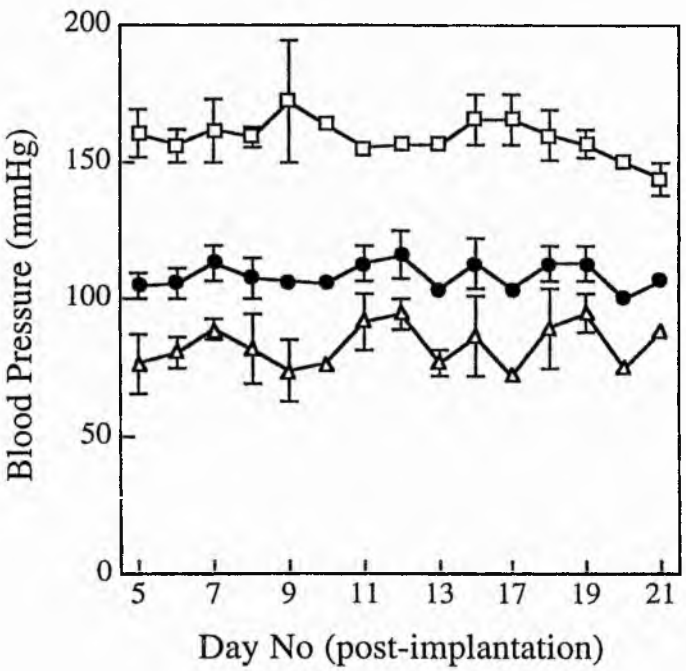


Fig. 8.18 *Systolic (open squares), mean (closed circles) and diastolic (open triangles) blood pressures for control, tumour bearing rats (n= 2).*

It is clear from fig. 8.18 that systolic blood pressure fluctuated around 160mmHg, mean blood pressure around 105mmHg and diastolic around 80-85mmHg. Furthermore, the injection of sterile saline did not appear to have any effect on blood pressure.

However, a single injection of LPS on day 11 post-implantation resulted in a considerable hypotension within 24hrs. This was manifested as a decrease in systolic blood pressure of ~42% from ~162 to 94mmHg. Within 48hrs after LPS treatment (day 13) the blood pressure had returned to pre-injection levels (see fig. 8.19).

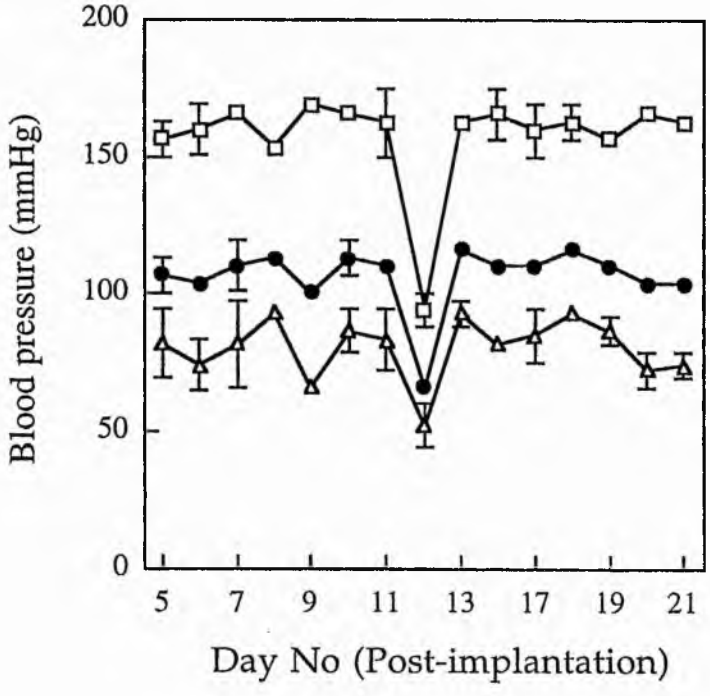


Fig. 8.19 *Systolic (open squares), mean (closed circles) and diastolic (open triangles) blood pressures for LPS-treated, tumour bearing rats (grp. 4a;n= 2).*

A decrease in blood pressure was also seen in animals treated with LPS + L-NAME, but the hypotensive effect of LPS was attenuated in animals treated with L-NAME. Thus, 24hrs after LPS was injected, systolic blood pressure had dropped by ~26% from ~153 to 112mmHg (see fig. 8.20). According to Glauser *et al.*, (1991), a decrease in systolic blood pressure of > 40mmHg indicates a state of haemodynamic shock associated with bacterial infection. Therefore, the

administration of L-NAME 1 day prior to LPS treatment did not prevent the subsequent LPS-induced hypotension.

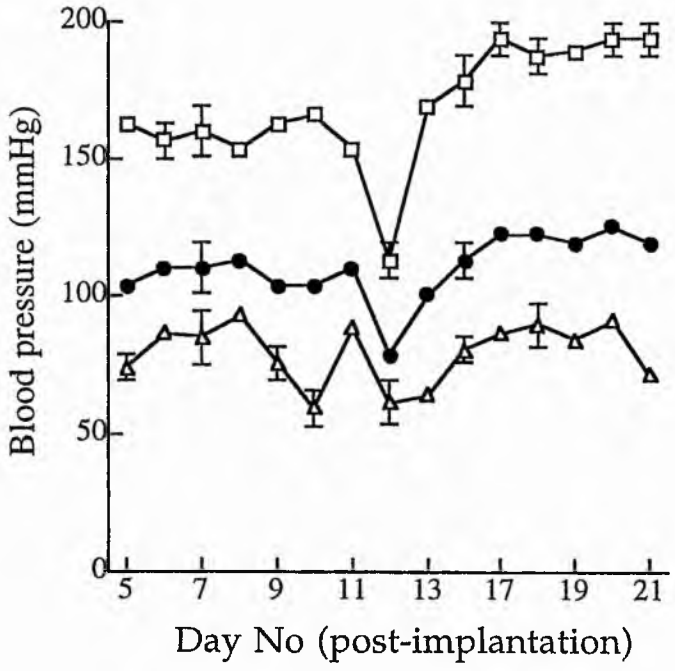
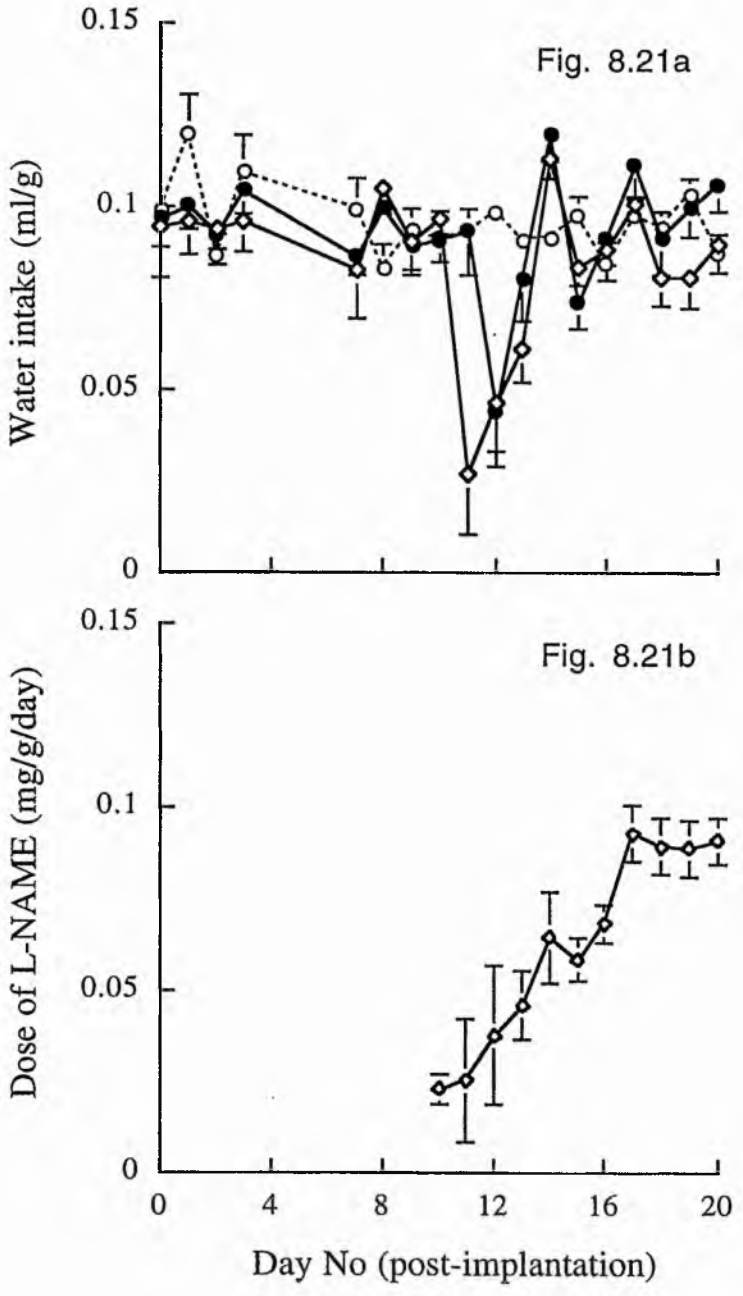


Fig. 8.20 *Systolic (open squares), mean (closed circles) and diastolic (open triangles) blood pressures for LPS + L-NAME-treated, tumour bearing rats (grp. 4c;n= 2).*

The hypotensive effect of LPS was followed by a 'rebound' hypertension in animals also receiving L-NAME. Thus, 72hrs following LPS treatment (at day 14) the systolic blood pressure was increased by ~25mmHg above control levels before LPS treatment (from ~153 up to 178mmHg). The L-NAME-induced hypertension continued to increase until a plateau was reached at ~day 17, at which point the systolic blood pressure had risen to ~194mmHg. The results are shown in Appendix table 8.6.

8.3.8. Drinking rates and weights of experimental animals. Generally, LPS-treated animals showed a transient (lasting up to ~24hrs) decrease in drinking rate. Fig. 8.21a shows the decrease (~53%) in animals treated with LPS on day 11 (grp. 4a). The transient reduction in drinking rates was greater in animals receiving LPS + L-NAME (grp. 4c; ~62%) and resulted in an initially low ingested dose of L-NAME which subsequently increased to reach ~0.9mg/Kg

(see fig. 8.21b). The data for drinking rates and weights are shown in Appendix Tables 8.7-8.15.



Figs. 8.21a & b *Showing the effects of LPS (closed circles) and LPS + L-NAME (open diamonds) treatment on the amount of water consumed per day as compared to controls (open circles; fig. 8.21a, n= 4-5 rats for each group). The dose of L-NAME ingested is shown in fig. 8.21b.*

It has been shown that the anti-tumour effects of L-canavanine, also known to inhibit NOS, may be attributed to its inherent toxicity, resulting in caloric deprivation and a subsequent loss in body weight (Thomas *et al.*, 1986). None of

the animals in this study suffered any permanent loss in body weight (see figs. 8.22a-f).

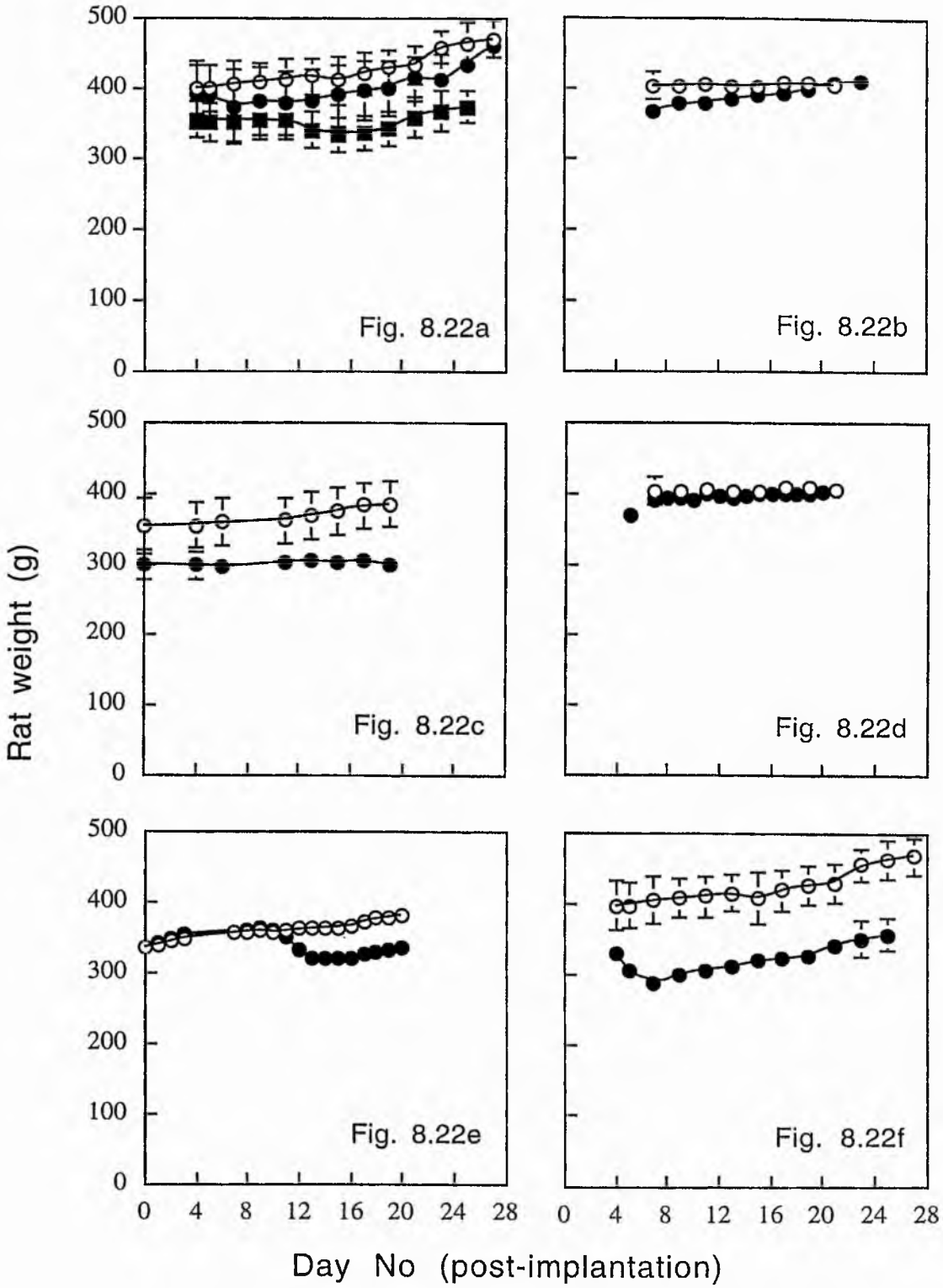


Fig. 8.22a-f *Showing the change in body weight for control (1a- 1d; open circles) and treated (closed circles) animals. LPS-treated (on day -1 fig. 8.22b; grp 2a), day 5 (fig. 8.22a) or day 11(closed squares; fig. 8.22a), L-NAME-treated (fro day 0; fig. 8.22c) AG-treated (from day 10; fig. 8.22d), L-NAME +*

LPS treated (fig. 8.22e; grp. 4c) and AG + LPS treated (fig. 8.22f; grp. 3b). Refer to text for an explanation of the different groups in each graph.

However, LPS treatment alone (on day 11; grps. 2c & 4a) or in combination with L-NAME, caused a small decrease in body weight (~5 & 11% respectively).

8.4 Discussion

8.4.1 LPS-induced delay of tumour growth is dependent on the time of administration. A single intraperitoneal injection of LPS significantly suppressed tumour growth when administered on day 11 and to a lesser extent day 5 post-implantation. No effect was seen when LPS was administered 24hrs prior to tumour implantation. LPS induces an immune response: specifically, it induces the expression of the inducible isoform of NOS and generally increases NO production as evidenced by the enhanced accumulation of nitric oxide oxidation products (>18 fold above control levels; see chapter 3, Section 3.5.2). One consequence of this is a transient hypotensive effect lasting for at least 48-72hrs, presumably due to the NO-induced hyporeactivity which occurs in the blood vessels. Normally this would be expected to decrease the driving force of blood entering the tumour and also increase the tumouricidal potential of macrophages resident in the tumour. The combined effect would be to decrease the flow of nutrients and oxygen into the tumour and increase cell killing for a period of at least 2-3 days. This time scale compares favourably with the delay in tumour growth (~4-5 days).

8.4.2 The effects of L-NAME or AG on solid tumour growth. Chronic oral administration of L-NAME or AG delayed tumour growth, the effect of the former being greater than that of the latter. Previous studies have attributed the effect of L-NAME to constricting tumour-supply vessels (Kennovin *et al.*, 1993), effectively starving the growing tumour of nutrients and oxygen (Wood *et al.*, 1993). This interpretation is supported by the fact that tumour growth resumes at control rates when L-NAME is removed from the drinking water, first shown by Kennovin *et al.*, (1994) and confirmed in the present study (Section 8.3.6, fig. 8.19). Presumably, AG also constricts tumour-supply vessels, but unlike L-NAME, will not inhibit cNOS and therefore will not have a generalised hypertensive effect. This is borne out by physiological experiments which show that AG reverses LPS-induced hyporesponsiveness and hypotension without

causing hypertension (Wu *et al.*, 1995b). Physiologically therefore, the only difference between the L-NAME treated group (grp. 4b) and the AG treated group (grp. 3a) is the sustained hypertension in animals treated with L-NAME. Although there is no direct evidence given in the present study, L-NAME and AG would inhibit macrophage NO production as well as that from vascular smooth muscle cells.

8.4.3 The effects of combined treatment with NOS inhibitors and LPS. The effect of LPS on tumour growth in animals chronically fed with L-NAME was completely different from that of animals fed with AG. In the former case an LPS injection given on day 11 potentiated the growth retarding effect of L-NAME, whereas a similar injection of LPS given on day 5 abolished the effects of AG. The only physiological difference between these two groups of animals is the fact that one remains normotensive (grp. 3b) while the other first experiences a transient hypotension (1-2 days), and then encounters a sustained hypertension lasting for 10-11 days. This is presumably due to the non-selective nature of L-NAME, inhibiting both cNOS and iNOS (Rees *et al.*, 1990). Significantly, removal of L-NAME, which probably restores animals to the normotensive state, correlates with the resumption of normal tumour growth also seen at this time.

8.4.4 iNOS expression in solid tumours. It is likely that much of the iNOS being expressed within the tumour is derived from tumour cells. Indeed it is possible that iNOS-derived NO actually assistes in growth as evidenced by the retarding effect of NOS inhibitors. This is presumably due to the vasodilator effect of NO, facilitating blood flow into the tumour. The poor immune reponse by the host, conspicuous by the relative lack of iNOS-expressing macrophages within the tumour, may be attributed to the origins of the tumour implant since this is derived from a spontaneous tumour which arose in an isogeneic rat.

The immune response triggered by a single injection of LPS was sufficient to significantly delay tumour growth with the maximum effect occuring during the

latter stages of growth (ie. the necrotic phase). This was presumably mediated by LPS-induced activation of macrophages and the subsequent release of cytotoxic levels of NO within the tumour (see fig. 8.6). However, the LPS-induced increase in tumour vascular resistance is not inhibited by scavengers of NO (Tozer *et al.*, 1995), indicating that NO may not be responsible for all of the anti-tumour effects associated with LPS treatment.

8.4.5 Conclusions. The factor which determines whether blood flow through a solid tumour is increased or decreased by vasoconstrictors appears to be the presence or lack of VSM in the tumour vasculature. Tumour vessels with VSM will constrict and blood flow through the tumour will consequently decrease (Jirtle, 1978), while those without VSM will not and blood flow will instead be determined by the elevated systemic blood pressure and associated increase in interstitial fluid pressure within the tumour (Suzuki *et al.*, 1981). In the present study tumour growth was retarded in hypertensive (ie. L-NAME-treated) and hypotensive (ie. LPS-treated) rats, with a greater effect seen in tumours which were beginning to outgrow their vascular supply. These could conceivably have a higher proportion of vessels, either recruited from the host or neovasculature, which contain smooth muscle. These results suggest that an altered haemodynamic state *per se* has a major influence on the growth of solid tumours, although further studies are needed in order to resolve whether this is true in animals which have not been subjected to agents which modify NO levels (eg. spontaneous hypertensive rats).

Chapter 9
Discussion

9.1 Background to the present study. Our understanding of NO and its implications in clinical disease has progressed considerably since its discovery as an endothelium-derived relaxing factor and mediator of the proinflammatory/cytotoxic response in immune-activated macrophages (Furchgott and Zawadki, 1980; Hibbs Jr. *et al.*, 1988). As a result, NO has been established as a pervasive physiological mediator of health and disease: in its capacity as a vasodilator, the small amounts of NO produced by the constitutively active NOS isoform (cNOS) are essential for normal physiological function, while the high levels of NO produced by the inducible isoenzyme (iNOS) under pathophysiological conditions, make it a very important part of the host's non-specifc immune system. However, it has recently been shown that the amount of NO produced during an immune response is often so great as to actually supress the specific T-cell-mediated immune system and even kill the activated macrophages responsible for the anti-tumourogenic response. (Lepoivre *et al.*, 1995; Orucevic & Lala, 1995; Drapier & Hibbs Jr., 1988). This paradoxical lymphoid immunosuppression in animals with enhanced nonspecific resistance to tumour development (mediated in part by increased NO production) further emphasises the 'double-edged sword' nature of NO and ultimately makes it difficult to predict the outcome of an immune-mediated upregulation of NO synthesis *in vivo*. In this respect, the role(s) of NO in cancer, and in particular its influence on the growth of solid tumours, is unclear, although it is largely governed by the quantities of NO being produced: large amounts of NO can have an anti-tumourigenic effect (Hibbs, Jr., 1988), while smaller amounts can favour

the development of solid tumours by improving their blood flow. Evidence supporting the latter is briefly outlined below.

