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An investigation of the mechanisms underlying hypotension and vascular hyporeactivity in septic shock

by

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For Mum, Dad and Laura

Abstract

Septic shock is a common condition carrying a high mortality. The clinical features are a high cardiac output circulation with hypotension and vascular hyporeactivity and evidence of organ failure. I developed an organ culture *in vitro* model and a fluid-resuscitated *in vivo* model of sepsis to investigate potential underlying mechanisms for the vascular hyporeactivity, namely inducible nitric oxide synthase (iNOS) overproduction and ATP-sensitive K^+ (K_{ATP}) channel activation. I also studied the phenomenon of vascular supersensitivity to vasopressin/terlipressin in sepsis. I could demonstrate a temporal variation in vascular hyporeactivity to lipopolysaccharide (LPS) in rat mesenteric artery (RMA) *in vitro* over a 46 h period. This was reversed by a variety of iNOS inhibitors (aminoguanidine, L-NAME, 1400W and GW273629) and the guanylyl cyclase inhibitor, ODQ. Likewise, inhibition of the K_{ATP} channel via its pore-forming subunit, rather than its sulphonylurea receptor (SUR), also reversed LPS-induced hyporeactivity *in vitro*, though vasopressin had no effect. *In vivo,* iNOS inhibition and terlipressin all raised blood pressure in the septic animals over and above the pressor effect achieved in non-septic animals; K_{ATP} channel inhibition raised blood pressure in shams more than septic animals. However, these agents failed to improve macro- or microcirculatory blood flow, nor did they attenuate the metabolic acidosis. In patients with norepinephrine-resistant septic shock, I reported the first use of terlipressin in reversing hypotension and reducing norepinephrine requirements. Although terlipressin represents a significant advance in our ability to treat septic shock, care should be taken when using it.

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Abbreviations

ABF	–	aortic blood flow
Ach	–	acetylcholine
AMG	–	aminoguanidine
ATP	–	adenosine triphosphate
$BaCl_2$	–	barium chloride
BK_{Ca}	–	large conductance calcium activated K^+ channel
cAMP	–	cyclic adenosine 3,5-monophosphate
cGMP	–	cyclic guanosine 3,5-monophosphate
ChTx	–	charybdotoxin
COX	–	cyclo-oygenase
DAG	–	diacylglycerol
DMSO	–	dimethyl sulphoxide
ENDO	–	endothelium
Glib	–	glibenclamide
HBSS	–	Hanks Balanced Salt Solution
HMA	–	human marginal artery
$HmvO_2$-	–	hepatic microvascular oxygenation
IbTx	–	iberiotoxin
IL-1β	–	interleukin 1 beta
iNOS	–	inducible nitric oxide synthase
IP_3	–	inositol triphosphate
iv	-	intravenous

K_{ATP}	–	ATP-sensitive K^+ channel
K_{IR}	–	inward rectifier K^+ channel
K_V	–	voltage-gated K^+ channel
LPS	–	lipolysaccharide
L-NAME	–	N(G)-nitro-l-arginine methyl ester
MAP	–	mean arterial pressure
Min	–	minute
NO	–	nitric oxide
NE	–	norepinephrine
ODQ	–	1H-[1,2,4]-oxadiazole[4,3-alpha]quinoxalin-1-one
PGI_2	–	prostacyclin
PKA	–	protein kinase A
PKC	–	protein kinase C
PKG	–	protein kinase G
RBF	–	renal blood flow
RMA	–	rat mesenteric artery
sGC	–	soluble guanylyl cyclase
SUR	–	sulphonylurea receptor
TEA^+	–	tetraethyammonium ion
TNF-α		tumour necrosis factor alpha
Tolb	–	tolbutamide
TP	–	terlipressin
VP	–	vasopressin

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Chapter 1

Introduction

1.1 Sepsis

Sepsis, the exaggerated systemic host response to infection (Bone *et al.*, 1992), is the leading cause of death in the critically ill. In the United States, sepsis affects 750,000 people per year, of whom 210,000 die (Murphy *et al.*, 2000 and Angus *et al.*, 2001). Data for England and Wales suggest that 51 per 100,000 population per year admitted to intensive care units meet criteria for severe sepsis (i.e. sepsis with related organ dysfunction) in the first 24 hours of admission. Of these patients, 35% died before intensive care unit discharge and a further 12% died during their hospital stay (Padkin *et al.*, 2003).