Solid tumours derive their blood supply from the host and via the induction of an angiogenic stimulus which facilitates the development of neovasculature, without which the tumour would not grow beyond ~2-3mm in diameter (Folkman, 1972). Angiogenically-derived blood vessels possess a number of characteristics that distinguish them from normal vessels, in particular their frailty, tortuosity, arteriovenous anastomoses, lack of innervation and susceptibility to collapse (Jain, 1988; Baillie *et al.*, 1995). In addition to structural abnormalities, in many solid tumours the apparent lack of a lymphatic system to assist interstitial fluid drainage results in abnormally high interstitial fluid pressures (Jain, 1994). Since these pressures often exceed intravascular pressures blood vessel patency can be compromised. The majority of vessels within a tumour lack smooth muscle (Chan *et al.*, 1984) and the high interstitial pressures within experimental tumours predispose tumour blood flow to being critically dependent on the systemic blood pressure of the host animal.

Initial research focused on identifying chemical agents which improve blood flow into the tumour to allow enhanced uptake of anti-cancer drugs. However, it has recently become evident that agents which can selectively reduce blood flow also have a strategic therapeutic role.

Reports that iNOS is expressed in solid tumour vasculature (Buttery *et al.*, 1993) and that host vessels which had previously supplied a solid tumour (ie. TEAs) display a diminished sensitivity to vasoconstrictor agents which can be abolished by specific inhibitors of NOS (Kennovin *et al.*, 1993), indicate that NO has a role in modulating blood flow through the solid tumour. This is confirmed by *in vivo* studies which show that specific, non-selective inhibitors of NOS (ie. L-NMMA and L-NAME) can reduce blood flow in experimental solid tumours (Andrade *et al.*, 1992; Meyer *et al.*, 1995) by increasing vascular resistance (ie.

vasoconstriction; Tozer *et al.*, 1995). Kennovin *et al.*, (1994), were first to report that chronic oral administration of L-NAME in the drinking water significantly retards the growth of subcutaneously transplanted tumours in the rat and, furthermore, that this effect only persists for as long as the drug is administered. Removal of L-NAME from the drinking water returns the growth rate of tumours back to those on control animals (ie. animals which had not been fed with L-NAME). It is clear that agents such as NOS inhibitors which constrict arteriole-supply vessels can effectively starve the tumour of its vital nutrients, thereby inhibiting its growth (Hirst & Wood, 1989; Wood *et al.*, 1993; Peterson, 1991).

It is well established that exposure to bacterial lipopolysaccharide (LPS) can induce a state of endotoxic shock in laboratory animals similar to that (ie. septic shock) seen in humans (Suffredini et al., 1989; Kilbourn & Billiar, 1996). Furthermore, arteries removed from animals in endotoxic shock (ie. LPS-treated animals) display a diminished pressor response to catecholamines, similar to that seen with tumour-supply vessels, and like them, the hyporesponsiveness can be reversed with NOS inhibitors (Julou-Schaeffer *et al.*, 1990; Schott *et al.*, 1993; Joly *et al.*, 1994).

The inherent difficulties associated with propagating tumours and isolating their supply arteries compromises the collection of consistent results and reliable data. It is conceivable that vessels isolated from LPS-treated animals will provide a more reliable alternative for studying the potential of NOS inhibitors to selectively abolish this abnormal hyporesponsive effect and thereby ultimately provide valuable information regarding the potential for a given NOS inhibitor to suppress tumour growth. Using an established model of endotoxic shock in the rat (see Chapter 3), this study therefore, offers a comparative look at some of the physiological properties of tumour-supply arteries and arteries isolated from LPS

treated rats and also assesses the potential for various inhibitors, administered alone or in combination with LPS, to retard the growth of solid tumours.

9.2 The effects of LPS treatment in the rat. A single bolus injection of bacterial LPS (*E. coli* serotype 055:B5; 4mg/Kg; i.p.) was sufficient to induce ~18 fold increase in the accumulated levels of nitrate and nitrite in rat blood plasma within 24hrs of the injection. This effect was transient and within a further 48hrs (at t= 72hrs) nitrate and nitrite had returned to control levels. The increase in circulating nitrate and nitrite (maximal at ~t= 20-24hrs) was evident after a lag time of ~6-8hrs in relation to iNOS expression in RTAs (maximal expression at ~t= 6hrs). Moreover, iNOS expression was almost undetectable at t= 24hrs. A similar lag was also seen for the transient LPS-induced hyporesponsiveness to phenylephrine in perfused segments of RTA, which increased with time after LPS exposure, becoming maximal after ~18-24hrs and disappearing within 72hrs. Thus, the time-course for LPS-induced hyporesponsiveness to PE closely resembles that for the LPS-induced increase in blood plasma nitrate and nitrite levels. Furthermore, both of these parameters parallel the LPS-induced systemic hypotension *in vivo* which is also maximal after ~24hrs and recedes within ~44-48hrs after treatment. In summary, the inducible isoform of NOS is expressed for ~20-24hrs in RTAs following a single bolus injection of LPS and the symptoms of the ensuing increased NO production persist for up to another 48hrs after this.

9.3 Hyporesponsiveness to PE of vessels isolated from LPS-injected rats and those which formerly supplied a solid tumour. The accumulated data for the initial D.R. curves to PE revealed a highly significant difference between control RTA responses (n= 56) and LPS-treated RTA responses (at t= 24hrs, n=29; see fig. 9.1 and Appendix table 9.1). Similarly, TEAs were found to be significantly less responsive to PE as compared to CEAs (see fig. 7.4). Interestingly, 2 way ANOVA reveals no significant difference between LPS

RTAs (at t=24hrs) and TEAs (F=1.60, df= 9, 513_e, p= 0.113) nor control RTAs and CEAs (F= 0.89, df= 9, 735_e, p= 0.538; see fig. 9.2). It must be assumed that this is purely coincidental since each type of artery is exposed to a different internal perfusion rate which will ultimately determine their contractile response.

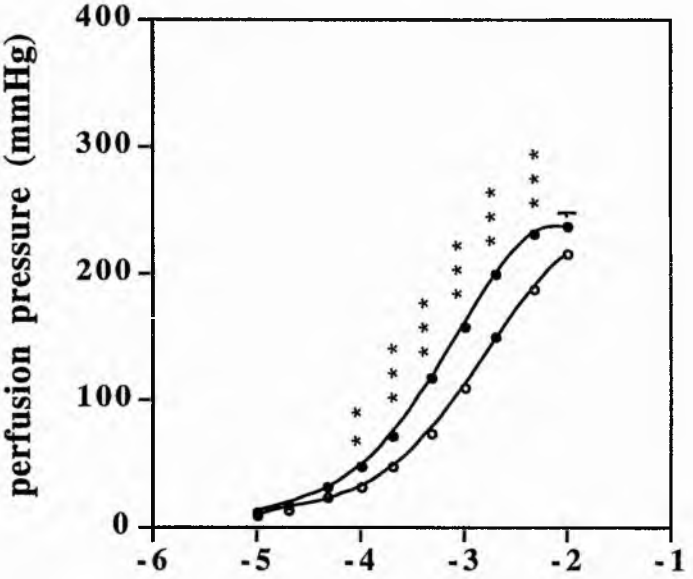


Fig. 9.1 *Represents the initial D.R. to PE data for accumulated control (closed circles, n=56) and LPS-treated (t= 24hrs; open circles, n=29) RTAs.*

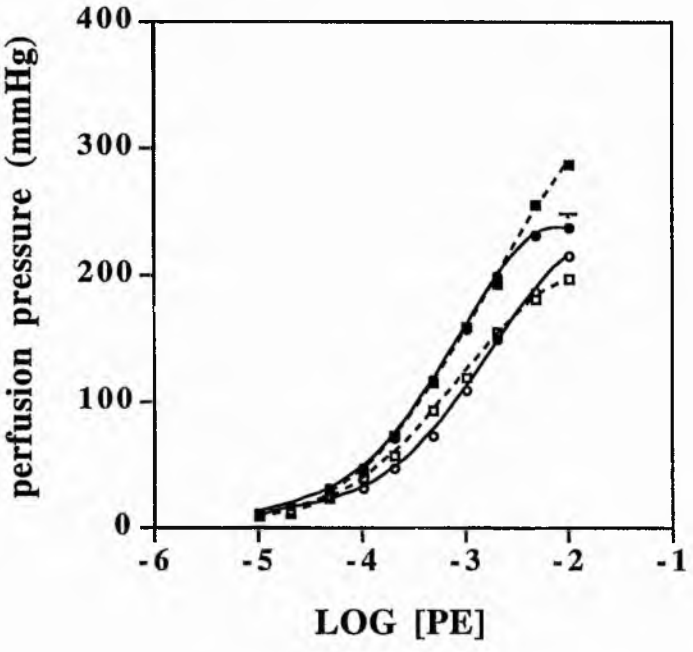


Fig. 9.2 *Compares the initial D.R. to PE for TEAs (open squares, n=29) /CEAs (closed squares, n=28) and LPS-treated (open circles) and control (closed circles) RTAs.*

From this evidence, it could be suggested that LPS-treated RTAs and TEAs display a similar hyporesponsiveness. However, on closer examination they appear to differ: TEAs have a comparatively greater hyporesponsiveness at higher concentrations of PE and as a result a more defined 'plateau' is seen at the top of the D.R. curve. A plot of *f* values produced a negative slope (see fig. 7.17). In contrast, LPS-treated RTAs proved to be more hyporesponsive at lower concentrations of PE and as a result, when the *f* values were plotted with a linear curve fit, a positive slope was obtained (see Fig. 3.20). It can therefore be concluded that the TEA-associated hyporesponsiveness conforms to the theoretical non-competitive-type of shift relative to CEAs, while that associated with LPS-treated RTAs more closely resembles a competitive shift (see Chapter 3, Section 3.6.1). This indicates a fundamental difference in the types hyporesponsiveness exhibited by these two types of vessel. This is perhaps not surprising when we consider that LPS and tumour growth result in the synthesis & release of a large number of different chemicals. LPS stimulates a cascade of various types of cytokine and inflammatory mediators, including iNOS, while growing tumour releases a concoction of chemicals into its surrounding enviroment to stimulate angiogenesis and generally promote growth (Klagsbrun & D'Amore, 1991; Rees *et al.*, 1990; Zuckerman *et al.*, 1989; Kilbourn & Billiar, 1996).

9.4 NOS inhibitors abolish the Hyporesponsiveness to PE of vessels isolated from LPS-injected rats and those which formerly supplied a solid tumour.

NO-induced insensitivity to α-adrenergic agents is thought to explain the failure of vasopressor agents in some patients with septic shock. In the present study two relatively non-selective NOS inhibitors, L-NMMA, L-NAME and the more isoform selective inhibitor, AG, were found to fully reverse the diminished sensitivity to PE in LPS-treated RTAs and TEAs with a general rank order of potency L-NMMA > L-NAME > AG (see Chapters 4 & 7). However, unlike L-NMMA or L-NAME, AG did not further potentiate responses beyond the

control groups (ie. control RTAs or CEAs), confirming its preferential inhibition of the inducible isoform (Griffiths *et al.*, 1993; Joly *et al.*, 1994; Wu *et al.*, 1995). In a separate set of experiments (see Chapter 6) a number of novel NO scavengers (JM1006, 1226 and 6245) were also shown to reverse the LPS-induced hyporesponsiveness in RTAs. These results together show that the hyposensitivity of vessels taken from LPS treated rats and those which had previously supplied a solid tumour is mediated at least in part by NO derived from the inducible isoenzyme of NOS.

9.5 *De Novo* Protein Synthesis in TEAs and LPS-Treated RTAs *in vitro*. It has previously been shown that arteries supplying a solid tumour are sensitive to the protein synthesis inhibitor, cycloheximide (Kennovin *et al.*, 1994), whereby the hyposensitivity associated with these vessels is abolished within ~2hrs of initiating treatment. This suggests that TEAs contain vasoactive proteins which are rapidly synthesised and are involved in sustaining the hyporeactive effect. The study by Kennovin *et al.* (1994), also found that CEAs undergoing prolonged perfusions (> 7hrs) developed a similar cycloheximide-sensitive hyporeactivity to PE as that seen in TEAs. Prolonged exposure to bacterial contamination within the *ex vivo* apparatus is thought to be responsible for inducing iNOS in these vessels and may explain this effect.

A comparable time-dependent hyporesponsiveness was not seen in control RTAs after 9hrs perfusion (results not shown). The reason for this is not clear, although it is conceivable that the considerably larger diameter of RTAs compared with EAs (~150-200μm and 40-70μm respectively) may mean that more NO must be produced in RTAs than in EAs for a comparable effect to be seen. These results do not contradict those of Rees *et al.*, (1990), who showed that rat aortic rings developed a cycloheximide-sensitive hyporesponsiveness after 8hrs using the organ bath technique, as a non-perfusion setup will undoubtedly favour the

tendency for bacterial comtamination to accumulate around the vessel and thereby increase the likelihood of inducing iNOS activity.

Indomethacin on its own caused a marked suppression of contractile responses in TEAs and CEAs, with a slightly greater effect in TEAs compared to CEAs (although not significant with n=5; see fig. 7.), suggesting that under normal conditions cyclooxygenase enzymes are responsible for producing a vasoconstrictor (eg. thromboxane A_2) in these vessels. A similar enhanced production of thromboxane A_2 or endoperoxides is thought to explain the augmented suppression of noradrenaline-induced contractions in rings from LPS-treated rats (Wu *et al.*, 1994). Further experiments with TEAs are required to identify the mediator involved in this effect. However, since cycloheximide was reported to have no effect on CEAs within 2hrs of treatment (Kennovin *et al.*, 1994) it must be concluded that the potentiating effect of cycloheximide, which is similar to that for AG, is not due to inhibition of cyclooxygenase protein synthesis, but more likely to inhibition of iNOS synthesis.

Responses to PE were similarly enhanced by cycloheximide in LPS-treated (24hrs) RTAs but not in controls. They were significantly increased by 2hrs and maximally so by 3hrs. After 4hrs perfusion with cycloheximide alone the subsequent addition of L-NMMA did not significantly potentiate the responses in either control or LPS-treated RTAs.

Indomethacin on its own did not alter responses in these vessels, however, it did completely abolish the cycloheximide-mediated augmentation of responses in LPS RTAs (see fig. 5.). The LPS-induced activation of iNOS and the subsequent release of NO is known to stimulate iCOX activity (Salvemini *et al.*, 1993). By inhibiting cyclooxygenase activity (iCOX), indomethacin may remove the influence of vasodilators (eg. prostacyclin) as well as vasoconstrictors (eg. thromboxane A_2/B_2). If both catergories are active in RTAs it is likely that treatment with indomethacin would not cause any visible alteration to PE-

induced contractile responses. This may explain why indomethacin abolished the effects of cycloheximide in RTAs, without having any apparent effect when added on its own. A similar case may also be true for rat aorta (Wu *et al.*, 1994). This could suggest that like iNOS, vasoactive products of the iCOX are rapidly turned over (ie. continously synthesised) during the LPS-induced immune response and therefore the effect of cycloheximide is a composite one inhibiting both iNOS and iCOX activity.

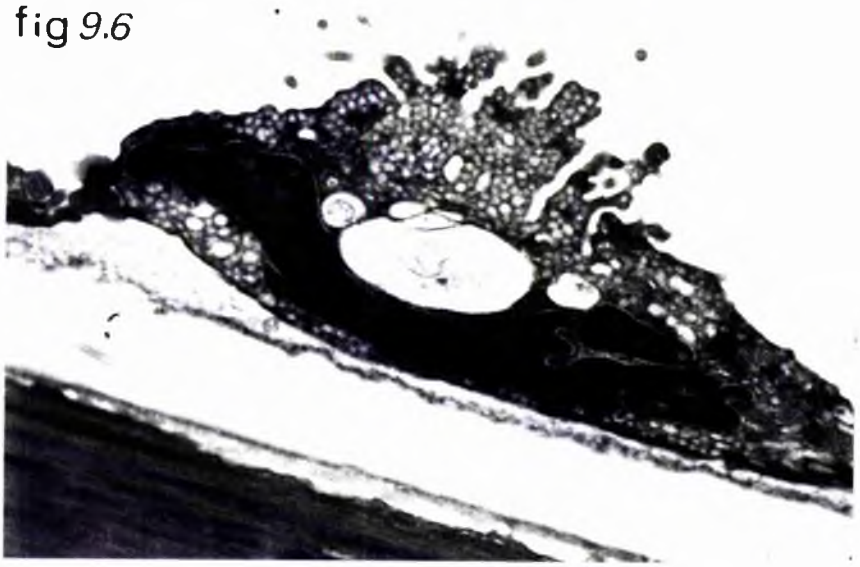
Assuming that cycloheximide inhibits iNOS activity in LPS-treated RTAs, the subsequent effect of L-NMMA presumably reflects the inhibition of eNOS activity. The effect of L-NMMA is masked by the shift caused by cycloheximide alone (see fig. 5.), although basal NO production may actually be reduced in these vessels. In the presence of cycloheximide + indomethacin, L-NMMA causes a considerable shift similar to that for untreated LPS RTAs (see fig. 5.), suggesting that eNOS-derived NO production is upregulated and does not rely on *de novo* protein synthesis for sustained activation. The presence of indomethacin therefore appears to enhance basal NO production in LPS-treated RTAs. The relative lack of effect by cycloheximide alone or in combination with indomethacin in control RTAs implies that iNOS or iCOX are not actively expressed in these vessels.

It can be concluded therefore, that the vasoactivity of rat epigastric arteries (EAs), whether they be associated with a tumour or not (ie. TEAs and CEAs respectively), is to some extent regulated by vasoactive products of the cyclooxygenase pathway. The ability of indomethacin to suppress EA responses to PE suggest that the predominant product is a vasoconstrictor. Similarly, RTA responses are also influenced by products of the COX pathway, although in contrast to EAs, this only applies to RTAs isolated from LPS-treated rats (synonymous with TEAs). This suggests the involvement of the inducible isoform of COX. Furthermore, the ability of indomethacin to abolish the

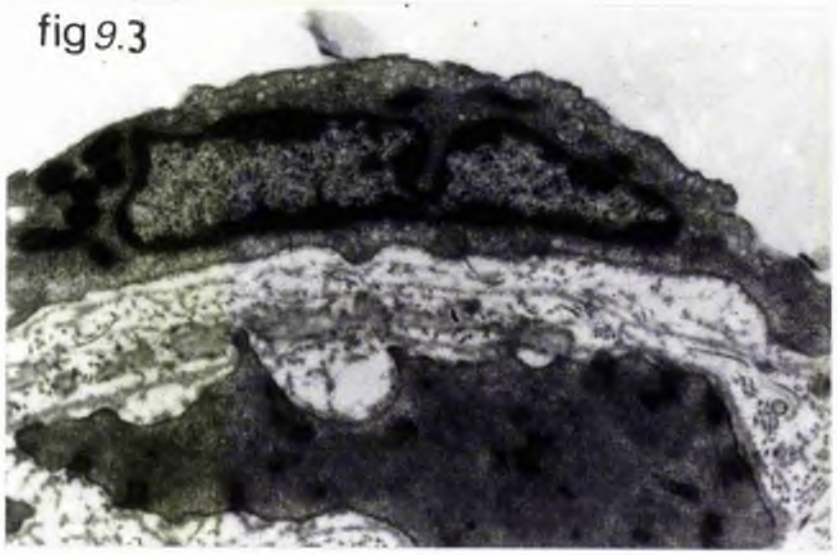
vasoconstricor effect of cycloheximide implies that a vasodilator is being inhibited and *not* a vasoconstrictor, as in the case of EAs. LPS-induced NO production can increase prostaglandin synthesis (Salvemini *et al.*, 1993). The fact that NOS inhibitors can fully reverse the LPS-induced hyporesponsiveness in isolated RTAs, does not identify NO as the sole mediator of this effect since recent evidence shows that NOS inhibitors, including L-NAME, can also inhibit prostaglandin production (Salvemini *et al.*, 1995).

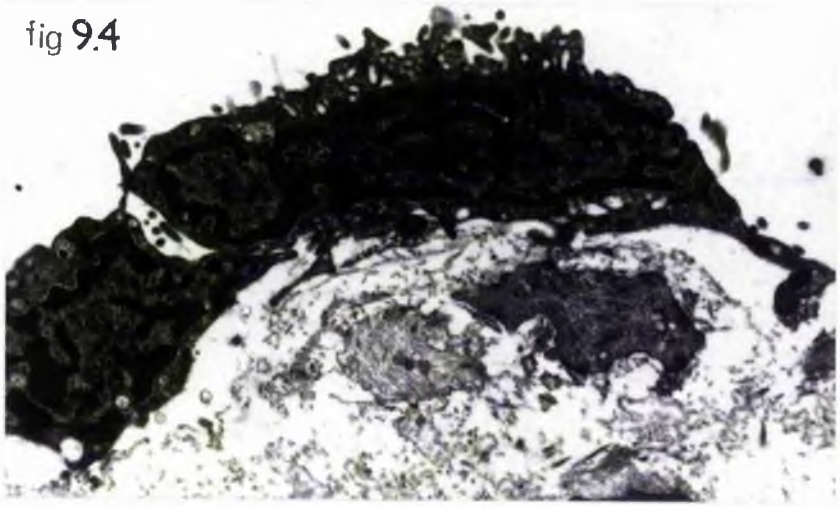
fig *9.5*

fig *9.6*



9.6 Perturbation of Endothelial Lining. Several TEAs and LPS-treated RTAs were found to be structurally different from their respective controls by having deformed endothelial lining (see Figs. 9.3-9.6). Transmission electron microscopy revealed disruption of the endothelial cells in a few TEAs and LPS-treated RTAs with small cellular processes extending out from their surface.





Figs. 9.3-9.6 are photographs (E.M. magn., x15,000) of endothelial cells from a control and an LPS-treated RTA (Figs. 9.3 & 9.4 respectively, above) and a CEA and a TEA (Figs. 9.5 & 9.6 respectively, opposite). Endothelial cells on the LPS-treated RTA and TEA commonly showed signs of abnormality with tiny cellular processes extending out from their endothelial surface. Their respective controls on the other hand showed no signs of endothelial perturbation.

Perturbed endothelial lining has been reported previously following LPS exposure and is thought to be responsible for the accompanying increased vascular permeability to proteins. (Hutcheson *et al.*, 1990). Furthermore, a more recent study implicates iNOS in this effect (Boughton-Smith *et al.*, 1993). Enhanced permeability is also a characteristic of tumour vasculature (Jain, 1994) and may reflect the expression of iNOS and/or vascular permeability factor in these vessels (Dvorak *et al.*, 1991; Maeda *et al.*, 1994). It has yet to be shown whether host vessels recruited into a solid tumour also exhibit hypermeability. Evidence here, although not conclusive, does raise the possibility that arteries recruited into a tumour are modified morphogically as well as physiologically and furthermore, that these properties are linked by the same common mediator, iNOS-derived NO production.

9.7 NOS expression in TEAs, their associated tumours and RTAs isolated form LPS-treated animals. Western blot analyses revealed that RTAs isolated from rats 6hrs after an injection with LPS expressed high levels of iNOS, while control (*untreated*) animals did not. iNOS expression diminished with time after LPS treatment and was absent by 72hrs. The enzyme was also highly expressed in lung tissue removed from LPS-treated animals 24hrs after being treated with LPS. Immunohistochemical analyses revealed that it was localised to macrophage-like cells and was generally not seen in the epithelial lining of major airways. iNOS was also weakly expressed in heart and liver extracts (not shown). Staining with antiserum specific to eNOS (see chapter 3, section 3.4.2) in RTAs at t= 6 , 24 and 72hrs after LPS treatment confirmed the expression of eNOS in the endothelial lining of these vessels. The expression of eNOS therefore persists throughout the LPS-mediated induction of iNOS.

TEAs also expressed eNOS as well as iNOS. iNOS expression was generally localised to their smooth muscle layer, although some sections revealed intensely staining cells on the endothelial layer. In contrast, CEAs did not express iNOS

(see Figs. 7.11 & 7.12). Tumours grown on the back of isogeneic BD-9 rats for 23-27 days also expressed iNOS. iNOS expression was greatest at the periphery of tumours (see Fig. 8.3), with little or no staining nearer their necrotic centres.

9.8 The effects of LPS and NOS inhibitors administered singly or in combination on the growth of solid tumours. A single injection of LPS (4mg/Kg, i.p.) significantly retarded tumour growth when administered 5 days or 11 days after tumour implantation. The maximum delay of growth was ~5 days after which the rate of growth returned to control levels. Interestingly, no effect was seen when LPS was given 24hrs prior to implantation suggesting that the transplantation of tumour cells into the host was not impeded by the LPS-induced immune response. Chronic oral administration of either L-NAME or AG in the drinking water of rats (concentration = 1mg/ml) significantly delayed tumour growth, although the inhibitory effect of L-NAME was considerably greater than that of AG. Growth rates returned to control levels when L-NAME was removed from the drinking water. A single injection of LPS to animals being fed L-NAME *potentiated* the growth retarding effects of L-NAME but *inhibited* those of AG. The precise reason for this difference is not clear, but it is conceivable that the hypertension caused by L-NAME after the effect of LPS had worn off may further act to suppress tumour growth. Presumably, the *preferential* inhibition of iNOS by AG would mean that these animals would not experience hypertension. This supports the view that altered haemodynamic state in the host *per se* is a prime determinant of solid tumour growth.

Bacterial endotoxin is currently undergoing clinical trials as a potential therapy against cancer (Engelhardt *et al.*, 1991) and interleukin-2 (IL-2), which also mimics the haemodynamic changes which occur in septic shock (Ognibene *et al.*, 1986), has been shown to have considerable anti-cancer properties (Rosenberg *et al.*, 1989). The results of the present study indicate that LPS adminstered on its own, although able to significantly retard tumour growth,

unfortunately also increases the incidence of lung metastases. Further studies are needed to confirm this.

9.9 The effects of NOS inhibitors on spontaneous vasoactivity in isolated TEAs. Spontaneous rythmic fluctuations in perfusion pressure are a unique characteristic of some isolated TEAs (~20% in the present study). They were never seen in CEAs. The rythmical increases in perfusion pressue are thought to reflect spontaneous vasoconstriction in TEAs and *in vivo* may be responsible for the intermittent blood flow through tumours in animals (Intaglietta *et al.*, 1977; Peters *et al.*, 1980) and/or humans (Chaplin &Trotter, 1992). Indeed, studies on the temporal and spatial changes in tumur blood flow at the microregional level have shown that blood vessels in experimental tumours can experience periods of non-perfusion lasting several minutes (Trotter *et al.*, 1989; Jirtle, 1988) which may ultimately lead to acute hypoxia within these tumours (Chaplin *et al.*, 1987). The present study shows that aminoguanidine (AG; 1mM) potentiates the frequency and amplitude of TEA-associated oscillations and this effect can be reversed with L-arginine. Moreover, the effect of AG was not further potentiated when L-NAME (80-250μM) was added to the Krebs + AG solution. These results show that the L-arginine/NO pathway is involved in this phenomenon and that NO probably acts to suppress spontaneous vasomotor activity.

Because NOS inhibitors increase the pulsatile vasoconstriction of feeding vessels and reduce blood flow into a solid tumour, areas within the tumour not receiving adequate nutrients will increase considerably and as a result the number of acutely hypoxic tumour cells will also increase. It is therefore likely that there exists a potentially important therapeutic advantage to using NOS inhibitors in combination with bioreductive agents which specifically target hypoxic cells.

10. Conclusions. The inherently inadequate blood supply into solid tumours was initially seen as an obstacle to the delivery of anti-cancer drugs. Increasing opinion and overwhelming evidence now see it as a remarkable therapeutic window through which tumours may be starved to death with agents which act to reduce tumour blood flow. It is unlikely that constriction of feeding arterioles is sufficent on its own to facilitate tumour regression. It is more likely that a combination of vasoconstrictor and bioreductive agents will prove to be more effective.

The present study has confirmed much of the work by Kennovin *et al.*, (1993, 1994) and provided immunohistochemical evidence that solid tumours and their host-derived supply vessels express iNOS. It also shows that inhibition of iNOS significantly retards tumour growth, indicating the pro-tumourogenic role of NO in the absence of any artificial stimulation of the immune response.

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Appendices

Appendix 3.1

Estimating NOS activity using Western Blot Analysis.

3.1.1 Preparation of tissue extracts (ie. Heart, liver, lung, tail artery and tumour)

Tissue was dissected out from the rat, wearing gloves and using sterile forceps /scalpel, placed into small universal containers and immediately frozen in liquid nitrogen until homogenizing. An estimate of total tissue volume was made.
N.B. Sterile technique was maintained throughout the procedure.

3.1.2 Tissue Homogenization

Lysis buffer- (TE buffer):- 0.05M Tris(hydroxymethyl)methylamine (Tris; F.W.= 121.14, BDH, AnalaR) = 6.05g, 0.001M Ethylenediaminetetraacetic acid (EDTA; Disodium, F.W.= 372.24, BDH AnalaR) = 0.372g into a litre of distilled water (DH2O). Adjust pH to 7.4 using conc. HCL.

Immediately before use add protease inhibitors:-
0.1M Dithiothreitol (DTT; SH reducing agent, prevents S-S polymers forming). MW=154.3 ie 86mg/5.6mls DH2O.
0.1M Phenylmethylsulphonyl fluoride (PMSF) MW=174.2. ie 87mg/5mls (in either methanol or isopropanol).
Trypsin inhibitor(TI) (from Sigma) (1mg/ml DH2O). This has been previously aliquoted and stored at -20°C.
Antipain (from Sigma; 1mg/mlDH2O). This is aliquoted into 50μls and stored at -20°C.
Pepstatin A (500μg/ml). Dissolve 5mg/10mls of 1% Acetic acid. This is stored at -20°C until use.

		Final Conc.
To 10mls of TE buffer add	100μl of 0.1M DTT	0.001M
	100μl of 1mg/ml TI	10μg/ml
	100μl of 0.1M PMSF	0.001M
	10μl of 1mg/ml Antipain	1μg/ml
	20μl of 500μg/ml Pepstatin A	1μg/ml

Tissue extracts were removed from their universal containers and placed into ready cooled beakers with 5x vol. of ice cold TE buffer to tissue vol. The tissue was then diced up using sterilized scissors and homogenized over ice with an Ultra-turrax T25 (Janke & Kunkel; IKA) for 3-5mins at a speed of 24,000 revs./min.

3.1.3 Ultracentrifuging tissue homogenate

The tissue homogenate was then pipetted from the beaker into ice cold Beckman ultracentrifuge tubes (1/2" x 2" approx. vol. = 5mls) and immediately ultracentrifuged at 100,000 xG for 1hr (at 4°C; Using the SW55Ti Beckman rotars, a rotor speed of approx. 23,000 rpm. = relative centrifugal field (xg) of 100,000 at r_{max}).
It was important when doing this that the centrifuge tubes were filled almost to the brim and were balanced on the rotar. The outsides of the tubes must by dry before being placed into the rotor buckets.

After centrifugation the supernatant was carefully pipetted into clean vials and stored at -80°C.

3.1.4 Protein estimation of tissue extracts.

Protein contents of tissue samples were estimated using the Bradford method (Merck), measuring range 0.1-1.4 mg/ml and the Biuret method (Merck) for measuring over a range of 1-10mg/ml.
Spectrophotometric measurments were carried out using disposable plastic cuvettes (5mls) and gloves were worn at all times.

Bradford Method (measuring range 0.1-1.4mg/ml)

A stock of standard solution(10mg/ml) was prepared by adding 100mg of bovine serum albumin; Sigma) (BSA) into 10mls of DH20 and is diluted over a range of 0.1mg/ml to 1.4 mg/ml in steps of 0.2mg/ml. This standard solution was aliquotted and stored at -20°C.
Each cuvette contained 50μl of sample solution (diluted into TE buffer in a ratio of 1: 5, ie 10μl into 40μl) or 50μl of standard solution (0.1-1.4mg/ml) and 2.5 mls of Bradford reagent. The reference cell contained an equal volume of TE buffer + 2.5mls of Bradford reagent. Solutions were left to sit for 2 minutes before readings were taken. Absorbance was measured at 595nm.

Biuret Method (measuring range 1-10g/l)

A stock of standard solution was prepared by dissolving exactly 1g of BSA into 100mls of DH20. This was then diluted: 8g/l, 6g/l, 4g/l, 2g/l, 1g/l. The stock was aliquotted and stored at -20°C.
Each cuvette contained 0.5mls of sample solution (diluted in TE buffer, 1: 5 ratio) or 0.5mls of standard solution (1-10g/l) and 2mls of Biuret reagent. The reference cell was similar to that for the Bradford method. The cuvetted solutions were allowed to sit at room temp. for 30 mins. before absorbance readings were taken. Absorbance was read at 546nm.

Evaluation of results

An estimation of protein concentrations in the tissues was made from a calibration curve: plotting the measured absorbance readings against the corresponding protein standards. A linear curve fit was applied to the graph and the gradient equation ($\underline{Y}$=mX+c) and r^2 value was noted. The $\underline{Y}$ value was be replaced for the absorbance readings obtained with the sample solution to calculate the X value. The approximate protein concentration in the tissue sample was estimated by multiplying the value of X by five, thus allowing for the previous dilution factor. (See above; The r^2 value must ≥ 0.995)
BSA has been suggested to result in a slight underestimation in protein concentration.

3.1.5 Preparing Samples

Lung, liver and heart samples were prepared at a protein concentration of 2mg/ml by diluting in TE buffer ; so if 20μl of sample was loaded onto the gel then 40μg of protein would theoretically be present. Artery samples were prepared as near to 1mg/ml as is possible and tumour samples are prepared at 5mg/ml. The final protein concentration used depends on how much protein was estimated to be in the samples initially.
Once diluted, 2 volumes of a particular sample were added to 1 volume Laemmli sample buffer [x3] containing 15% b-mercaptoethanol (BME; Sigma). The BME

was added immediately prior to use. The sample was then boiled in this solution for 5-10 mins to denature the proteins and break disulphide bonds.

Laemmli Buffer [x3]:

Glycerol	3ml
20% SDS	1.3ml
0.625MTris	3ml (pH=6.8)
0.1% Pyronin Y	0.9ml
DH2O	1.3ml

Samples were then either applied to the gel or stored at -20°C.
N.B. Samples were [2/3] at this stage : a sample diluted to 1mg/ml was now 2/3mg/ml; so 15μl of sample/gel lane = 10μg of protein.

3.1.6 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.1.6.1 Preparing Gels;- Running gel / Stacking gel. (Volumes are for two gels).
Using two clean (25ml) conical flasks into which prepare:-

	Running gel (7.5%)	Stacking gel (4%)
DH2O	15.7mls	5.9mls
acrylamide (29.2%)/ bis-acrylamide (0.8%)	8 mls	1.5 mls
1.5M Tris buffer (pH 8.8)	8 mls	-
0.5M Tris buffer (pH 6.8)	-	2.5 mls
10% SDS	0.32 mls	0.1 ml

N.B. Sodium dodecylsulfate (Lauryl sulfate) electrophoresis reagent (Sigma, F.W. 288.4).

A loosely fitting lid was placed onto each flask and they were placed into a vacuum chamber for 1hr (R.T.). This procedure removed the soluble oxygen from the solutions, thus inhibiting polymerization (This procedure may not be necessary with concentrations of ammonium persulfate; APS) >5%).

Polyacrylamide gels are composed of chains of polymerised acrylamide which become cross-linked by a bifunctional agent such as N,N'-methylene-bis-acrylamide. The separation of proteins onto the gel depends on the concentration of polyacrylamide and the amount of cross-linking.
A higher ratio of acrylamide monomer to N,N'- methylene-bis- acrylamide cross-linker allows most proteins to migrate faster and will also promote easier transfer of immunoblots.

3.1.6.2 Preparation of gel plates

The glass gel plates were prepared while the gel solutions were being degassed.

1) The glass plate were washed in decon 90 (Decon lab. LTD, E. Sussex) and rinsed in DH20. This was followed by a further wash in 70% alcohol and a final rinse in DH20.
2) The plates were assembled vertically in the MV2-DC apparatus (Anachem LTD) with the large plate at the back. Side spacers were then placed between the plates and tightened together in the front of the rack, ensuring that the spacing card could easily fit into the gap between them. The side clamps were tightened, the sample combs (1mm width) placed between the plates and a mark* was made 1cm below the teeth level. The combs were then removed and the plates were placed to the back of the rack.

3.1.6.3 Gels

Running gel: Pipette 2mls of running gel into a universal container and add 80μl of 10% APS followed by 8μl of N,N,N',N'-Tetramethylethylenediamine (TEMED).
1ml of this was then pipetted into the corners of each gel, ensure that the bottom of each gel was covered. This sealed the bottom before the rest of the running gel was added.
To the rest of the running gel add 160μl of 10% APS and 15μl of TEMED. Polymerization starts within ~10 minutes during which time the gel was pipetted between the glass plates up to the level of the mark*. It is important not to aerate this gel. A thin layer of water-saturated isobutanol (10%) was carefully pipetted over the running gel to create a barrier to oxygen, inhibiting the polymerization of the acrylamide. Allow the gel to set for ~30minutes.
N.B. It is important to remove air bubbles from the bottom of the running gel to prevent the gel front from being distorted.

After the gel was set, the isobutanol solution was poured off and the top of the running gel was washed with DH20, drained and dried with blotting paper.

Stacking gel: To the flask containing the stacking gel add 130μl of 10% APS and 20μl of TEMED. Using a 1ml pipette the remaining area between the plates was filled by pouring this gel solution directly onto the polymerized running gel and placing the appropriate comb (ie curtain or 24 tooth comb) into it without trapping air bubbles underneath any of the comb teeth. The stacking gel was allowed to set for ~ 10minutes.

During this time the samples, molecular weight markers (M_rM) and electrode buffer (E.B.) were be prepared.

M_rM: Calibration proteins for SDS gel electrophoresis were bought from combithek, Boehringer Mannheim Biochemica and Amersham, Life Science. The combithek contains eight calibration proteins [0.1-0.2/0.2ml buffer each with 50% glycerin (v/v)] over a M_r range of ~14,000 to 340,000. 10μl of each protein was mixed together in 80μl of laemmli sample buffer. 5μl of this mixture was applied per gel lane. This protein mixture is stable for 4months when stored at -20°C.
Rainbow M_rM from Amersham were bought ready mixed containing seven proteins [1mg/ml] over a M_r range of 14,300 to 220,000. This mixture was diluted before use in laemmli sample buffer, 1:1 with 20μl/gel lane for a large gel (16-20cm).

E.B(pH8.3):	(2litres)[x10]	0.1% SDS	(require 20g)
		0.025M Tris	(require 60g)
		Glycine	(require 288g)

Dissolved in 2l of DH20 and diluted 1:10 for use.

3.1.6.4 Preparing the gels for samples.

The gelled plates were lifted from the preparatory platform and placed into the electrophoresis chamber and clamped in. The combs were removed and E.B. was poured into the top reservoir between the gels, completely filling the reservoir and spilling over between the glass plates. The teeth of the stacking gel usually needed straightening at this stage and was carried out using a needled syringe and squirting E.B. into each well. The wells were washed thoroughly to remove any unpolymerised acrylamide. E.B. was also placed into the lower reservoir to cover

the electrodes running along the bottom of the chamber and any air bubbles were removed from the bottom of the gel using the syringe.

3.1.6.5 Loading samples

Thawed samples were pipetted slowly into each gel lane (15μl-20μl) to ensure the sample was kept as a tight layer and a note was made of the sample order along the gel. A M_rM was run with each gel. Care was taken not to spill samples into neighbouring lanes. Samples may sometimes need to be centrifuged at 13,000g for 5 minutes before loading.

3.1.6.6 Electrophoresis

Electrophoretic conditions vary depending on the size, thickness and number of gels being run. The current increases in proportion to the number of gels or gel thickness. eg. 2 gels require twice the current of 1 but the same voltage (V) . Longer gels require proportionally higher voltages or longer running time. During this procedure the gel plates are kept cool by water circulating around them.
Gels were usually run at 120-150V which allows ~ 1hr for the dye front to reach the bottom of the gel. The power was then turned off and the plates were removed from the electrophoretic chamber.

3.1.6.7 Staining gels or Western blotting.

The plates were laid flat on a clean surface and carefully prised apart using a spatula. This usually meant removing the side spacers and levering from this gap. Once the plates were separated, the gel was nicked at one corner with a clean razor blade. This nick was closest to the 1st gel lane and is important when identifying proteins from the western blot.

Fixing and staining gels with coomassie brilliant blue
Coomassie brilliant blue R250 (Sigma, FW = 826) is a triphenylmethane textile dye which is suitable for detecting proteins on PA gels. A stock of 2 litres was made up as follows:- 1.5g of coomassie blue was dissolved into 900mls of methanol: DH20 (1:1 v/v) and 200mls of glacial acetic acid. This solution was then filtered through a No. 1 Whatman filter to remove any particulate matter.
The gel was immersed in at least 5 volumes of stain and agitated for a period of >4hrs. at R.T. The stain was then drained off, back into the stock, and the gel was destained.
The destain solution consists of exactly the same ingredients as the stain but lacks coomassie blue. The gel was soaked in destain overnight changing the solution at least 4 times.
After destaining the gel can be stored indefinitely in DH20 (containing 20% glycerol) in a sealed container. For a permanent record the stained gel can be photographed.

Western blotting. (Using the WB2 Western blotting unit from Anachem Origo)
After the initial electrophoretic separation of the SDS-polyacrylamide complexed proteins in the running gel a further electrophoretic transfer of the proteins from the acrylamide gels to the transfer membranes is performed.

The plates were removed from the chamber and separated carefully using a spatula. A corner relating to the 1st gel lane was nicked off using a clean razor. The gels were then carefully lifted off the glass and soaked in transfer buffer for 20 minutes.