Numerous trials conducted using agents that inhibit the inflammatory cascade have generally failed to show any survival benefit. Examples include corticosteroids (Bone *et al.*, 1987), endotoxin antibodies (Ziegler *et al.*, 1991), tumour necrosis factor (TNF) antagonists (Abraham *et al.*, 1995 and Fisher *et al.*, 1996), interleukin-1 receptor antagonists (Fisher *et al.*, 1994), cyclo-oxygenase (COX) pathway antagonists (Bernard *et al.*, 1997) and inducible nitric oxide synthase (iNOS) inhibitors (Lopez *et al.*, 2004 and Watson *et al.*, 2004). However, recently published studies have demonstrated decreased mortality and morbidity as a result of interventions applied to patients with sepsis. Recombinant human activated protein C, a naturally-occurring anticoagulant, is the first anti-inflammatory agent shown to have statistically significant effectiveness in the treatment of sepsis with a 19.4% reduction in the risk of death, and an absolute risk reduction of 6.1% (Bernard *et al.*, 2001). van den Berghe and colleagues (van den Berghe *et al.*, 2001) demonstrated that intensive insulin therapy in which the blood

glucose level was maintained at 4.4 – 6.1 mM resulted in lower morbidity and mortality among high-risk surgical patients compared to conventional therapy (10-11.1 mM). Such therapy also reduced the frequency of episodes of sepsis by 46% and the mortality from bacteraemia (12.5% vs 29.5%). Insulin therapy reduced the rate of death from multiple organ failure among patients with sepsis, regardless of whether they had a history of diabetes. Rivers reported that an early and aggressive therapeutic strategy that optimized cardiac preload, afterload and contractility in patients with severe sepsis and septic shock in a Detroit emergency room improved their likelihood of survival (Rivers *et al.*, 2001). Likewise, early administration of "low-dose" corticosteroids to septic patients with persistent shock requiring ventilation also resulted in improved mortality (Annane *et al.*, 2002).

I chose to examine the mechanisms underlying vasodilatory septic shock. Sepsis usually leads to a high cardiac output, low systemic vascular resistance circulation with a depressed blood pressure (Parillo, 1993). In such patients, therapy with an α_1 adrenergic agonist is often required to reverse the hypotension. However, hypotension may be resistant to high doses of these vasopressor agents, a state known as "*vascular hyporeactivity*". Despite extensive research, the pathophysiology underlying septic shock in humans remains poorly understood. I set out to develop experimental models of sepsis to examine the effects of modulating the following mechanisms in vasodilatory septic shock – the nitric oxide (NO) pathway, potassium channels, and vasopressin. I also briefly investigated the inducible cyclo-oxygenase (COX-2) pathway. Other vasodilatory mechanisms may also be involved in sepsis, for instance,

increased production of calcitonin gene-related peptide (CGRP) (Fatehi–Hassanabad *et al.,* 1996). However, time constraints prevented study of these pathways.

A major reason for the current failure to have fully characterised mechanisms underlying vascular hyporeactivity and hypotension is that animal models are not necessarily representative of the clinical condition of septic shock (reviewed in Fink and Heard, 1990 and Deitch, 1998). Thus, one of my aims was to develop more reproducible *in vitro* and *in vivo* animal models.

1.2 Endotoxin

Gram-negative bacterial infections are responsible for approximately 50% of cases of septic shock (reviewed by Thiemermann, 1997). Administration of endotoxin (lipopolysaccharide; LPS), a cell wall component ubiquitous to Gram-negative bacteria, to animals and human volunteers has been used extensively to mimic Gram-negative sepsis (Thiemermann, 1997 and Deitch, 1998). Cells of the innate immune system recognise microorganisms and initiate responses through pattern-recognition receptors called toll-like receptors (TLRs) (Modlin *et al.*, 1999; Vasselon, *et al*, 2002 and Underhill *et al.*, 2002). Insight into the role of TLRs in combating infection has been provided by studies using C3H/HeJ mice (Modlin *et al.*, 1999) which are resistant to endotoxin because of a mutation in the toll-like receptor gene (TLR4). Despite their resistance to LPS, these mice have increased mortality in authentic sepsis (Hagberg *et al.*, 1985 and Hotchkiss *et al.*, 1999). TLR4 mutations have also been identified in humans and may make people more susceptible to infection (Arbour *et al.*, 2000). Thus

total blockade of LPS may be detrimental. Indeed, trials using monoclonal endotoxin antibodies to treat patients with sepsis have been unsuccessful (Warren *et al.*, 1993).

1.3 Pro-inflammatory cytokines

Several cytokines are considered to be involved in the pathophysiology of sepsis e.g. interleukin1-β (IL1-β), interleukin-6, tumour necrosis factor α (TNF-α), interferon-γ and adenosine (reviewed in Landry and Oliver, 2001). Although cytokines are considered to be culprits, they may have beneficial effects in sepsis. Studies in animal models of peritonitis demonstrated that blocking TNF-α worsens survival (Eskandari *et al.*, 1992 and Echtenacher *et al.*, 2001). In clinical trials, a TNF receptor fusion protein, which binds and neutralises TNF-α, increased mortality (Fisher *et al.*, 1996), despite findings of protection against death in animal models of bacterial sepsis (Mohler *et al.*, 1993). Combination immunotherapy against TNF-α and interleukin-1 receptors was also fatal in a neutropenic model of sepsis (Opal *et al.*, 1996). The role of TNF-α in combating infection has recently been underscored by the finding that sepsis and other infectious complications developed in patients with rheumatoid arthritis treated with TNF antagonists (Keane *et al.*, 2001). However, a meta-analysis of 10 sepsis studies with over 6500 patients showed an absolute reduction in mortality of 3.5% using anti-TNF drugs (Marshall, 2003). In addition, carriers of the TNFB2 allele are at increased risk of lethal septic shock (reviewed by Lin, 2004). Thus there may be subgroups of the population who will benefit from therapy directed against TNF. Other biologically active agents may also prove beneficial when used in specific strategies, but this is dependent on our understanding of sepsis improving.

1.4 Nitric Oxide

In many animal models, endotoxin-induced vascular hyporeactivity is associated with enhanced formation of nitric oxide (NO) within the blood vessel (Julou-Schaeffer *et al.*, 1990). This molecule is synthesised from the semi-essential amino acid L-arginine by a family of nitric oxide synthase (NOS) isoenzymes (Moncada & Higgs 1995). There are three isoforms of NOS enzymes: nNOS - a neuronal isoform, eNOS - an endothelial isoform and iNOS a transcriptionally inducible isoform. nNOS and eNOS are present in endothelial cells, certain neurones, platelets, endocardium and skeletal muscle as constitutively active enzymes. These two isoforms are calcium-calmodulin dependent and generate small amounts of NO (nanomolar range) that acts as a physiological mediator. In response to bacterial endotoxins, exotoxins, or pro-inflammatory cytokines, iNOS is transcriptionally up-regulated in a wide variety of cells, producing substantially amounts (micromolar range) of NO in a calcium-independent manner (Vallance and Charles, 1998). Expression of iNOS has been shown to occur in endothelial, vascular smooth muscle and myocardial cells, macrophages and circulating leucocytes. The adventitia appears to represent the main site of NO production in the vasculature (reviewed by Muller *et al.*, 2000). Furthermore, pro-inflammatory cytokines (interleukin-1β and interferon-γ) may also increase NO production by upregulating the expression of GTP cyclohydrolase 1 (GTPCH-1). Induction of GTPCH-1 leads to the generation of tetrahydrobiopterin, an essential cofactor for all NOS isoforms (Bhagat & Vallance, 1999). However, eNOS expression has also been shown to be upregulated in certain septic models (Bhagat *et al.*, 1999). Indeed, enhanced NO production may result

from activation of eNOS in the early stages of the septic insult (<1 hour (h)) followed by expression of iNOS commencing after 2 - 3 h (Thiemermann, 1994 & 1997).

Once formed, NO can activate soluble guanylyl cyclase which results in vascular smooth muscle relaxation via the formation of guanosine 3'-5' cyclic-monophosphate (cGMP) (see Fig 1.1). Consistent with this, inhibition of NOS or soluble guanylyl cyclase has been shown to reverse vascular hyporeactivity and hypotension *in vivo* and *in vitro* (Julou-Schaeffer *et al.,* 1990; Hall *et al.*, 1996; Scott *et al.,* 1996; Kilbourn, 1999; Levy *et al.*, 2004 and Martin *et al.*, 2004). This mechanism is likely to be responsible for the immediate effects of NO. The NO derived from iNOS- may interact with physiological substrates such as thiols to yield biologically active or inactive S-nitrosothiols during endotoxemia. It is possible that these relatively stable, vasoactive compounds may act as a storage system for NO, perhaps within the medial layer of vascular smooth muscle, and thus mediate longer lasting effects of NO. Dinitrosyl non-heme iron complexes (DNIC), which occur in different cell types or tissues during activation of the iNOS pathway, may also be involved in this putative mechanism (reviewed in Muller *et al.*, 2000). After incubation with LPS, four times as much DNIC was present in the media compared to the adventitia (Kleschyov *et al.*, 1997). These may represent NO stores with which low molecular weight thiols interact to produce vasorelaxation (Mulsch *et al.*, 1991). In LPS treated tissues, the vasorelaxant effect of the low molecular weight thiol, N-acetylcysteine, correlated with preformed NO stores as it was inhibited by the non-selective NOS inhibitor, L-NAME (Muller *et al.*, 1996). Interestingly the relaxation observed with N-acetylcysteine was not associated with a