Transfer buffer (10 litres)

Tris (AnalaR grade, pH 7.5 - 8)	30g
Glycine (Electrophoresis grade, pH 8.3)	144g
Methanol (AnalaR grade)	2 l
(MilliQ DH20	make up to 10 l)

0.1% (w/v) SDS can also be added. This anionic detergent binds to the denatured proteins which then become negatively charged and are therefore more efficiently electrolysed across from the cathode (-ve) to the anode (+ve).

At this stage the area of the gel was measured and the equivalent area of Whatman chromatography paper (3mm) cut out (x4/gel). The same size of nitrocellulose transfer membrane (BDH) was prepared and together with the blotting paper was pre-soaked in transfer buffer for 15 minutes. The fibre pads of the transfer apparatus were also pre-soaked.
N.B. If only one gel was being blotted then the smaller pads were used and a pre-cooled buffer saver block was placed into the transfer tank.

The transfer tank was half filled with transfer buffer and continually stirred to maintain uniform temp.
Once the gel had soaked for 20 minutes the half of the transfer cage corresponding to the cathode side was placed into a basin of transfer buffer and the following was placed on top of this pre-soaked fibre pad:

Two pre-soaked blotting papers.
The gel.
The pre-soaked transfer membrane.
Two pre-soaked blotting papers.
The other pre-soaked fibre pad.
The other half of the transfer cage corresponding to the +ve side.

It is important to roll out any air bubbles between the layers. This was done using a pre-wet glass rod.
The layers were then clamped together and placed into the transfer tank.The power was switched on and set to 200mA. Transfer was carried out over 2hrs.

3.1.6.8 Detecting proteins on Western blot membranes :- Using [1]Ponceau 'S' red stain (PonS), 3,3' [2]diaminobenzidine Tetrahydrochloride (DAB) or [3]enhanced chemiluminescence (ECL).

1 Prewet membrane in PBS_a.

2 Block overnight in 10% marvel (in PBS- 0.5%Triton X-100; PBS-0.5%T).

3 Wash (x3) briefly in PBS-0.5%T then for a further 15 mins.

4 Probe with 1^o antibody. Make up in 1% marvel (PBS-0.5%T). Incubate on rotator for 2hrs at R.T.

5 Wash x4-x5 mins. in PBS-0.5%T.

6 Probe with 2^o antibody in 1% marvel for 2-4hrs, R.T. on rotator.

7 Wash briefly in PBS-0.5%T (x2) then for 15mins., followed by a furhter 2 washes for 5 mins.

8 Wash in PBS_a

9 Visualise with **[1] PonS**, **[2] DAB**, or **[3] ECL.**

10 Staining procedure[1]: PonS [x10] stock

a) Trichloroacetic acid (TCA) AnalaR grade (BDH) 30g
5-Sulfosalicylic acid dihydrate (Sigma,F.W.254.2) 30g
Dissolve into 100mls DH20 then add 2g of Ponceau S sodium salt (Sigma, practical grade F.W. 760.6) and dissolve.
b) Rinse blot in PonS solution for 2-10 mins. with agitation.
c) Wash off stain with DH20 and mark the postions of the MWM with a pencil and store damp wrapped in clingfilm at 4°C.
This procedure can be used to show the presence of proteins on the blot before immuno-probing for a particular protein.

Staining procedure[2]: Nickel enhanced DAB

a) A 10mg tablet of DAB (Sigma, stored at -20°C) was thawed to R.T. and dissolved into 15mls of Tris (50mM, pH 7.6).
b) Add 1.7mls of 0.3% nickel chloride (Sigma, $NiCl_2$ Hexahydrate) and filter into a clean flask using No1 whatman.
c) Pipette in 16.7μl of hydrogen peroxide (30%, Sigma) immediately before application to blot.
d) This solution was pipetted into the bag containing the blot and the bag was then sealed.
e) The solution was left for 1-5mins. on the rotator.
f) Then rinse briefly in PBSa and allow the blot to air dry before being wrapped in clingfilm.
g) Blots were photographed within a few days as stain may fade.

Staining procedure[3]: ECL

ECL Western blotting is a light emiting non-radioactive method for detecting immobilized specific antigens, conjugated directly or indirectly with horseradish peroxidase(HRP)-labelled antibodies. Enhanced chemiluminescence is achieved by performing an HRP/hyrogen peroxide catalyzed oxidation of luminol in the presence of chemical enhancers such as phenols. The maximum light emission (l=428nm) can be detected by a short exposure to the blue-light sensitive autoradiography film (Hyperfilm ECL).

3.1.6.9 Calculating the Molecular weight of Immunolabelled Proteins.

The logarithms of molecular weight (M.W., daltons; Da) values of certain **M_rM** (Boehringer CombitheK; Boehringer Mannheim Biochemica; see appendix section 2.1.6.3) were plotted against Rf values; where Rf = Migration distance of the **M_rM** sample, measured from the start of the running gel (mm), divided by the length of the entire gel; from the start of the running gel to the gel front (indicated by Pyronin Y; mm). If the procedure had been correctly carried out, a linear calibration curve ($r^2 \geq 0.995$) was obtained from which the molecular weights of the samples were plotted.

Boehringer Combithek **M_rM**;

Calibration Proteins	**M.W.**
α_2-Macroglobulin	170,000
ß-Galactosidase	116,353

Fructose-6-phosphate	85,204
Glutamate dehydrogenase	55,562
Aldolase	39,212
Triose phosphate isomerase	26,626

10μl of each of the above standards was added to a 1.5ml eppendorf tube containing; 42μl of sterile milli-Q-water, 53μl of laemmli buffer [x3]. 5μl of 15% BME was then added and the samples were boiled for 2-10mins, cooled to R.T. and stored at -20°C. For use, 5μl of $\mathbf{M_rM}$ sample was applied per well.

From the gradient, $\underline{Y}=mX'+c$, of the plotted graph where X' can be substitued for the Rf value of the unknown immunolabelled protein to give a value for $\underline{Y}$. $\underline{Y}$ = the logarithmic M.W. of the unknown, so the anti-logarithimic value of $\underline{Y}$ = M.W. of the unknown immunolabelled protein. For iNOS the expected M.W. is ~ 130,000Da.

Appendix 3.2

Staining Histological sections using the Indirect Imunofluorescence Method

3.2.1 Tissue Preparation

1. Lung, liver, heart and tumour extracts were isolated from experimental animals and, either fixed by submersion in 1% paraformaldehyde (Fisons Lab. Reagent) overnight at 4°C or, dissected into small blocks and quick frozen in isopentane/liquid nitrogen or CO_2 snow. All arterial preparations (ie. RTAs, TEAs and CEAs) were perfuse fixed (1% paraformaldehyde) for 10-15mins at 37°C and then submersed for a further 4-6hrs at 4°C.

2 Fixed lung, liver, heart and tumour extracts were then dehydrated in 10% sucrose solution (made up in PBS_C) overnight, before being embedded in paraffin. Arteries were also submersed in 10% sucrose (2-3hrs) before being embedded in paraffin. Frozen blocks were wrapped in clingfilm and tinfoil before being stored at -70°C.

3 5-7μm sections were cut from a cryostat and thaw-mounted onto polysine slides . N.B. Frozen sections were embedded in O.C.T-Tissue-Tek, BDH for sectioning.

4 Mounted frozen sections were stored in dry, air-tight container at 4°C until use. (Used within 7-10 days of mounting). Paraffin embedded sections were deparaffinized and hydrated through a series of alcohols. They were sat in PBS_C immediately before use. A well was formed around the sections using bostik glue to contain the antibody solutions.

3.2.2 Staining Procedure

5 Rinsed briefly in fresh PBS_C before being washed in 0.1% Nonidet P40 (NP-40; nonionic detergent, Sigma) in PBS_C for 10mins at R.T.

6 Rinsed off NP-40 solution in PBS_C (2mins (x2) and placed coverslips into 0.02% sodium borohydride ($NaBH_4$; F.W.= 37.83, Sigma) solution (made up in DH2O) for 10mins. and rinse with DH2O. This prevents binding of fluorescent label to aldehydes and alcohols within the tissues.

7 Blocked non-specific staining using an appropriate 5% serum solution (ie. serum derived from the same animal as the 2° antibody was raised in, diluted in PBS_C). Alternatively used 0.1% PBS_C/bovine serum albumin (BSA). Blocked for 1hr at R.T.

8 Rinsed off blocking serum in PBS_C for 2mins (x2).

9 Diluted the 1° antibody in 5% blocking solution and flash-spun the mixture in the centrifuge at 13,000 RPM. Applied 20μl of 1° antibody to each slide and incubated at 4°C in a moist chamber overnight, making sure they were kept level.

10 Washed in 5% blocking solution for 5mins (x3).

11 Applied appropriate 2° antibody, (antibodies should be raised against animal in which 1° antibody was raised (eg. anti-rabbit for 1° rabbit polyclonal). Diluted 2° in blocking solution and flash-spun the mixture in the centrifuge at 13,000 RPM. 50μl of the 2° antibody solution was applied to each slide and they were incubated for 30-45mins at 37°C in a closed, moist chamber.

12 Washed off 2° antibody with PBS_a for 20mins. (x4-5).

13 Rinsed in DH2O.

14 Applied a small drop of mounting agent (gelvatol) to each slide and placed a coverslip onto each. Care was taken to remove any bubbles without pressing too hard onto the coverslips.

15 Slides were kept at 4°C in a sealed, dried container until the mounting agent had set before photos were taken.

3.2.3 Materials

PBS_a (per litre; [X10]) =	NaCl	80g	
	KCl	2g	
	KH_2PO4	2g,	
	Na_2HPO4	21.6g	Dissolved into 1l of DH2O.

PBS_c = [1x] PBS_a + 1mM CaCl + 0.5mM MgCl.

Isopentane- BDH; GPR, F.W.= 72.15.

Gelvatol was prepared fresh every two or three days in the following way;

Add 25g of Airvol into 100mls of 0.14M NaCl, 0.01M KH_2PO_4 / $NaHPO_4$; pH, 7.2 solution.
Mix solution and add vinol (ploy vinyl alcohol resin) slowly. Continue to mix for 16hrs at R.T. Add glycerol (AnalaR grade) and mix for a further 16hrs. Centrifuge at 12,000 rpm for 15mins at R.T. and then decant supernatant into air-tight containers for storage at -20°C. Beofre use, thaw out and add 100mg 1,4-diazabicyclo[2,2,2]octane (DABCO; F.W.= 112.2, Sigma)/ ml of gelvatol. Mix well and centrifuge (5mins at 3,000 rpm) before application to slides.

Appendix 3.3

Staining sections using the ABC method

Slide-mounted tissue sections were prepared as above, see appendix section 3.2.1.

3.3.1 Staining Procedure

1 Frozen sections were air-dried and then fixed in 1% paraformaldehyde for 5mins. Deparaffinized sections and frozen sections were then washed in PBS_a before being incubated for 30mins in 0.3% hydrogen peroxide in methanol to reduce the high background staining which may occur due to endogenous peroxidase activity.

2 Rinsed off blocking serum in PBS_c for 2mins (x2).

3 Blocked non-specific staining using 5% goat serum solution (ie. serum derived from the same animal as the 2^o antibody was raised in, diluted in PBS_c). Blocking was carried out for 1hr at R.T.

4 Blotted excess blocking solution from the slides and pipetted 20μl of 1^o antibody; 2464 (1:750-1:1000; diluted in 5% blocking solution and flash-spun the mixture in the centrifuge at 13,000 RPM.) on each section. Slides were incubated for 1hr at R.T.

5 Rinsed off in PBS_c for 2mins (x2).

6 Sections were then incubated in the dark for 30mins in biotinylated 2^o antibody; goat anti-rabbit IgG, (50μl/section; pre-diluted in 5% blocking serum) at R.T.

7 Rinsed off in PBS_c for 5mins (x2).

8 Incubated sections for a further 30mins in the dark with VECTASTAIN® *Elite* ABC Reagent; avidin-biotinylated horseradish peroxidase macromolecular complex.

9 Rinsed off in PBS_c for 2mins (x2).

10 Sections were subsequently incubated for 2-5mins in peroxidase substrate solution:
A 10mg tablet of DAB (Sigma, stored at -20°C) was thawed to R.T. and dissolved into 15mls of Tris (50mM, pH 7.6).
b) 1.7mls of 0.3% nickel chloride (Sigma, $NiCl_2$ Hexahydrate) was added to this and filtered into a clean flask using No1 whatman.
c) 16.7μl of hydrogen peroxide (30%, Sigma) was pipetted into this immediately before application to the slides. N.B. All of the above solutions were made up in tinfoil-covered beakers.

11 Sections were rinsed carefully under the tap water and then counterstained with eosin (submersion 1-2mins), before finally being dehydrated through a series of ethanol dilutions; 50%-100% (30sec -2mins in each), cleared using histoclear (3mins submersion) and mounted with coverslips using DPX solution.

3.3.2 Materials

3,3' Diaminobenzedine tetrahydrochloride tablet- DAB; Sigma. Suspected carcinogen; gloves were worn during experiment.

DPX- 10g of distrene 80, 5ml of dibutylphalate and 35ml of xylol; BDH.

Vectastain® *Elite* ABC kit- Goat anti-rabbit IgG; Vector Labs, CA, USA.

Appendix 3.4

Staining for NADPH-Diaphorase

Slide-mounted tissue sections were prepared as above, see appendix section 3.2.1 although, 10-15μm sections were used in this procedure.

3.4.1 Staining Procedure

Solutions of nitroblue tetrazolium (Sigma; 0.4mg/ml; [0.48mM]) and the reduced form of ß-nicotinamide adenine dinucleotide phosphate (ß-NADPH) diaphorase (Sigma; 2mg/ml; [2.39mM]) were prepared separately in freshly made 0.1M PBS solution, pH 7.4. Solutions were made in tinfoil-covered beakers and when they had dissolved, following stirring with a glass rod, they were mixed together and applied to the sections. Sections were incubated for 20-35mins in the dark at 37°C. The sections were then rinsed in PBS solution, dehydrated, cleared and mounted with coverslips as for appendix section 3.3.1 (No 11).

3.4.2 Materials

0.1M PBS solution, pH 7.4 (500mls); 6.8g disodium hydrogen orthophosphate dihydrate (BDH, AnalaR, F.W.= 177.99) was added with 1.25g sodium dihydrogen orthophosphate dihydrate (BDH, AnalaR, F.W.= 155.99) to 500mls of DH2O and dissolved at R.T.

Appendix 3.5

SPECTROPHOTOMETRIC DETERMINATION OF NITRITE AND NITRATE LEVELS USING NITRATE REDUCTASE ENZYME.

SOLUTIONS

Phosphate buffer solution pH 7.5 made up with :

1 Potassium Dihydrogen Orthophosphate (AnalaR) **M.W. = 136.09**
2 DiPotassium Hydrogen Orthophosphate trihydrate (AnalaR) **M.W. = 228.23**

Make up 500mls, [400mM] stock solution of each and from these make up 100mls of buffer by mixing solution 1 with solution 2 in the ratio of 2:8 respectively. Check that the pH is 7.3. The PBS is kept in the fridge until needed.

By adding 22.5μl of the mixed PBS to each tube a final concentration of 40mMPBS (pH 7.3) is present.

Diazochromophore agents

Solution A is 1% sulfanilamide in 5% conc. phosphoric acid in distilled water.
Solution B is 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in distilled water.
Make up stock solutions of both (500mls), which can be kept separately in the fridge for up to 2months.

β- Nicotinamide Adenine Dinucleotide Phosphate, Reduced form (β-NADPH)
M.W. = 833
NADPH is made up fresh before each experiment, by adding 4 mg of NADPH into 1 ml of
distilled water giving a solution of 4.8mM. By adding 22.5μl of this into each test tube a final concentration of 480μM NADPH is present.

Flavin adenine dinucleotide (FAD) solution
M.W. = 829.5

The FAD is made up to 100x the final concentration. A final concentration of 48.2μM is used.
The FAD can be kept frozen at -20°C.
Prepare 4mg of FAD in 10mls of distilled water, this gives a 482μM solution.
22.5μl of this is then added into each test tube to give a final concentration of 48.2μM.

Nitrate Reductase enzyme
Nitrate reductase(NR) from the Aspergillus species is in powder form. The batch size is 20Units and we require 70mU of NR per 127μl sample, so by diluting the 20U into 2mls of distilled water it can be calculated that 7μl of this solution will contain 70mU. This stock enzyme solution is kept frozen at -20°C.

Standards; No_2^- and No_3^- solutions

Using sodium nitrite (**M.W.= 69**) and Sodium nitrate (**M.W. = 84.99**) (both AnalaR) **2mM** stock solutions are made of each (in distilled water).

PROTOCOL (1): To DETERMINE No_X^- LEVELS IN 18 hr and 24 hr LPS SERUM SAMPLES.

Prepare 6 **1ml** tubes into which a final total of **0.672mls** of solution is going to be added. Make up the NADPH solution fresh each day, and prepare serial dilutions of internal standard i.e. No_3^- at **2mM, 1mM, 0.7mM, 0.5mM , 0.1mM** from the stock solutions. (with > 2mls for each concentration).

Inorder to ensure that any precipitate in the serum sample is removed, it is centrifuged at 13,000 rpm for 15mins before use.

Into 5 of the tubes add the following:

127μl of sample. (i.e. serum).

22.5μl of 400mM mixed PBS (pH 7.3).

22.5μl of NADPH solution

22.5μl of FAD solution

Internal Standardization; Into each of the 5 tubes add the following:

22.5μl of $N0_3^-$ of the previously made dilutions:-		**Amount Present**
1	**2mM**	**45nmoles**
2	**1mM**	**22.5nmoles**
3	**0.7mM**	**15.75nmoles**
4	**0.5mM**	**11.25nmoles**
5	**0.1mM**	**2.25nmoles**

THEN ADD 7μl of Nitrate Reductase enzyme TO EACH.

Incubate aliquots for 2hrs at 37°C.

Then add **224μl of Solution A and 224μl of Solution B to each tube and incubate for a further 15mins at 37°C.**
Read absorbances on the spectrophotometer at a wavelength of 540nm.
REFERENCE CELL

The reference cell for $N0_X^-$ levels in serum should contain 127μl of serum sample, 22.5μl of NADPH solution, 22.5μl of PBS solution, 224μl of solution A, 22.5μl of 2mM $N03^-$ internal standard and 253.5μl of distilled water (which takes into account the 22.5μl of FAD solution, 224μl of solution B and 7μl of N.R.).

PROTOCOL (2): To DETERMINE No_2^- LEVELS IN 18 hr and 24 hr LPS SERUM SAMPLES.

Prepare 6 **1ml** tubes into which a final total of **0.672mls** of solution is going to be added. Make up the NADPH solution fresh each day, and prepare serial dilutions of internal standard i.e. No_2^- at **2mM, 1mM, 0.7mM, 0.5mM , 0.1mM** from the stock solutions. (with > 2mls for each concentration.)

Inorder to ensure that any precipitate in the serum sample is removed, it is centrifuged at 13,000 rpm for 15mins before being read.

Into 5 of the tubes add the following:

127μl of sample. (i.e. serum).

22.5μl of 400mM mixed PBS (pH 7.3).

52μl of distilled water.

Internal Standardization; Into each of the 5 tubes add the following:

	22.5μl of $N0_2^-$ of the previously made dilutions:-	**Amount Present**
1	2mM	45nmoles
2	1mM	22.5nmoles
3	0.7mM	15.75nmoles
4	0.5mM	11.25nmoles
5	0.1mM	2.25nmoles

Incubate aliquots for 2hrs at 37°C.

Then add **224μl of Solution A and 224μl of Solution B to each tube and incubate for a further 15mins at 37°C.**

Read absorbances on the spectrophotometer at a wavelength of 540nm.

REFERENCE CELL

The reference cell for $N0_2^-$ levels in serum should contain 127μl of serum, 22.5μl of PBS solution, 224μl of solution A, 22.5μl of 2mM $N0_2^-$ internal standard and 276μl of distilled water (which takes into account the 224μl of solution B) .

PROTOCOL (3): THE STANDARD CURVE FOR No_3^- READINGS (FOR 18hr AND 24hr LPS SAMPLES).

Prepare 6 **1ml** tubes into which a final total of **0.672mls** of solution is going to be added. Prepare serial dilutions of internal standard i.e. **No_3^-** at **2mM, 1mM, 0.7mM, 0.5mM , 0.1mM** from the stock solutions. (with > 2mls for each concentration.).

Into the 5 tubes add the following:

127μl of distilled water.

22.5μl of 400mM mixed PBS (pH 7.3).

22.5μl of NADPH solution

22.5μl of FAD solution

Internal Standardization; Into each of the 5 tubes add the following:

22.5μl of $N0_3^-$ of the previously made dilutions:		**Amount Present**
1	**2mM**	**45nmoles**
2	**1mM**	**22.5nmoles**
3	**0.7mM**	**15.75nmoles**
4	**0.5mM**	**11.25nmoles**
5	**0.1mM**	**2.25nmoles**

THEN ADD 7μl of Nitrate Reductase enzyme TO EACH.

Incubate aliquots for 2hrs at 37°C.

Then add 224μl of Solution A and 224μl of Solution B to each tube and incubate for a further 15mins at 37°C.