rise in cGMP or affected by inhibition with cGMP-dependent protein kinase. It was, however, attenuated by the non-selective potassium channel inhibitor tetrabutylammonium (3mM), and by elevated concentrations of KCl as the contractile agent (Muller *et al.*, 1998). Thus the N-acetylcysteine-induced vasorelaxation, although related to preformed NO stores, was mediated by potassium channel activation rather than through cGMP. The precise mechanism involved remains unknown, but could involve oxidation of thiol residues.

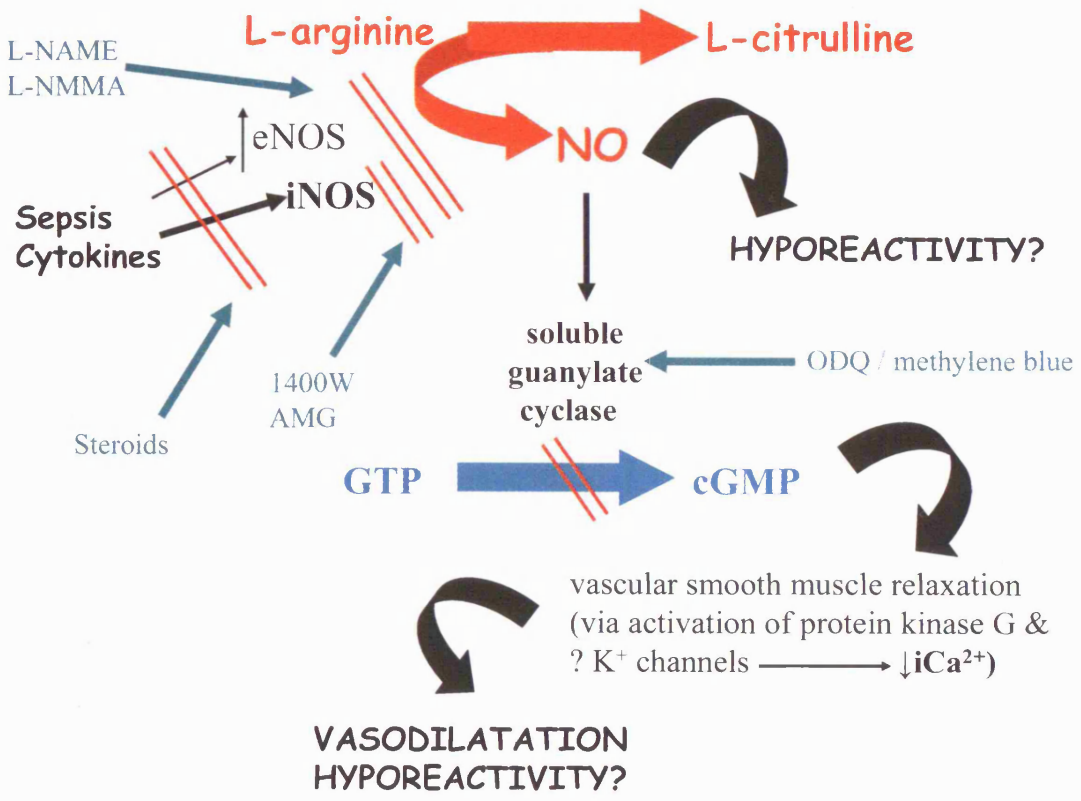


Figure 1.1 The pathway of nitric oxide (NO) formation and action within a vascular smooth muscle cell. NO either diffuses into the cell from the endothelium or adventitia, or is synthesised from L-arginine via NO synthase (NOS). The NO then activates soluble guanylyl cyclase (sGC) to catalyse the formation of cGMP from GTP, which then activates protein kinase G. Inhibitors of various aspects are shown in green. (AMG-aminoguanidine; ODQ-1H-[1,2,4]-oxadiazole[4,3-alpha]quinoxalin-1-one; L-NAME-N(G)-nitro-l-arginine methyl ester; L-NMMA- N^G-monomethyl-L-arginine).

1.5 Human Studies

Septic humans produce much lower quantities of NO than rodents. iNOS induction has been particularly difficult to demonstrate in many human cell types (reviewed by Vallance & Charles, 1998). However, there is plentiful evidence for enhanced NO production in septic shock patients. For example, NO levels of up to 150 micromolar

were detected in blood from ebola virus victims, with NO being much higher in fatal cases and with increasing disease severity (Sanchez *et al.*, 2004). Moreover, positive iNOS immunoreactivity has been detected in a handful of human studies. It has been demonstrated in the internal mammary artery after incubation with a mixture of cytokines and high doses of LPS for 24-48 hours *in vitro* (Chester *et al.*, 1998) and in omental arteries taken from eight septic patients undergoing bowel resection (Stoclet *et al.,* 1999). In addition, iNOS activity was detected in muscle, fat and artery but not in skin of putrescent areas in 13 patients with septic shock from cellulitis (Annane, 2000). They could also not detect iNOS activity in surrounding inflamed areas. Interestingly, polymorphisms within the iNOS promoter regions were found in Ghanaian children with severe malaria (Cramer *et al.*, 2004).

The observation that N^G-monomethyl-L-arginine (L-NMMA) restored blood pressure in septic shock patients (Bakker *et al.*, 2004) prompted a large-scale phase III study with this agent. However, the study was halted prematurely after an interim analysis revealed an *increase* in deaths in the L-NMMA treated group (Lopez *et al.*, 2004). As mentioned above, NO is required for a large range of normal physiological functions. It plays an important role in the regulation of organ blood flow distribution and autoregulation, and it inhibits the adhesion of platelets and neutrophils to the endothelium. In addition, the induction of NOS by cytokines or endotoxin in macrophages greatly contributes to the bactericidal effects of these cells (reviewed by Thiermermann 1994 and Hauser *et al.*, 2004). Thus, NO has been described as both a 'friend and foe' in septic shock (Springall, 1995). Given its widespread actions, it is not surprising that non-specific

inhibition of its synthesis in sepsis has been associated with a variety of deleterious effects and an increase in mortality.

1.6 Vascular Smooth Muscle

The vascular smooth muscle is located between the endothelium, on the luminal side, and the fibroblast-like cells of the adventitia. Vascular smooth muscle cells (VSMCs) are excitable cells that are the mechanical effectors of blood vessel diameter. A wide variety of agents exert their effect upon blood vessels via vascular smooth muscle. These include (i) factors released from the endothelium such as NO, prostacyclin (PGI_2), endothelin (e.g. Rubanyi & Polokoff, 1994) and endothelium-derived hyperpolarising factor (EDHF) (Gryglewski *et al.*, 1986; Palmer *et al.*, 1987 and Edwards *et al.*, 1998); (ii) neurotransmitters released from the adventitia, such as substance P, norepinephrine, and NO (Hirst & Edwards, 1989 and Guterman, 1999); and (iii) factors circulating within the bloodstream such as angiotensin II and thromboxane (Hirst & Edwards, 1989). The pH and temperature may also regulate vascular smooth muscle function (Hill *et al.*, 2001). Smooth muscle cells can themselves generate vasoactive factors after an inflammatory insult, including PGI_2, endothelin and NO (Moncada, 1982 and Moncada *et al.*, 1991).

1.6.1 Vascular smooth muscle contraction

All muscle cells use calcium (Ca^{2+}) as a signal for contraction. VSMCs are regulated by a variety of neurotransmitters and hormones. These interact with a network of signal transduction pathways that ultimately affect contractility either by affecting Ca^{2+} levels