Read absorbances on the spectrophotometer at a wavelength of 540nm.

REFERENCE CELL

The reference cell for $N03^-$ standards contains, 22.5μl of PBS solution, 224μl of solution A, 224μl of solution B, 22.5μl of 2mM internal standard, 22.5μl of NADPH solution and 156.5μl of distilled water (which takes into account the 22.5μl of FAD and 7μl of N.R.) .

PROTOCOL (4): THE STANDARD CURVE FOR No_2^- READINGS (FOR 18hr AND 24hr LPS SAMPLES).

Prepare 6 **1ml** tubes into which a final total of **0.672mls** of solution is going to be added. Prepare serial dilutions of internal standard i.e. No_2^- at **2mM, 1mM, 0.7mM, 0.5mM , 0.1mM** from the stock solutions. (with > 2mls for each concentration.).

Into the 5 tubes add the following:

179μl of distilled water

22.5μl of 400mM mixed PBS (pH 7.3).

Internal Standardization; Into each of the 5 tubes add the following:

22.5μl of $N0_2^-$ of the previously made dilutions:-		**Amount Present**
1	**2mM**	**45nmoles**
2	**1mM**	**22.5nmoles**
3	**0.7mM**	**15.75nmoles**
4	**0.5mM**	**11.25nmoles**
5	**0.1mM**	**2.25nmoles**

Incubate aliquots for 2hrs at 37°C.

Then add **224μl of Solution A and 224μl of Solution B to each tube and incubate for a further 15mins at 37°C**

Read absorbances on the spectrophotometer at a wavelength of 540nm.

REFERENCE CELL

The reference cell for $N02^-$ levels in the standard should contain 22.5μl of PBS solution , 224μl of solution A, 22.5μl of 2mM internal standard and 403μl of distilled water (which takes into account the 224μl of solution B).

PROTOCOL (5): To DETERMINE No_X^- LEVELS IN CONTROL SERUM AND 6hr / 12hr LPS SERUM SAMPLES.

Prepare 6 **1ml** tubes into which a final total of **0.672mls** of solution is going to be added. Make up the NADPH solution fresh each day, and prepare serial dilutions of internal standard i.e. No_3^- at **0.5mM, 0.4mM, 0.3mM, 0.1mM** from the stock solutions. (with > 2mls for each concentration).
N.B. 2 mls of serum is freeze-dried and reconstituted into 0.5mls, so the final result has to be divided by 4 .
Inorder to ensure that any precipitate in the serum sample is removed, it is centrifuged at 13,000 rpm for 30 mins, precipiatate removed and then a further 10 mins; and precipitate removed again before use.

Into 5 of the tubes add the following:

100μl of sample. (i.e. serum) + 27μl of distilled water.

22.5μl of 400mM mixed PBS (pH 7.3).

22.5μl of NADPH solution

22.5μl of FAD solution

Internal Standardization; Into each of the 5 tubes add the following:

22.5μl of NO_3^- of the previously made dilutions:-	Amount Present
1 0.5mM	11.25nmoles
2 0.4mM	9 nmoles
3 0.3mM	6.75 nmoles
4 0.1mM	2.25nmoles

THEN ADD 7μl of Nitrate Reductase enzyme TO EACH.

Incubate aliquots for 2hrs at 37°C.

Then add **224μl of Solution A and 224μl of Solution B to each tube and incubate for a further 15mins at 37°C**

Read absorbances on the spectrophotometer at a wavelength of 540nm.
REFERENCE CELL
The reference cell for NO_3^- levels in serum should contain 100μl of serum, 22.5μl of PBS solution, 224μl of solution A, 22.5μl of 0.5mM NO_3^- internal standard, 22.5μl of NADPH and 280.5μl of distilled water (which takes into account the 22.5μl 0f FAD, 7μl of N.R. and 224μl of solution B).

PROTOCOL (6): To DETERMINE No_2^- LEVELS IN CONTROL SERUM AND 6hr / 12hr LPS SERUM SAMPLES.

Prepare 5 **1ml** tubes into which a final total of **0.672mls** of solution is going to be added. Make up the NADPH solution fresh each day, and prepare serial dilutions of internal standard i.e. No_2^- at **0.5mM, 0.4mM, 0.3mM, 0.1mM** from the stock solutions. (with > 2mls for each concentration.)
N.B. 2 mls of the control serum is freeze-fried over-night, and is reconstituted into 0.5mls (ie 4x original concentration). So the final result has to be divided by 4.
Inorder to ensure that any precipitate in the serum sample is removed, it is centrifuged at 13,000 rpm for 30 mins, precipiatate removed and then a further 10 mins; and precipitate removed again before use.

Into 5 of the tubes add the following:

100μl of sample. (i.e. serum).

22.5μl of 400mM mixed PBS (pH 7.3).

79μl of distilled water.

Internal Standardization; Into each of the 5 tubes add the following:

22.5μl of $N0_2^-$ of the previously made dilutions:-	**Amount Present**
1 0.5mM	**11.25nmoles**
2 0.4mM	**9nmoles**
3 0.3mM	**6.75nmoles**
4 0.1mM	**2.25nmoles**

Incubate aliquots for 2hrs at 37°C.

Then add **224μl of Solution A and 224μl of Solution B to each tube and incubate for a further 15mins at 37°C.**

Read absorbances on the spectrophotometer at a wavelength of 540nm.

REFERENCE CELL

The reference cell for $N02^-$ levels in serum should contain 100μl of serum, 22.5μl of PBS solution, 22.5μl of 2mM $N0_2^-$ internal standard, 224μl of solution A and 303μl of distilled water (which takes into account the 224μl of solution B).

PROTOCOL (7): THE STANDARD CURVE FOR No_3^- READINGS (for control and 6hr/12hr LPS samples).

Prepare 5 **1ml** tubes into which a final total of **0.672mls** of solution is going to be added. Prepare serial dilutions of internal standard i.e. **No_3^-** at **.0.5mM, 0.4mM, 0.3mM, 0.1mM** from the stock solutions. (with > 2mls for each concentration.).

N.B. 2 mls of serum is freeze-dried and reconstituted into 0.5mls, so the final result has to be divided by 4 .

Inorder to ensure that any precipitate in the serum sample is removed, it is centrifuged at 13,000 rpm for 30 mins, precipiatate removed and then a further 10 mins; and precipitate removed again before use.

Into the 4 tubes add the following:

127μl of distilled water.

22.5μl of 400mM mixed PBS (pH 7.3).

22.5μl of NADPH solution

22.5μl of FAD solution

Internal Standardization; Into each of the 5 tubes add the following:

22.5μl of $N0_3^-$ of the previously made dilutions:	**Amount Present**
1 0.5mM	**11.25nmoles**
2 0.4mM	**9nmoles**
3 0.3mM	**6.75nmoles**
4 0.1mM	**2.25nmoles**

THEN ADD 7μl of Nitrate Reductase enzyme TO EACH.

Incubate aliquots for 2hrs at 37°C.

Then add **224μl of Solution A and 224μl of Solution B to each tube and incubate for a further 15mins at 37°C.**
Read absorbances on the spectrophotometer at a wavelength of 540nm.
REFERENCE CELL

The reference cell for $N03^-$ standards contains, 22.5μl of PBS solution, 224μl of solution A, 224μl of solution B, 22.5μl of 2mM internal standard, 22.5μl of NADPH solution and 156.5μl of distilled water (which takes into account the 22.5μl of FAD and 7μl of N.R.).

PROTOCOL (8): THE STANDARD CURVE FOR No_2^- READINGS (for control and 6hr/12hr LPS samples)

Prepare 5 **1ml** tubes into which a final total of **0.672mls** of solution is going to be added. Prepare serial dilutions of internal standard i.e. No_2^- at **0.5mM, 0.4mM, 0.3mM, 0.1mM** from the stock solutions. (with > 2mls for each concentration.).

Into the 4 tubes add the following:

179μl of distilled water

22.5μl of 400mM mixed PBS (pH 7.3).

Internal Standardization; Into each of the 5 tubes add the following:

22.5μl of $N0_2^-$ of the previously made dilutions:-		**Amount Present**
1	**0.5mM**	**11.25nmoles**
2	**0.4mM**	**9nmoles**
3	**0.3mM**	**6.75nmoles**
4	**0.1mM**	**2.25nmoles**

Incubate aliquots for 2hrs at 37°C.

Then add **224μl of Solution A and 224μl of Solution B to each tube and incubate for a further 15mins at 37°C**

Read absorbances on the spectrophotometer at a wavelength of 540nm.

REFERENCE CELL

The reference cell for $N02^-$ levels in the standard should contain 22.5μl of PBS solution , 224μl of solution A, 22.5μl of 2mM internal standard and 403μl of distilled water (which takes into account the 224μl of solution B).

Appendix tables

Appendix table (3.1) to Fig. 3.5.

Blood Pressure & Heart Rate before and after LPS injection

Time	Systolic			Diastolic			Mean			Heart Rate		
(hrs)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
-96	163.00	8.23	9	86.80	2.60	9	108.70	2.80	9	-	-	-
-48	152.00	10.60	9	89.70	2.30	9	111.50	2.90	9	-	-	-
0	150.30	3.59	5	89.00	4.09	5	109.56	4.38	5	313.90	31.00	6
6	156.90	7.27	10	75.70	4.38	10	102.80	2.88	10	299.30	13.50	10
12	140.30	4.00	10	73.00	3.60	10	91.50	3.46	10	275.20	11.60	10
18	129.30	5.88	9	76.90	4.40	9	97.90	3.90	9	251.30	15.90	9
24	115.50	5.76	10	71.90	2.78	10	86.90	3.13	10	283.40	16.70	10
Student's unpaired t-test p values												
t= 6hrs	0.480	10		0.062	10		0.339	10		0.505	10	
t= 12hrs	0.108	9		0.009	8		0.076	10		0.358	10	
t= 18hrs	0.019	8		0.064	9		0.198	9		0.011	9	
t= 24hrs	0.000	10		0.005	10		0.016	10		0.646	10	

Appendix table (3.2) to Fig. 3.6.

Nitrate and nitrite levels in blood plasma

Time post-LPS	NO_X (μmoles/l)	S.E.	n	Mean % conversion	unpaired t-test
(control) 0	26.4	6	6	88.7	from 0hr
6	50.6	18.67	4	93.6	0.182
12	81.8	27.9	2	93.1	0.018
18	384.8	97.7	4	90.9	0.002
24	463.1	50.3	10	93.1	0.000
48	247.0	41.6	4	93.1	0.000
72	19.7	6.28	4	91.2	0.284

Appendix table (3.3) to Fig. 3.7

The time-dependent hyporesponsive effect of LPS.

[PE]	Control			6hr			12 hr		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	11.38	1.08	18	3.32	0.84	16	5.78	3.47	4
$2x10^{-5}$	18.26	1.88	18	8.81	1.15	16	5.78	1.49	4
$5x10^{-5}$	34.12	3.49	18	23.58	3.20	16	13.80	4.00	4
$1x10^{-4}$	51.40	5.34	18	47.22	5.00	16	26.01	4.60	4
$2x10^{-4}$	83.45	7.12	18	70.90	6.00	16	50.30	8.20	4
$5x10^{-4}$	133.80	8.39	18	134.75	8.70	16	80.90	11.60	4
$1x10^{-3}$	182.00	10.30	18	182.56	8.12	16	154.30	16.50	4
$2x10^{-3}$	226.00	9.83	18	243.95	9.19	16	189.05	15.70	4
$5x10^{-3}$	262.70	11.60	7	296.20	8.86	16	234.14	8.80	4
$1x10^{-2}$	286.30	13.50	2	-	-	-	258.99	7.40	4

[PE]	18 hr			24 hr			72 hr		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	3.20	0.69	12	3.23	0.70	15	16.18	4.52	4
$2x10^{-5}$	6.47	1.48	12	5.85	1.00	15	19.65	6.70	4
$5x10^{-5}$	15.90	3.30	13	12.48	2.40	15	35.25	9.21	4
$1x10^{-4}$	26.49	5.90	13	22.65	3.00	15	53.17	10.55	4
$2x10^{-4}$	41.60	9.80	13	34.37	3.81	15	69.36	17.37	4
$5x10^{-4}$	79.40	12.27	13	56.47	4.90	14	123.11	31.86	4
$1x10^{-3}$	123.00	11.87	13	75.47	5.00	14	179.11	23.32	4
$2x10^{-3}$	165.70	13.30	13	111.47	6.35	14	232.93	25.35	4
$5x10^{-3}$	218.30	12.99	12	149.35	9.60	15	281.15	28.20	4
$1x10^{-2}$	221.00	13.78	8	176.79	10.65	15	282.02	30.20	4

48 hr data

[PE]	$1x10^{-5}$	$2x10^{-5}$	$5x10^{-5}$	$1x10^{-4}$	$2x10^{-4}$	$5x10^{-4}$	$1x10^{-3}$	$2x10^{-3}$	$5x10^{-3}$	$1x10^{-2}$
Mean	1.15	10.98	49.71	62.42	83.23	150.8	206.4	241.6	266.5	265.3
S.E.	0.66	1.73	6.08	9.85	7.49	16.16	11.91	12.32	19.12	12.39
n	4	4	4	4	4	4	4	4	4	4

Appendix table (3.4) to Fig. 3.8

SNAP	Control			LPS (24 hr)			t-test
(M)	Mean	S.E.	n	Mean	S.E.	n	unpaired
$1x10^{-7}$	13.90	2.30	8	3.50	1.50	4	0.014
$1x10^{-6}$	23.80	4.35	7	5.30	1.70	4	0.013
$1x10^{-5}$	40.35	5.56	8	11.10	1.70	4	0.005
$1x10^{-4}$	61.80	2.26	8	30.30	8.80	4	0.001
$1x10^{-3}$	69.30	2.10	8	68.89	5.90	4	0.932

Appendix tables (4.1) to Figs. 4.2-4.5

L-NMMA & L-Arg Data Tables

Control Table

[PE]	Krebs			L-NMMA (80 μM)			L-Arg (1 mM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	10.63	1.42	10	25.43	5.07	10	12.25	1.75	10
$2x10^{-5}$	15.02	2.39	10	38.37	6.67	10	17.34	3.30	10
$5x10^{-5}$	26.82	5.68	10	67.27	8.79	10	40.60	5.40	10
$1x10^{-4}$	41.38	8.23	10	92.48	10.76	10	56.80	7.70	10
$2x10^{-4}$	70.70	11.41	10	122.53	13.75	10	83.20	10.30	10
$5x10^{-4}$	115.83	13.91	10	178.25	18.09	10	149.50	14.30	10
$1x10^{-3}$	156.06	14.13	10	215.47	16.02	10	186.80	13.40	10
$2x10^{-3}$	209.00	14.95	10	249.69	18.80	10	234.20	13.40	10
$5x10^{-3}$	246.61	14.09	10	254.00	0.00	10	240.40	25.40	10
$1x10^{-2}$	256.63	16.18	10	-	-	-	263.50	27.70	10

6 Hour Table

[PE]	Krebs			L-NMMA (80 μM)			L-Arg (1 mM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	20.49	6.70	7	33.04	8.35	7	18.17	4.29	7
$2x10^{-5}$	22.80	7.81	7	33.37	6.71	7	17.51	3.53	7
$5x10^{-5}$	33.05	10.80	7	55.51	8.47	7	30.07	5.14	7
$1x10^{-4}$	51.88	11.65	7	74.34	10.23	7	49.23	4.62	7
$2x10^{-4}$	70.71	10.79	7	98.79	10.93	7	72.03	4.69	7
$5x10^{-4}$	109.03	9.00	7	138.76	9.63	7	107.71	5.44	7
$1x10^{-3}$	137.70	8.47	7	182.70	11.51	7	139.75	6.90	7
$2x10^{-3}$	179.10	13.16	7	210.12	14.40	7	175.43	10.50	7
$5x10^{-3}$	219.70	24.20	7	233.58	18.56	7	194.93	12.29	7
$1x10^{-2}$	-	-	-	-	-	-	-	-	-

18 Hour Table

[PE]	Krebs			L-NMMA (80 μM)			L-Arg (1 mM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	6.50	3.20	5	21.65	6.90	5	8.50	2.20	5
$2x10^{-5}$	12.02	4.35	5	30.50	6.50	5	13.40	4.20	5
$5x10^{-5}$	19.80	7.00	5	45.70	7.70	5	20.30	6.50	5
$1x10^{-4}$	30.50	9.40	5	76.70	13.70	5	24.90	8.40	5
$2x10^{-4}$	41.60	11.60	5	114.20	17.10	5	44.80	12.80	5
$5x10^{-4}$	78.10	18.40	5	174.30	17.20	5	63.30	18.20	5
$1x10^{-3}$	122.90	21.40	5	219.60	20.50	5	82.70	20.50	5
$2x10^{-3}$	173.80	20.20	5	245.07	19.60	5	112.80	21.70	5
$5x10^{-3}$	227.90	21.40	5	254.70	22.60	5	154.00	23.40	5
$1x10^{-2}$	228.40	15.60	5	242.10	27.90	5	154.90	20.90	5

24 Hour Table

[PE]	Krebs			L-NMMA (80 μM)			L-Arg (1 mM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	6.60	1.35	9	17.90	3.70	9	8.00	2.50	9
$2x10^{-5}$	11.04	1.70	9	24.40	3.80	9	11.00	2.27	9
$5x10^{-5}$	22.30	3.70	9	50.30	4.60	9	26.90	6.20	9
$1x10^{-4}$	32.30	4.60	9	74.70	7.60	9	42.10	6.60	9
$2x10^{-4}$	52.40	7.50	9	103.70	16.02	9	65.70	9.40	9
$5x10^{-4}$	80.90	11.70	9	142.50	18.50	9	97.80	13.70	9
$1x10^{-3}$	106.80	1.40	9	175.00	16.90	9	125.90	17.00	9
$2x10^{-3}$	142.00	15.80	9	211.60	18.00	9	159.00	20.00	9
$5x10^{-3}$	172.30	14.40	9	244.00	13.70	9	201.60	17.70	9
$1x10^{-2}$	205.50	12.90	9	246.30	16.90	9	213.03	22.10	9

Statistical tables for Fig. 4.2-4.5

Group	Control	6 Hr	18 Hr	24 Hr
[PE] (M)	Student's paired t-tests comparing Krebs and L-NMMA curves			
$1x10^{-5}$	0.022	0.014	0.078	0.005
$2x10^{-5}$	0.012	0.115	0.020	0.001
$5x10^{-5}$	0.000	0.044	0.015	0.001
$1x10^{-4}$	0.000	0.099	0.036	0.000
$2x10^{-4}$	0.000	0.094	0.008	0.003
$5x10^{-4}$	0.006	0.112	0.008	0.001
$1x10^{-3}$	0.010	0.005	0.015	0.000
$2x10^{-3}$	0.066	0.008	0.021	0.001
$5x10^{-3}$	-	0.339	0.166	0.000
$1x10^{-2}$	-	-	0.272	0.005

Group	Control	6 Hr	18 Hr	24 Hr
[PE] (M)	Student's paired t-tests comparing Krebs and L-arginine curves			
$1x10^{-5}$	0.466	0.676	0.131	0.815
$2x10^{-5}$	0.612	0.488	0.661	1.000
$5x10^{-5}$	0.022	0.820	0.775	0.491
$1x10^{-4}$	0.028	0.816	0.606	0.196
$2x10^{-4}$	0.115	0.910	0.680	0.137
$5x10^{-4}$	0.025	0.918	0.919	0.221
$1x10^{-3}$	0.077	0.821	0.783	0.221
$2x10^{-3}$	0.060	0.641	0.042	0.361
$5x10^{-3}$	-	0.102	0.005	0.194
$1x10^{-2}$	-	-	0.004	0.705

Appendix tables (4.2) to Figs. 4.6-4.10

Control Table

[PE]	Krebs			L-NAME (80 μM)			L-Arg (1 mM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	11.70	1.63	9	29.89	1.50	9	23.15	3.98	9
$2x10^{-5}$	20.59	2.76	9	39.79	3.56	9	23.27	3.20	9
$5x10^{-5}$	39.21	3.33	9	60.30	7.23	9	39.47	4.45	9
$1x10^{-4}$	58.88	5.52	9	80.93	10.85	9	58.00	6.48	9
$2x10^{-4}$	91.37	8.10	9	119.10	12.69	9	92.00	9.90	9
$5x10^{-4}$	143.85	10.57	9	178.40	17.09	9	162.50	14.50	9
$1x10^{-3}$	201.53	13.01	9	229.74	17.40	9	206.60	17.60	9
$2x10^{-3}$	237.85	12.39	9	266.09	14.20	5	243.40	16.41	9
$5x10^{-3}$	282.81	17.17	3	282.58	12.47	5	246.34	27.71	5
$1x10^{-2}$	299.82	0.00	3	-	-	-	-	-	-

6 Hour Table

[PE]	Krebs			L-NAME (80 μM)		
(M)	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	1.15	0.60	7	25.74	5.88	7
$2x10^{-5}$	8.66	2.56	7	30.03	6.74	7
$5x10^{-5}$	29.45	5.68	7	68.98	12.81	7
$1x10^{-4}$	60.63	7.20	7	86.47	16.49	7
$2x10^{-4}$	86.62	5.20	7	123.78	14.64	7
$5x10^{-4}$	156.50	8.18	7	193.40	13.12	7
$1x10^{-3}$	195.70	2.56	7	243.29	11.16	7
$2x10^{-3}$	251.70	7.48	7	268.92	13.56	3
$5x10^{-3}$	297.90	11.50	7	278.98	16.56	3
$1x10^{-2}$	-	-	-	-	-	-

18 Hour Table

[PE]	Krebs			L-NAME (80 μM)			L-Arg (1 mM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	6.08	2.53	9	25.69	9.29	9	16.55	6.24	9
$2x10^{-5}$	10.68	3.40	9	30.88	8.36	9	18.94	5.91	9
$5x10^{-5}$	18.90	3.89	9	50.13	12.86	9	32.98	8.53	9
$1x10^{-4}$	32.62	7.50	9	74.08	12.62	9	50.59	11.32	9
$2x10^{-4}$	50.60	12.80	9	114.03	12.10	9	72.70	14.70	9
$5x10^{-4}$	95.93	13.70	9	177.30	13.20	9	120.60	18.10	9
$1x10^{-3}$	141.49	9.79	9	234.18	11.90	9	158.12	19.60	9
$2x10^{-3}$	184.90	10.60	9	251.40	8.87	5	198.47	19.50	9
$5x10^{-3}$	235.94	11.90	8	280.00	11.16	5	242.80	15.60	9
$1x10^{-2}$	247.95	14.00	4	-	-	-	233.38	25.40	4

24 Hour Table

[PE]	Krebs			L-NAME (80 μM)			L-Arg (1 mM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	6.40	1.70	9	15.15	2.28	9	15.60	3.60	9
$2x10^{-5}$	10.70	2.90	9	25.60	3.60	9	21.00	4.60	9
$5x10^{-5}$	23.37	4.50	9	47.00	5.50	9	38.70	6.60	9
$1x10^{-4}$	36.20	7.10	9	66.50	6.30	9	53.90	8.70	9
$2x10^{-4}$	52.10	10.70	9	105.30	9.00	9	75.00	11.40	9
$5x10^{-4}$	95.00	16.30	9	152.60	11.90	9	107.80	13.60	9
$1x10^{-3}$	140.00	17.00	9	198.80	11.47	9	134.30	18.30	9
$2x10^{-3}$	186.20	17.80	9	245.50	8.50	9	153.10	18.00	9
$5x10^{-3}$	225.00	16.00	9	271.00	9.30	8	180.90	20.80	8
$1x10^{-2}$	251.70	12.40	9	276.00	14.80	5	195.60	14.80	8

72 Hour Table

[PE]	Krebs			L-NAME (80 μM)			L-Arg (1 mM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	12.30	4.70	6	45.50	8.37	6	23.50	5.40	6
$2x10^{-5}$	28.90	9.60	6	45.50	8.90	6	35.40	6.20	6
$5x10^{-5}$	40.10	8.49	6	74.00	11.20	6	46.20	7.90	6
$1x10^{-4}$	62.80	11.30	6	100.50	13.10	6	73.50	11.20	6
$2x10^{-4}$	98.30	15.40	6	128.00	18.10	6	104.80	17.50	6
$5x10^{-4}$	149.80	17.90	6	181.80	13.10	6	159.00	25.80	6
$1x10^{-3}$	194.50	18.00	6	227.70	12.20	6	189.00	17.50	6
$2x10^{-3}$	232.60	18.50	5	278.90	12.20	6	214.00	14.80	6
$5x10^{-3}$	278.50	20.10	4	314.40	14.20	4	256.60	11.30	6
$1x10^{-2}$	292.50	14.60	4	312.80	10.19	3	266.30	11.60	5

Statistical tables for Fig. 4.6-4.10

Group	6 Hr	18 Hr	24 Hr	72Hr
[PE] (M)	Student's unpaired t-tests comparing Krebs curves with control			
$1x10^{-5}$	0.001	0.08	0.045	0.888
$2x10^{-5}$	0.012	0.038	0.026	0.344
$5x10^{-5}$	0.063	0.001	0.013	0.916
$1x10^{-4}$	0.493	0.013	0.023	0.735
$2x10^{-4}$	0.228	0.017	0.010	0.675
$5x10^{-4}$	0.930	0.014	0.023	0.761
$1x10^{-3}$	0.734	0.002	0.011	0.753
$2x10^{-3}$	0.175	0.005	0.030	0.811
$5x10^{-3}$	0.202	0.108	0.14	0.902
$1x10^{-2}$	-	-	-	-

Group	Control	6 Hr	18 Hr	24 Hr	72 Hr
[PE] (M)	Student's paired t-tests comparing Krebs and L-NAME curves				
$1x10^{-5}$	0.000	0.008	0.048	0.001	0.019
$2x10^{-5}$	0.001	0.012	0.034	0.018	0.155
$5x10^{-5}$	0.010	0.009	0.036	0.008	0.043
$1x10^{-4}$	0.029	0.131	0.020	0.002	0.030
$2x10^{-4}$	0.012	0.040	0.014	0.000	0.093
$5x10^{-4}$	0.009	0.073	0.013	0.000	0.071
$1x10^{-3}$	0.685	0.067	0.001	0.000	0.093
$2x10^{-3}$	0.009	0.447	0.051	0.001	0.103
$5x10^{-3}$	0.500	0.207	0.253	0.01	0.262
$1x10^{-2}$	-	-	-	0.208	0.376

Group	Control	6 Hr	18 Hr	24 Hr	72 Hr
[PE] (M)	Student's paired t-tests comparing Krebs and L-arginine curves				
$1x10^{-5}$	0.008	-	0.082	0.004	0.113
$2x10^{-5}$	0.466	-	0.103	0.077	0.416
$5x10^{-5}$	0.957	-	0.152	0.011	0.428
$1x10^{-4}$	0.915	-	0.190	0.008	0.212
$2x10^{-4}$	0.960	-	0.255	0.003	0.704
$5x10^{-4}$	0.413	-	0.314	0.107	0.681
$1x10^{-3}$	0.869	-	0.334	0.586	0.798
$2x10^{-3}$	0.806	-	0.370	0.034	0.663
$5x10^{-3}$	-	-	0.627	0.074	0.436
$1x10^{-2}$	-	-	0.659	0.016	0.143

Appendix table (4.3) to Figs. 4.11 &4.12

Control Tables

[PE]	Krebs			AG (1 mM)			L-NAME (80 μM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	9.80	2.38	8	19.26	8.80	6	36.10	9.00	5
$2x10^{-5}$	22.80	6.10	8	22.30	9.80	6	36.90	7.20	5
$5x10^{-5}$	41.30	11.40	8	38.10	15.70	6	64.20	11.00	5
$1x10^{-4}$	67.60	12.90	8	57.00	16.60	6	84.60	12.40	5
$2x10^{-4}$	97.30	15.20	8	73.59	18.10	6	110.00	13.00	5
$5x10^{-4}$	155.70	16.60	8	123.69	21.80	6	153.50	9.80	5
$1x10^{-3}$	196.80	16.70	8	158.40	20.70	6	214.00	14.30	5
$2x10^{-3}$	245.00	9.20	8	226.00	23.20	6	261.25	12.40	5
$5x10^{-3}$	293.30	8.46	8	278.90	11.00	6	319.50	5.80	5
$1x10^{-2}$	313.60	9.00	6	309.80	7.00	6	332.90	17.70	4

18 hours

[PE]	Krebs			AG (1 mM)			L-NAME (80 μM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	10.08	2.05	11	20.80	4.80	11	49.50	7.50	7
$2x10^{-5}$	17.40	3.80	11	20.59	4.70	11	40.90	8.20	7
$5x10^{-5}$	27.70	3.90	11	47.29	5.11	11	83.89	8.40	7
$1x10^{-4}$	51.90	4.60	11	66.40	6.29	11	104.00	9.46	7
$2x10^{-4}$	73.90	5.57	11	92.90	7.00	11	154.90	12.90	7
$5x10^{-4}$	111.60	8.35	11	146.28	10.10	11	206.40	16.60	7
$1x10^{-3}$	154.06	8.50	11	193.70	9.70	11	249.70	18.16	7
$2x10^{-3}$	199.25	9.50	11	248.00	11.30	11	314.70	18.20	7
$5x10^{-3}$	237.90	7.80	11	299.00	11.00	11	319.40	11.90	7
$1x10^{-2}$	281.83	11.00	10	285.90	21.20	3	-	-	-

72 hours

[PE]	Krebs			AG (1 mM)			L-NAME (80 μM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	10.90	1.10	4	12.10	3.10	4	19.26	8.10	3
$2x10^{-5}$	13.80	2.10	4	15.60	2.50	4	29.30	4.68	4
$5x10^{-5}$	23.80	6.00	3	25.40	6.10	3	56.30	9.47	3
$1x10^{-4}$	34.60	4.80	3	47.90	10.20	3	91.30	11.40	4
$2x10^{-4}$	60.80	10.70	3	93.40	23.80	3	138.70	14.40	4
$5x10^{-4}$	100.90	19.20	3	140.00	21.20	3	161.80	11.50	2
$1x10^{-3}$	156.40	20.40	3	195.00	17.40	3	223.00	23.00	4
$2x10^{-3}$	228.80	10.90	3	231.20	11.50	3	249.60	20.80	4
$5x10^{-3}$	292.80	19.40	3	273.90	15.40	3	265.80	6.90	4
$1x10^{-2}$	312.10	21.40	3	307.50	4.60	3	263.50	11.00	4

Group	Control	18 Hr	72 Hr	Krebs 18Hr vs Con	18 hr(+AG) vs Con Krebs
[PE] (M)	paired t-tests comparing Krebs and AG curves			unpaired	unpaired
$1x10^{-5}$	0.305	0.079	0.752	0.934	0.087
$2x10^{-5}$	0.800	0.510	0.547	0.440	0.773
$5x10^{-5}$	0.804	0.016	0.840	0.220	0.607
$1x10^{-4}$	0.988	0.037	0.405	0.215	0.928
$2x10^{-4}$	0.783	0.004	0.300	0.124	0.773
$5x10^{-4}$	0.611	0.001	0.112	0.020	0.613
$1x10^{-3}$	0.529	0.001	0.060	0.025	0.870
$2x10^{-3}$	0.665	0.000	0.695	0.004	0.851
$5x10^{-3}$	0.280	0.000	0.760	0.000	0.705
$1x10^{-2}$	0.767	0.716	0.177	0.066	0.191

Appendix Tables (5.1) for Figs.5.1-5.4

Cycloheximide raw meaned Data

Zero Hour Table

[PE]	Control			LPS (24 hr)		
(M)	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	7.32	2.63	6	6.93	1.19	6
$2x10^{-5}$	9.64	2.18	6	11.94	2.69	6
$5x10^{-5}$	21.96	6.33	6	23.12	5.02	6
$1x10^{-4}$	37.76	10.31	6	40.46	7.55	6
$2x10^{-4}$	52.79	13.33	6	55.11	11.73	6
$5x10^{-4}$	107.52	26.43	6	92.90	16.05	6
$1x10^{-3}$	135.66	25.48	6	127.57	19.17	6
$2x10^{-3}$	182.31	19.27	6	166.11	18.19	6
$5x10^{-3}$	209.54	13.05	5	207.70	17.19	6
$1x10^{-2}$	218.30	16.80	3	198.92	11.87	4

1 Hour Table

[PE]	Control			LPS (24 hr)		
(M)	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	14.64	4.68	6	16.18	3.10	6
$2x10^{-5}$	15.80	5.70	6	14.79	4.65	6
$5x10^{-5}$	34.68	8.56	6	31.44	4.36	6
$1x10^{-4}$	47.40	11.75	6	47.17	6.14	5
$2x10^{-4}$	71.68	15.50	6	69.37	7.56	5
$5x10^{-4}$	117.16	27.04	6	106.36	11.13	5
$1x10^{-3}$	163.02	28.67	6	151.69	10.70	5
$2x10^{-3}$	202.33	15.08	4	194.20	13.76	6
$5x10^{-3}$	234.97	6.33	5	205.05	13.07	3
$1x10^{-2}$	240.52	15.36	5	210.40	11.53	3

2 Hour Table

[PE]	Control			LPS (24 hr)		
(M)	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	6.93	2.19	5	18.88	4.02	5
$2x10^{-5}$	8.32	1.87	5	18.49	4.09	5
$5x10^{-5}$	17.11	5.30	5	32.75	7.19	5
$1x10^{-4}$	32.84	7.75	5	51.25	10.41	5
$2x10^{-4}$	47.63	9.62	5	78.62	14.84	5
$5x10^{-4}$	90.65	14.45	5	118.32	20.25	5
$1x10^{-3}$	129.04	20.21	5	176.65	19.90	5
$2x10^{-3}$	167.89	19.54	5	212.27	17.91	5
$5x10^{-3}$	236.49	14.42	4	199.48	13.46	4
$1x10^{-2}$	230.13	25.24	4	-	-	-

3 Hour Table

[PE]	Control			LPS (24 hr)		
(M)	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	5.55	1.38	5	32.83	6.67	5
$2x10^{-5}$	7.86	1.56	5	32.83	7.32	5
$5x10^{-5}$	16.65	4.22	5	45.78	8.34	5
$1x10^{-4}$	29.13	5.30	5	78.29	11.86	5
$2x10^{-4}$	45.32	7.43	5	110.97	15.76	4
$5x10^{-4}$	85.10	16.46	5	143.82	17.79	5
$1x10^{-3}$	110.54	13.19	5	185.44	19.30	5
$2x10^{-3}$	153.56	18.12	5	215.50	25.67	5
$5x10^{-3}$	200.74	15.62	5	205.85	11.46	4
$1x10^{-2}$	229.89	11.77	5	-	-	-

4 Hour Table

[PE]	Control			LPS (24 hr)		
(M)	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	8.78	4.92	5	15.26	4.65	6
$2x10^{-5}$	13.87	6.07	5	22.19	0.92	6
$5x10^{-5}$	20.34	8.96	5	31.91	1.69	6
$1x10^{-4}$	31.44	11.52	5	55.95	5.50	6
$2x10^{-4}$	43.01	12.02	5	80.46	5.33	6
$5x10^{-4}$	76.30	20.70	5	135.96	10.98	6
$1x10^{-3}$	114.24	20.60	5	159.00	9.71	5
$2x10^{-3}$	152.64	13.91	5	180.76	11.18	6
$5x10^{-3}$	200.75	9.82	5	214.30	10.41	6
$1x10^{-2}$	221.47	14.32	4	235.87	15.50	5

L-NMMA

[PE]	Control			LPS (24 hr)		
(M)	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	16.64	3.53	5	22.66	4.16	5
$2x10^{-5}$	19.88	4.54	5	19.88	3.58	5
$5x10^{-5}$	34.22	8.18	5	41.16	9.76	5
$1x10^{-4}$	58.72	6.64	5	60.58	14.10	5
$2x10^{-4}$	80.45	9.43	5	104.53	17.14	5
$5x10^{-4}$	124.38	12.65	5	143.38	16.25	5
$1x10^{-3}$	154.44	15.37	5	195.19	10.11	5
$2x10^{-3}$	185.42	17.92	5	228.51	10.68	5
$5x10^{-3}$	221.37	20.73	4	250.96	30.71	4
$1x10^{-2}$	250.85	30.27	4	-	-	-

Appendix Tables (5.2) to Figs. 5.5-5.8
Indomethacin & cycloheximide raw meaned Data

Combined data for control and LPS-treated RTAs.

[PE]	Control			LPS (18 hr)			t-test
(M)	Mean	S.E.	n	Mean	S.E.	n	unpaired
$1x10^{-5}$	8.82	1.83	12	5.25	1.03	11	0.147
$2x10^{-5}$	12.86	2.41	12	9.04	1.93	11	0.264
$5x10^{-5}$	28.32	4.42	12	17.45	3.52	11	0.086
$1x10^{-4}$	46.10	6.81	12	29.22	5.34	11	0.082
$2x10^{-4}$	66.62	8.68	12	46.25	6.98	11	0.102
$5x10^{-4}$	122.26	15.22	12	77.24	10.05	10	0.042
$1x10^{-3}$	158.97	14.84	12	116.26	12.10	11	0.048
$2x10^{-3}$	198.14	11.75	12	157.89	13.40	11	0.035
$5x10^{-3}$	212.08	8.26	10	190.27	12.45	11	0.169
$1x10^{-2}$	223.39	12.09	7	189.08	9.34	8	0.040

1 Hour Table

[PE]	Control			LPS (18hr)		
(M)	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	14.64	8.58	6	11.18	4.19	6
$2x10^{-5}$	16.59	7.96	6	5.09	2.44	6
$5x10^{-5}$	23.12	10.33	6	20.04	3.61	6
$1x10^{-4}$	37.37	14.21	6	31.22	5.87	6
$2x10^{-4}$	57.41	18.30	6	48.95	8.85	6
$5x10^{-4}$	99.80	25.15	6	80.56	13.81	6
$1x10^{-3}$	138.30	29.80	6	121.04	18.32	6
$2x10^{-3}$	162.20	28.65	6	158.42	25.10	6
$5x10^{-3}$	173.40	24.19	4	171.75	21.67	4
$1x10^{-2}$	184.96	23.59	4	176.37	19.97	4

2 Hour Table

[PE]	Control			LPS (18 hr)		
(M)	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	13.49	5.32	6	10.70	4.82	8
$2x10^{-5}$	13.48	4.44	6	8.38	3.05	8
$5x10^{-5}$	27.75	9.44	6	15.61	4.43	8
$1x10^{-4}$	42.39	13.58	6	31.22	7.48	8
$2x10^{-4}$	64.75	15.42	6	49.15	8.20	8
$5x10^{-4}$	113.70	21.64	6	88.18	14.07	8
$1x10^{-3}$	168.44	21.63	6	131.26	18.49	8
$2x10^{-3}$	198.88	15.38	6	186.00	23.01	8
$5x10^{-3}$	230.73	13.60	4	192.00	23.09	6
$1x10^{-2}$	241.13	12.65	4	-	-	-

3 Hour Table

[PE]	Control			LPS (18 hr)		
(M)	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	15.61	6.54	8	11.27	4.22	8
$2x10^{-5}$	15.90	3.22	8	9.25	3.03	8
$5x10^{-5}$	33.53	6.75	8	18.51	6.48	8
$1x10^{-4}$	48.56	8.66	8	30.36	7.66	8
$2x10^{-4}$	70.24	10.15	8	48.87	8.79	8
$5x10^{-4}$	108.10	11.73	8	79.22	12.10	8
$1x10^{-3}$	152.63	16.25	8	125.48	13.82	8
$2x10^{-3}$	190.78	14.32	8	176.26	22.37	8
$5x10^{-3}$	220.48	15.44	6	205.52	23.57	6
$1x10^{-2}$	244.38	17.35	6	-	-	-

4 Hour Table

[PE]	Control			LPS (18 hr)		
(M)	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	13.87	3.65	8	10.12	4.12	8
$2x10^{-5}$	19.07	4.29	8	8.38	3.73	8
$5x10^{-5}$	31.79	5.90	8	14.16	3.69	8
$1x10^{-4}$	56.60	8.70	8	28.05	7.65	8
$2x10^{-4}$	68.22	9.08	8	43.37	9.27	8
$5x10^{-4}$	110.42	13.63	8	81.25	12.36	8
$1x10^{-3}$	148.87	15.19	8	128.08	14.60	8
$2x10^{-3}$	180.95	11.45	8	184.75	19.24	8
$5x10^{-3}$	214.31	16.12	6	207.82	21.70	6
$1x10^{-2}$	233.19	14.13	6	-	-	-

L-NMMA

[PE]	Control			LPS (18 hr)		
(M)	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	37.33	13.24	7	30.45	8.09	6
$2x10^{-5}$	42.28	11.63	7	25.15	8.63	8
$5x10^{-5}$	57.81	9.46	7	46.84	11.19	8
$1x10^{-4}$	82.58	13.15	7	66.49	15.21	8
$2x10^{-4}$	106.37	14.28	7	94.54	18.25	8
$5x10^{-4}$	140.39	14.05	7	151.77	21.18	8
$1x10^{-3}$	186.31	23.73	7	190.79	27.43	8
$2x10^{-3}$	197.03	27.17	5	219.39	27.98	7
$5x10^{-3}$	216.90	18.67	5	216.50	10.60	5
$1x10^{-2}$	237.50	16.54	4	226.70	15.40	4

Statistical Tables (5.3) to Figs. 5.1-5.4

Cycloheximide data. Student's paired t-test analysis comparing the initial responses in the presence of Krebs only with responses after 1-4hrs perfusion and in the presence of L-NMMA.

Groups	Con	LPS	Con	LPS
[PE] (M)	t=1 Hr		t= 2 Hr	
$1x10^{-5}$	0.311	0.006	0.665	0.008
$2x10^{-5}$	0.330	0.087	0.587	0.010
$5x10^{-5}$	0.022	0.007	0.815	0.019
$1x10^{-4}$	0.052	0.019	0.388	0.020
$2x10^{-4}$	0.013	0.020	0.119	0.029
$5x10^{-4}$	0.118	0.078	0.546	0.032
$1x10^{-3}$	0.055	0.058	0.487	0.046
$2x10^{-3}$	0.610	0.057	0.963	0.029
$5x10^{-3}$	0.213	0.376	0.112	0.791
$1x10^{-2}$	0.586	0.558	0.766	0.854

Groups	Con	LPS	Con	LPS	Con	LPS
[PE] (M)	t=3 Hr		t= 4 Hr		LNMMA	
$1x10^{-5}$	0.495	0.011	0.938	0.670	0.164	0.013
$2x10^{-5}$	0.799	0.023	0.390	0.001	0.170	0.016
$5x10^{-5}$	0.849	0.017	0.509	0.001	0.188	0.038
$1x10^{-4}$	0.766	0.013	0.775	0.001	0.032	0.026
$2x10^{-4}$	0.392	0.007	0.916	0.001	0.042	0.008
$5x10^{-4}$	0.562	0.011	0.681	0.003	0.477	0.003
$1x10^{-3}$	0.457	0.007	0.973	0.083	0.319	0.029
$2x10^{-3}$	0.330	0.016	0.476	0.463	0.903	0.147
$5x10^{-3}$	0.739	0.321	0.466	0.695	0.747	0.391
$1x10^{-2}$	0.205	0.559	0.681	0.639	0.109	0.471

Statistical tables (5.4) for Figs.5.5-5.8

Indomethacin + cycloheximide Data. Student's paired t-test analysis comparing the initial responses in the presence of Krebs only with responses after 1-4hrs perfusion and in the presence of L-NMMA.

Groups	Con	LPS	Con	LPS
[PE] (M)	t=1 Hr		t= 2 Hr	
$1x10^{-5}$	0.379	0.087	0.352	0.221
$2x10^{-5}$	0.727	0.119	0.957	0.488
$5x10^{-5}$	0.889	0.036	0.683	0.388
$1x10^{-4}$	0.979	0.111	0.800	0.281
$2x10^{-4}$	0.748	0.150	0.608	0.274
$5x10^{-4}$	0.651	0.305	0.449	0.082
$1x10^{-3}$	0.897	0.607	0.344	0.064
$2x10^{-3}$	0.681	0.710	0.493	0.202
$5x10^{-3}$	0.329	0.601	0.706	0.689
$1x10^{-2}$	0.640	0.673	0.502	0.675

Groups	Con	LPS	Con	LPS	Con	LPS
[PE] (M)	t=3 Hr		t= 4 Hr		LNMMA	
$1x10^{-5}$	0.316	0.076	0.080	0.100	0.101	0.018
$2x10^{-5}$	0.703	0.194	0.366	0.434	0.106	0.041
$5x10^{-5}$	0.355	0.172	0.071	0.508	0.062	0.013
$1x10^{-4}$	0.404	0.056	0.058	0.306	0.062	0.021
$2x10^{-4}$	0.284	0.012	0.171	0.008	0.006	0.020
$5x10^{-4}$	0.279	0.187	0.196	0.408	0.065	0.024
$1x10^{-3}$	0.368	0.144	0.416	0.084	0.192	0.017
$2x10^{-3}$	0.470	0.086	0.671	0.032	0.509	0.050
$5x10^{-3}$	0.967	0.108	0.989	0.090	0.532	0.157
$1x10^{-2}$	-	-	0.125	0.416	0.304	-

Statistical Tables (5.5) for Figs.5.1-5.8

Student's unpaired t-test analysis comparing the Krebs data (ie. t= 0hr) for control and LPS-treated RTAs which have not previously been exposed to cycloheximide (*untreated*) with those exposed to cycloheximide (cyclo) only or indomethacin + cycloheximide (cyclo + indo). Reveals no significant difference between any of the groups.

Groups	Con	LPS	Con	LPS	Con	LPS
[PE] (M)	Krebs-untreated vs t= 0hr (Cyclo)		Krebs-untreated vs t= 0hr (Cyclo/Indo)		t= 0hr cyclo vs t=0hr cyclo/Indo	
$1x10^{-5}$	0.246	0.896	0.111	0.096	0.900	0.063
$2x10^{-5}$	0.150	0.773	0.771	0.041	0.281	0.057
$5x10^{-5}$	0.592	0.902	0.572	0.066	0.970	0.085
$1x10^{-4}$	0.790	0.350	0.742	0.224	0.958	0.083
$2x10^{-4}$	0.336	0.842	0.305	0.166	0.925	0.193
$5x10^{-4}$	0.763	0.555	0.226	0.344	0.536	0.162
$1x10^{-3}$	0.458	0.396	0.422	0.961	0.962	0.383
$2x10^{-3}$	0.293	0.344	0.243	0.485	0.853	0.760
$5x10^{-3}$	0.090	0.141	0.601	0.283	0.261	0.624
$1x10^{-2}$	0.221	-	0.151	-	0.950	0.901

Student's unpaired t-test analysis Comparing the L-NMMA data for *untreated* control and LPS-treated RTAs with that vessels exposed to cycloheximide only (cyclo) or cycloheximide + indomethacin (cyclo + indo).

Groups	Con	LPS	Con	LPS
[PE] (M)	LNMMA-untreated vs LNMMA (Cyclo)		LNMMA-untreated vs LNMMA (Cyclo/Indo)	
$1x10^{-5}$	0.275	0.446	0.712	0.142
$2x10^{-5}$	0.090	0.454	0.307	0.935
$5x10^{-5}$	0.033	0.349	0.483	0.766
$1x10^{-4}$	0.008	0.350	0.568	0.623
$2x10^{-4}$	0.065	0.975	0.440	0.710
$5x10^{-4}$	0.072	0.977	0.147	0.747
$1x10^{-3}$	0.032	0.426	0.306	0.627
$2x10^{-3}$	0.038	0.526	0.137	0.812
$5x10^{-3}$	-	0.813	-	0.201
$1x10^{-2}$	-	-	-	-

Appendix table (6.1) to Figs. 6.3a/b

Data for control RTAs

[PE]	Control Krebs			JM1006 (100μM)			L-NMMA (100μM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	5.4	1.3	8	20.48	5.7	8	22.4	5.7	6
$2x10^{-5}$	12.1	2.8	8	21.1	5.0	8	30.3	5.6	6
$5x10^{-5}$	28.3	6.8	8	54.18	6.4	8	62.7	9.4	6
$1x10^{-4}$	47.7	7.7	8	85.2	9.0	8	91.7	9.5	6
$2x10^{-4}$	74.0	9.6	8	116.9	13.5	8	127.9	15.3	6
$5x10^{-4}$	115.0	9.9	8	180.0	12.8	8	185.0	11.2	6
$1x10^{-3}$	167.0	13.4	8	228.3	16.9	8	231.6	12.3	6
$2x10^{-3}$	213.0	14.3	8	276.8	17.6	7	289.0	16.8	6
$5x10^{-3}$	270.0	14.3	8	306.0	12.1	4	328.0	17.4	6
$1x10^{-2}$	297.0	19.2	8	331.1	19.2	4	343.6	21.2	5

Data for LPS-treated RTAs

[PE]	LPS Krebs			JM1006 (100μM)			L-NMMA (100μM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	1.6	0.9	7	19.1	3.8	7	27.4	7.7	7
$2x10^{-5}$	3.9	1.3	7	22.4	6.3	7	24.7	4.4	7
$5x10^{-5}$	7.6	1.9	7	45.2	8.1	7	62.0	8.3	7
$1x10^{-4}$	18.8	4.2	7	62.0	8.4	7	85.1	10.0	7
$2x10^{-4}$	33.9	7.9	7	96.3	8.9	7	119.8	12.8	7
$5x10^{-4}$	75.2	9.6	7	149.8	12.4	7	165.3	11.7	7
$1x10^{-3}$	113.5	13.3	7	186.0	16.4	7	205.6	10.3	7
$2x10^{-3}$	147.8	8.7	7	210.6	24.6	7	250.8	12.0	7
$5x10^{-3}$	194.7	16.5	7	242.0	10.7	7	301.4	16.4	7
$1x10^{-2}$	216.2	10.8	7	263.0	8.5	7	299.4	17.7	7

Appendix table (6.2) to Figs. 6.4a/b

Data for control RTAs

[PE]	Control Krebs			JM1226 (100μM)			L-NMMA (100 μM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	4.8	1.7	9	36.9	6.5	9	25.43	3.3	8
$2x10^{-5}$	14.1	3.2	9	43.4	5.29	9	30.0	3.7	8
$5x10^{-5}$	28.3	4.7	9	79.6	9.97	9	58.0	5.5	8
$1x10^{-4}$	45.5	4.7	9	117.9	15.17	9	84.4	7.0	8
$2x10^{-4}$	66.8	6.6	9	159.7	16.9	9	114.2	10.4	8
$5x10^{-4}$	129.5	9.1	9	248.6	18.0	9	174.2	12.5	8
$1x10^{-3}$	191.1	10.1	9	288.7	17.7	5	213.38	14.4	8
$2x10^{-3}$	247.3	9.3	9	-	-	-	259.5	14.5	8
$5x10^{-3}$	299.0	11.1	9	-	-	-	291.5	13.8	8
$1x10^{-2}$	297.6	7.8	9	-	-	-	294.7	15.5	7

Data for LPS-treated RTAs

[PE]	LPS Krebs			JM1226 (100μM)			L-NMMA (100μM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	3.8	0.7	9	25.9	4.5	9	29.8	5.3	13
$2x10^{-5}$	7.2	1.1	9	33.4	3.9	9	28.6	4.4	13
$5x10^{-5}$	21.1	3.3	9	56.2	6.0	9	64.2	6.3	13
$1x10^{-4}$	35.4	5.6	9	89.1	9.0	9	90.6	8.7	13
$2x10^{-4}$	49.3	6.2	9	120.5	13.5	9	133.3	10.0	13
$5x10^{-4}$	89.1	9.5	9	181.4	13.0	9	179.2	9.5	13
$1x10^{-3}$	130.2	13.6	9	224.0	14.7	9	214.0	8.5	13
$2x10^{-3}$	167.2	13.4	9	260.0	15.0	8	248.0	8.8	13
$5x10^{-3}$	223.7	12.7	9	286.3	19.5	6	293.0	11.0	12
$1x10^{-2}$	224.2	9.12	9	273.4	21.5	4	296.6	12.0	12

Appendix Table (6.3) to Fig. 6.5

The effect of JM1226 on Blood pressure (mmHg)					
Group	**t**	**Systolic**	**Mean**	**Diastolic**	**n**
Controls					
inject saline	**0**	162 ±9	109 ±4	83 ±6	**7**
inject JM1226	**20**	151 ±6	92 ±8	62 ±11	**7**
	24	144 ±8	101 ±6	80 ±6	**7**
	29	156 ±6	105 ±10	80 ±12	**3**
	32	169 ±20	118 ±4	93 ±3	**3**
LPS-treated					
inject LPS	**0**	169 ±13	105 ±8	73 ±7	**7**
inject saline	**20**	114 ±6	75 ±7	56 ±8	**7**
	24	99 ±7	65 ±7	48 ±8	**7**
	29	126 ±14	87 ±7	68 ±6	**3**
	32	132 ±9	88 ±18	66 ±19	**3**
LPS + L-NAME					
inject LPS	**0**	164 ±13	115 ±6	91 ±6	**7**
inject JM1226	**20**	112 ±9	83 ±11	68 ±13	**7**
	24	149 ±8	104 ±10	83 ±11	**7**
	29	191 ±9	135 ±5	106 ±3	**3**
	32	183 ±19	122 ±15	106 ±5	**3**

Appendix table (7.1) to fig. 7.1

The Krebs curves using the combined L-NMMA and L-NAME data.

[PE]	ALL CEAs (Krebs)			ALL TEAs (Krebs)			**t-Test**
(M)	Mean	S.E.	n	Mean	S.E.	n	*unpaired*
$1x10^{-5}$	8.36	1.05	14	7.47	0.68	15	0.470
$2x10^{-5}$	11.93	1.19	14	10.75	1.02	15	0.450
$5x10^{-5}$	21.73	2.26	14	17.48	1.93	15	0.163
$1x10^{-4}$	37.43	4.50	13	30.13	4.01	15	0.236
$2x10^{-4}$	71.57	9.58	13	48.12	6.07	15	0.043
$5x10^{-4}$	125.13	16.31	13	83.43	8.55	15	0.026
$1x10^{-3}$	179.59	22.02	13	121.93	11.43	15	0.023
$2x10^{-3}$	221.98	17.75	13	165.54	15.31	14	0.023
$5x10^{-3}$	253.88	15.61	11	202.32	15.94	11	0.032
Anova	2-way: F= 2.85			df= 8, 230_e			p= 0.005 **

Appendix tables (7.2) to Figs. 7.5 & 7.6

Data for CEAs

[PE]	Krebs			L-NMMA (80 μM)			L-Arg (1 mM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	9.08	1.93	7	16.43	2.53	6	8.36	0.85	5
$2x10^{-5}$	12.04	2.18	7	29.93	2.90	6	15.13	2.36	5
$5x10^{-5}$	22.07	4.27	7	51.01	11.06	5	31.06	5.95	6
$1x10^{-4}$	36.98	7.50	7	69.16	10.70	6	48.04	7.34	7
$2x10^{-4}$	72.81	15.99	7	130.52	13.77	6	86.31	7.85	7
$5x10^{-4}$	113.87	24.00	7	222.12	18.59	6	146.10	15.60	7
$1x10^{-3}$	167.70	32.20	7	282.64	17.30	5	176.02	11.70	7
$2x10^{-3}$	240.29	26.60	7	292.00	7.97	5	192.98	18.60	5
$5x10^{-3}$	263.59	22.50	6	299.98	0.00	5	254.43	0.00	2
$1x10^{-2}$	-	-	-	-	-	-	-	-	-

Data for TEAs

[PE]	Krebs			L-NMMA (80 μM)			L-Arg (1 mM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	8.37	0.44	7	13.33	1.60	7	6.61	1.74	4
$2x10^{-5}$	11.19	0.98	7	25.52	5.46	7	11.75	2.17	6
$5x10^{-5}$	15.75	1.88	7	48.65	10.70	7	25.50	7.53	6
$1x10^{-4}$	24.12	3.86	7	89.10	14.90	7	45.62	13.72	6
$2x10^{-4}$	38.15	6.83	7	205.00	25.50	7	108.60	25.60	5
$5x10^{-4}$	68.03	11.70	7	251.01	26.60	6	172.58	27.15	5
$1x10^{-3}$	93.62	16.40	7	260.00	24.79	4	176.65	29.60	4
$2x10^{-3}$	143.34	26.40	7	283.00	16.76	3	160.23	1.47	2
$5x10^{-3}$	191.19	26.50	6	299.98	0.00	3	-	-	-
$1x10^{-2}$	-	-	-	-	-	-	-	-	-

Appendix Tables (7.3) to Figs. 7.7 & 7.8

Data for CEAs

[PE]	Krebs			L-NAME (80 μM)			L-Arg (1 mM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	7.5	0.79	8	15.72	2.66	10	10.25	2.98	5
$2x10^{-5}$	11.22	1.21	8	27.85	3.75	8	15.86	5.09	5
$5x10^{-5}$	20.28	2.05	7	59.57	6.36	8	28.96	8.36	5
$1x10^{-4}$	35.00	5.16	7	123.44	19.64	8	46.89	10.60	5
$2x10^{-4}$	66.31	9.96	7	193.53	28.29	8	77.23	18.53	5
$5x10^{-4}$	127.60	21.83	7	238.73	27.55	4	132.06	25.42	5
$1x10^{-3}$	180.60	29.50	7	240.78	29.70	3	163.78	28.22	5
$2x10^{-3}$	214.81	23.20	7	-	-	-	233.17	38.32	4
$5x10^{-3}$	251.70	20.32	6	-	-	-	246.63	32.26	5
$1x10^{-2}$	-	-		-	-	-	-	-	

Data for TEAs

[PE]	Krebs			L-NAME (80 μM)			L-Arg (1 mM)		
(M)	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
$1x10^{-5}$	7.67	0.97	10	18.64	3.80	10	12.93	6.69	4
$2x10^{-5}$	10.01	1.42	10	29.33	4.78	10	16.37	8.51	4
$5x10^{-5}$	17.78	2.70	10	61.28	4.80	8	17.97	9.47	7
$1x10^{-4}$	32.80	5.34	10	115.96	15.88	6	23.92	8.43	8
$2x10^{-4}$	52.21	7.72	10	191.34	23.06	7	53.44	19.78	8
$5x10^{-4}$	86.85	10.80	10	258.52	21.35	6	74.13	17.05	7
$1x10^{-3}$	130.49	13.40	10	276.13	23.84	4	148.55	24.09	6
$2x10^{-3}$	169.16	15.40	9	272.81	27.16	3	198.99	13.32	7
$5x10^{-3}$	200.38	14.98	7	-	-		221.90	12.29	6
$1x10^{-2}$	-	-		-	-		-	-	

Appendix Tables (7.4) to Fig. 7.9 &7.10

Data for CEAs							A	B
[PE]	**Krebs**			**AG (1mM)**			student's	student's
(M)	Mean	S.E.	n	Mean	S.E.	n	paired t-test	unpaired test
$1x10^{-5}$	6.55	1.1	6	12.94	1.56	5	0.003	0.010
$2x10^{-5}$	14.60	1.9	6	21.4	1.9	6	0.014	0.088
$5x10^{-5}$	26.90	2.1	6	41.61	5.9	6	0.035	0.742
$1x10^{-4}$	49.30	2.9	6	69.30	13.1	6	0.18	0.470
$2x10^{-4}$	86.7	4.9	6	95.20	9.87	5	0.448	0.021
$5x10^{-4}$	144.10	11.8	6	169.30	18.5	4	0.145	0.198
$1x10^{-3}$	194.04	12.8	6	234.00	8.8	4	0.056	0.517
$2x10^{-3}$	250.00	9.6	6	290.70	10.1	4	0.222	0.252
$5x10^{-3}$	301.00	9.9	6	310.00	19.2	5	0.659	0.508
$1x10^{-2}$	325.00	26.3	3	320.50	27.4	3	0.926	0.418

Column A shows the statistical significance between the CEA Krebs and CEA + AG values.
Column B shows the statistical significance between the CEA Krebs and TEA + AG values.

Data for TEAs							C	D
[PE]	**Krebs**			**AG (1mM)**			student's	student's
(M)	Mean	S.E.	n	Mean	S.E.	n	paired t-test	unpaired test
$1x10^{-5}$	4.60	1.0	5	19.4	2.7	5	0.014	0.24
$2x10^{-5}$	6.00	1.2	5	19.8	1.8	5	0.009	0.006
$5x10^{-5}$	24.90	6	5	28.6	4.4	5	0.540	0.750
$1x10^{-4}$	44.30	12.7	5	61.9	1.8	5	0.450	0.630
$2x10^{-4}$	62.42	17.7	4	128.2	15.4	5	0.026	0.150
$5x10^{-4}$	99.89	23.6	4	193.0	39.9	4	0.090	0.102
$1x10^{-3}$	119.70	24.6	4	213.5	27.89	4	0.045	0.018
$2x10^{-3}$	205.10	12.5	4	272.5	16.7	4	0.018	0.020
$5x10^{-3}$	245.00	15.5	4	312.1	4.8	4	0.016	0.012
$1x10^{-2}$	268.90	18.7	3	298.2	14.1	3	0.190	0.010

Column C shows the statistical significance between the TEA Krebs and TEA + AG values.
Column D shows the statistical significance between the TEA Krebs and CEA Krebs values.

Appendix Tables (8.1) to Figs. 8.11, 8.12 & 8.16b

Tumour Volume for Control group 1a and those injected with LPS on day 5 post-implantation (ie. grp. 2b).

Grp	Control (1)			2b		
Day	Mean	S.E.	n	Mean	S.E.	n
5	0.13	0.03	12	0.10	0.03	12
7	0.18	0.03	12	0.08	0.02	12
9	0.33	0.05	12	0.19	0.03	12
11	0.73	0.10	12	0.25	0.04	12
13	0.92	0.15	12	0.32	0.05	12
15	1.49	0.26	12	0.62	0.14	12
17	2.12	0.35	12	1.03	0.20	12
19	3.08	0.56	12	1.88	0.35	12
21	4.64	0.81	12	3.48	0.40	12
23	5.80	0.85	9	4.33	0.79	8
25	7.40	1.15	10	5.04	0.60	10
27	8.12	1.13	10	5.04	0.61	9

Tumour Volume for those injected with LPS on day 11 post-implantation (ie. grp. 2c) and those being fed AG from day 4 and subsequently injected on day 5 (ie. grp. 3b).

Grp	2c			3b		
Day	Mean	S.E.	n	Mean	S.E.	n
5	0.02	0.01	14	0.01	0.00	18
7	0.10	0.03	14	0.03	0.01	18
9	0.20	0.04	14	0.09	0.02	18
11	0.35	0.04	14	0.29	0.05	18
13	0.41	0.05	14	0.66	0.10	18
15	0.60	0.08	14	1.31	0.20	18
17	0.85	0.13	14	2.07	0.31	18
19	1.33	0.17	14	3.30	0.60	18
21	2.10	0.28	12	4.70	0.60	18
23	3.03	0.32	13	5.67	0.50	10
25	4.65	0.52	13	6.23	0.82	8

Appendix Tables (8.2) to Fig. 8.10

Tumour volumes for Control group 1b and those injected with LS on day 11 post-implantation (ie. grp. 2c).

Grp	Control (2)			2a		
Day	Mean	S.E.	n	Mean	S.E.	n
7	0.01	0.00	16	0.01	0.00	16
9	0.04	0.01	16	0.05	0.01	16
11	0.24	0.06	16	0.51	0.11	16
13	0.85	0.15	16	0.88	0.13	16
15	1.72	0.28	16	1.33	0.22	16
17	2.13	0.29	16	1.60	0.25	16
19	2.68	0.32	16	2.40	0.48	16
21	3.43	0.56	16	4.00	0.63	16
23	4.08	0.63	16	5.30	1.04	16

Tumour volumes for animals fed with AG from day 10 (ie. grp. 3a)

Grp	AG treated		
Day	Mean	S.E.	n
7	0.13	0.03	14
8	0.16	0.03	14
9	0.24	0.04	14
10	0.35	0.05	14
11	0.52	0.08	14
12	0.57	0.07	14
13	0.73	0.07	14
14	0.84	0.08	14
15	1.01	0.08	14
16	1.19	0.12	14
17	1.32	0.15	14
18	1.58	0.13	14
19	1.90	0.15	14
20	2.18	0.17	14

Appendix table (8.3)

Tumour Volumes for Control group 1c and those treated with L-NAME from day 0 post-implantation (ie. grp. 4b).

Grp	Control (3)			L-NAME Treated		
Day	Mean	S.E.	n	Mean	S.E.	n
7	0.28	0.05	12	0.08	0.01	16
9	0.75	0.12	12	0.31	0.05	16
11	1.38	0.31	12	0.43	0.07	16
13	2.22	0.42	12	0.72	0.14	16
15	3.32	0.62	12	0.95	0.12	16

Appendix Table (8.4) to Fig. 8.16a

Tumour Volumes for Control group 1d and those injected with LPS on day 11 post-implantation (ie. grp. 4a) and those receiving both LPS and L-NAME treatment

Grp	Control (4)			LPS			LPS + L-NAME		
Day	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
7	0.25	0.03	16	0.50	0.05	20	0.27	0.03	20
9	0.50	0.05	16	0.67	0.05	20	0.47	0.05	20
10	0.72	0.07	16	0.80	0.07	20	0.65	0.08	20
11	0.99	0.09	16	0.94	0.09	20	0.72	0.08	20
12	1.23	0.13	16	1.00	0.10	20	0.75	0.08	20
13	1.61	0.16	16	1.26	0.10	20	0.80	0.09	20
14	2.20	0.25	16	1.54	0.12	20	0.93	0.13	20
16	2.79	0.33	16	1.91	0.19	20	1.06	0.15	20
18	3.24	0.49	16	2.35	0.26	16	1.51	0.23	16
20	3.94	0.57	16	2.76	0.35	16	1.70	0.26	16
21	4.54	0.63	16	3.10	0.36	16	1.79	0.25	16
23	-	-	-	-	-	-	2.50	0.31	16
24	-	-	-	-	-	-	3.17	0.41	16
25	-	-	-	-	-	-	3.54	0.44	16

Appendix Tables (8.5) to Figs. 8.10-8.16b

Student's unpaired t-test analysis comparing the tumour volumes on a particular day. Column G is the result of a comparison between grp. 1d and grp. 4a. Column H is the result of a comparison between grp. 1d and grp. 4c. Column I is the result of a comparison between grp. 4a and grp. 4c.

	G	H	I
Day	**Control vs LPS**	**Control vs LPS + L-NAME**	**LPS vs LPS + L-NAME**
10	0.580	0.598	0.144
11	0.154	0.038*	0.409
12	0.069	0.002**	0.117
13	0.002**	0.0001***	0.137
14	0.001***	0.0001***	0.048*
16	0.001***	0.0001***	0.017*
18	0.018**	0.003**	0.196
20	0.017**	0.001***	0.079
21	0.025*	0.0001***	0.029*

Appendix Tables (8.6) to Figs. 8.18-8.20.
Systolic, Mean and Diastolic Blood Pressure Measurements (mmHg) for the Control Group (ie. grp. 1d).

Day	Systolic	Mean	Diastolic
5	160.00	104.20	76.32
6	155.96	105.40	80.12
7	161.45	112.60	88.15
8	158.96	107.30	81.45
9	171.89	106.00	73.30
10	163.54	105.20	76.00
11	155.00	112.50	91.31
12	156.25	115.60	94.00
13	156.25	103.10	76.70
14	165.60	112.50	86.02
17	165.63	103.13	71.97
18	159.40	112.50	89.00
19	156.25	112.50	94.40
20	150.00	100.00	75.00
21	143.75	106.25	87.52

Systolic Mean and Diastolic Blood Pressure Measurements (mmHg) for the LPS Treated Group (ie. grp. 4a)

Day	Systolic	Mean	Diastolic
5	156.25	106.25	81.23
6	159.40	103.13	73.50
7	165.63	109.40	81.23
8	153.13	112.50	92.30
9	168.75	100.00	65.63
10	165.37	112.50	85.93
11	162.50	109.38	82.84
12	93.75	65.63	51.56
13	162.50	115.63	92.14
14	165.60	109.40	81.26
17	159.38	109.40	84.37
18	162.50	115.63	92.25
19	156.25	109.40	86.06
20	165.63	103.13	71.86

21	162.50	103.13	73.45

Systolic, Mean and Diastolic Blood Pressure Measurements (mmHg) for the LPS + L-NAME Treated Group (ie. grp. 4c)

Day	Systolic	Mean	Diastolic
5	162.50	103.13	73.40
6	156.25	109.40	85.90
7	159.38	109.40	84.38
8	153.13	112.50	92.25
9	162.50	103.13	75.20
10	165.63	103.13	58.84
11	153.13	109.40	87.59
12	112.50	78.12	61.10
13	168.75	100.00	63.63
14	178.13	112.50	79.70
17	193.60	121.89	86.00
18	187.50	121.89	89.10
19	189.20	118.75	83.53
20	193.75	125.00	90.62
21	193.75	118.75	71.24

Appendix Tables 8.7

The drinking rates for control group 1a and animals injected on day 5 (grp. 2b) and 11 (grp. 2c) post-implantation.

Grp	Control (1)			2b			2c		
Day	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
3-4	42.93	3.80	3	44.80	2.40	3	32.70	9.20	4
4-5	65.60	3.41	3	67.20	2.00	3	23.70	5.60	4
5-7	67.00	25.50	3	55.60	28.00	3	42.10	5.50	4
7-9	65.20	6.00	3	73.80	14.40	3	50.25	2.90	4
9-11	70.70	5.90	3	71.40	7.70	3	36.20	10.70	4
11-13	90.56	13.60	3	98.50	10.70	3	29.10	2.42	4
13-15	66.46	15.90	3	65.30	18.30	3	38.00	1.37	4
15-17	78.80	7.90	3	80.00	14.00	3	48.50	1.18	4
17-19	70.56	4.60	3	76.80	8.60	3	68.90	2.80	4
19-21	58.50	19.20	3	60.70	20.90	3	49.10	4.40	4
21-23	79.50	7.70	3	85.50	14.80	3	55.30	4.00	4
23-25	75.50	3.70	3	93.90	10.50	3	58.86	2.36	4
25-27	87.60	17.60	3	68.10	8.40	3	-	-	-

The drinking rate and injested dose of AG for animals fed on AG from day 4 post-implantation and subsequently injected with LPS on day 5 (ie. grp. 3b).

Grp	3b			3b Doseage		
Day	Mean	S.E.	n	Mean	S.E.	n
3-4	14.60	0.10	5	-	-	-
4-5	30.60	0.60	5	0.08	0.006	5
5-7	41.80	4.90	5	0.145	0.018	5
7-9	41.90	4.10	5	0.140	0.017	5
9-11	46.50	2.40	5	0.158	0.010	5
11-13	43.90	1.80	5	0.140	0.009	5
13-15	47.30	2.10	5	0.149	0.007	5
15-17	51.80	2.00	5	0.150	0.009	5
17-19	68.40	2.00	5	0.200	0.007	5
19-21	49.30	3.50	5	0.140	0.011	5
21-23	48.90	2.70	5	0.140	0.150	5
23-25	47.60	2.30	5	0.130	0.012	5

Appendix Tables 8.8

Drinking rates for Control group 1b and animals injected with LPS one day prior to implantation (ie. grp. 2a).

Grp	Control (2)			2a		
Day	Mean	S.E.	n	Mean	S.E.	n
5-7	61.50	3.30	4	63.40	3.00	4
7-9	67.40	2.20	4	51.50	3.70	4
9-11	59.80	3.70	4	59.50	2.70	4
11-13	58.00	4.30	4	60.60	3.80	4
13-15	56.25	2.90	4	53.30	3.20	4
15-17	56.25	2.70	4	59.50	3.80	4
17-19	57.40	6.65	4	71.30	3.50	4
19-21	54.20	4.60	4	50.02	2.60	4

Drinking rates and injested dose of AG for animals fed AG from day 10 (ie. grp. 3a).

Grp	AG Treated			AG Treated Doseage		
Day	Mean	S.E.	n	Mean	S.E.	n
6-7	33.25	0.62	4	-	-	-
7-8	39.15	1.49	4	-	-	-
8-9	36.50	1.80	4	-	-	-
9-10	41.60	1.60	4	-	-	-
10-11	41.90	2.40	4	0.020	0.002	4
11-12	39.40	1.06	4	0.100	0.003	4
12-13	47.67	24.60	4	0.100	0.002	4
13-14	40.47	3.40	4	0.118	0.006	4
14-15	36.45	2.50	4	0.100	0.004	4
15-16	38.50	2.70	4	0.090	0.004	4
16-17	40.50	3.00	4	0.100	0.005	4
17-18	39.80	2.40	4	0.100	0.006	4
18-19	39.15	2.40	4	0.096	0.008	4
19-20	37.90	2.30	4	0.097	0.006	4

Appendix Table 8.9

Drinking rates for Control group 1c and animals fed with L-NAME from day 0 (ie. grp. 4b).

Grp	Control (3)			L-NAME Treated			L-NAME Dose	
Day	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.
0-2	67.20	3.47	3	48.12	2.90	4	0.078	0.008
2-4	53.90	2.70	3	29.90	2.50	4	0.069	0.012
4-6	63.50	6.30	3	38.70	6.21	4	0.101	0.026
6-11	127.80	9.00	3	79.60	4.04	4	0.076	0.007
11-13	67.30	1.60	3	44.00	4.10	4	0.067	0.005
13-15	61.40	6.20	3	38.70	6.00	4	0.067	0.061
15-17	70.20	5.90	3	41.70	6.30	4	0.058	0.010
17-19	67.80	5.30	3	45.40	6.40	4	0.081	0.008

Appendix Table (8.10) to Fig. 8.21a.

Mean drinking rates, weights and effective dosage for control group 1d. This group of animlas received water only.

Grp	Weight (g)			Intake (ml)			Effective Dose (ml/g)		
Day	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
0	335.52	8.82	5	30.30	1.14	5	0.090	0.004	5
1	338.40	8.38	5	33.76	1.30	5	0.100	0.003	5
2	343.40	8.27	5	31.30	1.10	5	0.091	0.003	5
3	347.80	8.32	5	34.20	1.17	5	0.099	0.005	5
7	355.36	8.69	5	31.26	2.04	5	0.089	0.007	5
8	357.60	8.37	5	34.76	1.95	5	0.097	0.006	5
9	359.00	8.36	5	32.52	0.81	5	0.091	0.002	5
10	357.62	7.12	5	30.34	2.21	5	0.085	0.006	5
11	358.40	7.75	5	35.32	1.41	5	0.099	0.004	5
12	363.60	6.44	5	32.82	1.73	5	0.090	0.004	5
13	362.20	7.61	5	31.34	2.40	5	0.087	0.006	5
14	361.60	7.95	5	39.48	2.25	5	0.110	0.008	5
15	363.20	7.37	5	28.48	1.97	5	0.079	0.005	5
16	364.80	7.64	5	34.34	2.47	5	0.094	0.006	5
17	370.20	8.19	5	38.65	4.03	4	0.102	0.010	4
18	377.75	6.64	4	31.30	2.49	4	0.083	0.006	4
19	376.23	6.75	4	42.28	3.28	4	0.113	0.010	4
20	381.25	7.32	4	35.70	3.65	4	0.093	0.008	4

Appendix Tables (8.11) to Fig. 8.21a

Mean drinking rates, weights and effective dosage in animals injected with LPS on day 11 (ie. grp. 4a).

Grp	Weight (g)			Intake (ml)			Effective Dose (ml/g)		
Day	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
0	320.08	12.16	5	31.14	3.51	5	0.096	0.008	5
1	323.80	11.98	5	32.48	3.49	5	0.100	0.007	5
2	327.00	11.72	5	29.94	2.26	5	0.092	0.004	5
3	331.40	11.67	5	34.26	2.74	5	0.104	0.007	5
7	337.60	11.29	5	29.12	2.40	5	0.086	0.005	5
8	337.60	10.87	5	33.56	2.12	5	0.099	0.004	5
9	337.70	10.46	5	30.00	1.49	5	0.087	0.006	5
10	339.30	11.33	5	30.90	2.95	5	0.091	0.006	5
11	337.40	11.08	5	15.14	4.43	5	0.093	0.012	5
12	345.60	16.98	5	22.72	3.90	5	0.044	0.011	5
13	334.98	14.64	5	27.08	2.92	5	0.082	0.010	5
14	325.60	13.11	5	39.08	3.54	5	0.119	0.007	5
15	324.60	13.10	5	24.06	3.04	5	0.073	0.007	5
16	324.00	13.10	5	29.90	3.40	5	0.091	0.007	5
17	325.38	13.01	4	36.50	4.54	4	0.111	0.009	4
18	328.50	14.45	4	30.25	4.72	4	0.091	0.010	4
19	328.00	14.12	4	32.88	4.21	4	0.099	0.008	4
20	333.00	14.24	4	35.45	4.03	4	0.106	0.007	4

Appendix Table (8.12) to Figs. 8.21a/b & 8.22e.

Mean drinking rates, weights and effective dosage in the animals receiving LPS + L-NAME (ie. group 4c).

Grp	Weight (g)			Intake (ml)			Actual Dose (ml/g)		
Day	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
0	335.96	7.84	5	33.36	3.69	5	-	-	-
1	341.00	6.86	5	33.99	2.37	5	-	-	-
2	347.80	5.79	5	34.06	2.20	5	-	-	-
3	353.40	5.41	5	38.40	1.72	3	-	-	-
7	357.24	5.28	5	31.34	3.77	5	-	-	-
8	360.60	4.93	5	38.74	1.87	5	-	-	-
9	362.00	4.97	5	34.42	2.24	5	-	-	-
10	359.24	4.60	5	24.40	1.52	5	0.020	0.004	5
11	350.20	4.87	5	9.00	6.04	5	0.026	0.017	5
12	332.00	7.35	5	13.02	6.68	5	0.038	0.019	5
13	319.60	10.30	5	19.46	3.58	5	0.060	0.009	5
14	321.00	10.31	5	33.58	4.10	5	0.104	0.013	5
15	321.40	9.76	5	15.78	2.37	5	0.048	0.006	5
16	320.20	9.47	5	32.00	1.99	5	0.068	0.005	5
17	327.13	11.41	4	30.68	3.33	4	0.093	0.008	4
18	328.75	12.09	4	29.18	1.81	4	0.090	0.008	4
19	331.75	10.07	4	29.38	1.93	4	0.089	0.008	4
20	334.25	10.55	4	30.13	1.40	4	0.091	0.006	4

Appendix Table (8.13) to Figs. 8.22a &8.22f

Meaned weights (g) of control animals (1a) and animals injected with LPS on day 5 post-implantation (ie. grp. 2b).

Grp	Control (1)			2b		
Day	Mean	S.E.	n	Mean	S.E.	n
4	397.70	34.40	3	389.10	49.00	3
5	398.60	32.16	3	388.70	43.60	3
7	405.00	32.97	3	373.10	52.10	3
9	408.00	27.40	3	379.50	48.10	3
11	411.70	28.50	3	377.30	46.00	3
13	416.30	26.00	3	381.80	45.20	3
15	409.70	35.70	3	389.00	44.40	3
17	420.30	29.40	3	395.40	42.40	3
19	428.00	26.40	3	399.30	40.10	3
21	431.10	27.50	3	413.30	32.10	3
23	456.60	22.30	3	410.00	38.00	3
25	463.50	28.50	3	432.00	11.00	3
27	469.00	26.00	3	458.50	11.50	3

Meaned weights of animals injected with LPS on day 11 (ie, grp. 2c) and fed with AG and subsequently injected with LPS on day 5 post-implantation.

Grp	2c			3b		
Day	Mean	S.E.	n	Mean	S.E.	n
4	354.40	25.90	4	331.60	16.20	5
5	351.70	27.13	4	307.00	16.50	5
7	350.90	26.67	4	288.40	16.27	5
9	352.30	24.50	4	299.10	16.62	5
11	353.80	27.10	4	307.80	16.10	5
13	339.90	25.90	4	312.20	15.40	5
15	332.30	25.50	4	320.30	14.08	5
17	335.80	23.80	4	324.50	15.70	5
19	341.70	23.50	4	327.80	15.60	5
21	358.00	29.60	4	343.10	14.10	5
23	367.00	27.50	4	325.26	25.58	5
25	373.00	23.17	4	358.90	22.90	5

Appendix Table (8.14) to Figs. 8.22b & 8.22d.

Meaned weights of control animals (grp. 1b) and animals injected with LPS one day prior to implantation.

Grp	Control (2)			2a		
Day	Mean	S.E.	n	Mean	S.E.	n
7	403.10	18.60	4	366.60	12.30	4
9	402.10	16.53	4	376.30	13.40	4
11	404.60	17.69	4	378.40	13.60	4
13	402.70	16.69	4	384.70	12.60	4
15	403.70	16.89	4	388.40	11.40	4
17	402.80	14.50	4	391.50	11.80	4
19	408.90	13.30	4	400.00	11.70	4
21	408.20	13.05	4	408.60	8.40	4
23	406.30	10.50	4	410.50	8.70	4

Meaned weights of animals fed with AG from day 10 post-implantation.

Grp	AG Treated		
Day	Mean	S.E.	n
5	368.70	12.20	4
7	389.60	10.50	4
8	393.60	9.80	4
9	394.00	9.70	4
10	391.00	6.70	4
11	398.80	8.36	4
12	395.50	8.25	4
13	394.20	8.75	4
14	394.70	9.19	4
15	399.20	9.36	4
16	399.20	9.50	4
17	399.90	10.00	4
18	398.50	10.90	4
19	399.30	11.20	4
20	400.60	10.60	4

Appendix Tables (8.15) to Fig. 8.22c

Meaned weight of control animals (grp. 1c) and those treated with L-NAME from day 0 (ie. grp. 4b).

Grp	Control (3)			L-NAME Treated		
Day	Mean	S.E.	n	Mean	S.E.	n
0	354.60	39.60	3	300.20	20.70	4
4	355.00	33.10	3	298.00	19.60	4
6	358.90	32.70	3	296.00	13.70	4
11	361.70	30.90	3	303.30	15.20	4
13	368.80	33.10	3	305.00	13.00	4
15	374.20	32.80	3	303.00	13.10	4
17	383.00	32.30	3	305.00	9.90	4
19	385.00	31.16	3	300.00	7.50	4

Appendix tables (9.1) to Figs.9.1 & 9.2

Comparing all the control and LPS-treated RTA data

[PE]	Control			LPS (24 hr)			t-test
(M)	Mean	S.E.	n	Mean	S.E.	n	unpaired
$1x10^{-5}$	10.57	1.19	56	8.13	1.62	25	0.248
$2x10^{-5}$	10.25	1.64	56	11.87	1.84	29	0.102
$5x10^{-5}$	30.28	2.85	56	21.52	2.85	29	0.054
$1x10^{-4}$	45.91	3.65	55	30.53	3.27	29	0.007
$2x10^{-4}$	69.97	4.66	56	45.36	4.21	29	0.001
$5x10^{-4}$	115.34	6.41	56	71.67	6.31	28	0.000
$1x10^{-3}$	156.91	7.29	56	108.83	7.00	29	0.000
$2x10^{-3}$	198.28	7.48	56	148.46	7.57	29	0.000
$5x10^{-3}$	230.04	8.34	42	185.13	7.86	29	0.000
$1x10^{-2}$	236.66	9.53	30	214.12	7.66	28	0.073

Comparing all the CEA and TEA data

[PE]	CEA			TEA			t-test
(M)	Mean	S.E.	n	Mean	S.E.	n	unpaired
$1x10^{-5}$	9.20	0.70	28	7.60	0.70	29	0.210
$2x10^{-5}$	12.85	1.00	28	10.56	1.00	27	0.135
$5x10^{-5}$	26.10	2.80	28	21.20	2.10	29	0.175
$1x10^{-4}$	41.40	3.60	27	37.70	3.90	29	0.500
$2x10^{-4}$	72.88	6.80	27	56.80	5.20	29	0.067
$5x10^{-4}$	114.00	8.90	27	92.40	7.50	28	0.059
$1x10^{-3}$	159.00	12.70	26	117.80	7.30	27	0.007
$2x10^{-3}$	192.60	11.80	21	154.30	9.40	23	0.015
$5x10^{-3}$	254.00	10.40	14	179.40	9.80	18	0.000
$1x10^{-2}$	286.40	6.70	5	195.10	15.80	8	0.001