in the cell, or the response of the contractile elements to calcium. Ca^{2+} levels are increased by extracellular entry via voltage-gated Ca^{2+} channels, receptor- operated and capacitative (store-operated) Ca^{2+} channels (transient receptor potential proteins), as well as by release from intracellular stores (Nelson and Quayle, 1995). At high cytosolic concentrations, Ca^{2+} forms a complex with calmodulin that activates a kinase, which phosphorylates the regulatory light chain of myosin. Phosphorylated myosin activates myosin ATPase by actin and the cycling of myosin cross-bridges along actin filaments, which contracts the muscles. Vasodilatation occurs when a kinase interacts with myosin light chain phosphatase (MLCP), which dephosphorylates myosin and prevents muscle contraction (Surks *et al.*, 1999). In smooth muscle, RhoA and Rho-kinase (ROCK) play an important role in Ca^{2+} sensitization, an event that controls vascular vessel tone. Ca^{2+} sensitization of vascular smooth muscle contraction involves Rho- and Rho-kinase-dependent suppression of myosin phosphatase activity (Hirata *et al.*, 1992). NO dilates hamster resistance arterioles by activating MLCP in a cyclic cGMP-dependent manner, thereby reducing the apparent Ca^{2+} sensitivity of the contractile apparatus. MLCP inactivation via the RhoA/Rho kinase pathway antagonizes this Ca^{2+}-desensitizing effect that, in turn, can be restored using RhoA/Rho kinase inhibitors (Bolz *et al.*, 2003). Thus cGMP-dependent inhibition of Rho Kinase may represent a mechanism of vasodilation in sepsis.

1.6.2 Vascular smooth muscle membrane potential

The membrane potential of vascular smooth muscle is a major determinant of vascular tone. All arteries exist in a partially contracted, pressurised state (vascular tone) and can

either hyperpolarise (make the membrane potential more negative) and dilate, or depolarise (make the membrane potential more positive) and thus induce contraction (Nelson and Quayle, 1995). There is a steep relationship between vascular tone and membrane potential; a few millivolts' (mV) depolarisation or hyperpolarisation can lead to significant alterations in blood vessel diameter (Nelson *et al.*, 1990). The resting membrane potential of vascular smooth muscle ranges from -50mV to -70mV, although it may vary with blood pressure, becoming depolarised as pressure increases (Nelson & Quayle, 1995). This, in turn, opens L-type voltage-gated calcium channels, thereby increasing cytosolic Ca^{2+} concentration and inducing vasoconstriction. Conversely, hyperpolarisation closes these channels, thus decreasing the cytosolic Ca^{2+} concentration and promoting vasodilation (Landry & Oliver, 2001). The membrane potential of vascular smooth muscle is controlled by a number of ion transporters and channels, such as K^+ and chloride (Cl^-) channels. The theoretical K^+ equilibrium potential (E_K) (-82 mV in a physiological gradient of 140mM $[K^+]_o$ and 5mM $[K^+]_i$) is close to the resting membrane potential, indicating that permeability through K^+ channels is a major factor in determining the resting membrane potential in smooth muscle.

1.7 K^+ channels and vascular smooth muscle function

Potassium (K^+) channels form the largest superfamily of ion channels, with at least 60 - ion conducting subunits described (Coetzee *et al.*, 1999). In blood vessels K^+ channels regulate the resting membrane potential of vascular smooth muscle cells (VSMC) and so control vascular tone and blood pressure (Standen & Quayle, 1998). These channels

when opened promote relaxation and, when closed, promote vasoconstriction. This is achieved predominantly via modulation of calcium (Ca^{2+}) entry through voltage-sensitive Ca^{2+} channels and IP_3 modulation (Nelson & Quayle, 1995).

1.7.1 Vascular K^+ channels

Four main types of K^+ channels have been described in vascular smooth muscle. These belong either to the voltage-gated K^+ channel family comprising of voltage-gated (K_V) and Ca^{2+} -activated K^+ channels (K_{Ca}) or the inwardly rectifying K^+ channel family comprising ATP-sensitive K^+ (K_{ATP}) and strong inward rectifier K^+ (K_{IR}) channels (Nelson & Quayle, 1995). More recently, K^+ channels belonging to a family of channels with two ion conducting pores (twin pore) have been identified in vascular smooth muscle (Gurney *et al.*, 2003), although the physiological role of these channels is far from clear.

(i) K_V channels

Voltage-dependent K^+ channels (K_V) have been identified in a variety of arteries including coronary, cerebral, mesenteric and pulmonary (Nelson & Quayle, 1995 and Clapp & Tinker, 1999). The structure consists of a 6-transmembrane domain (S1-6) protein with a pore domain (H5) between S5 and S6. Functional channels are formed through the association of 4 separate subunits with or without accessory proteins. The channel may be inhibited by 4-aminopyridine (4-AP) with a K_i in the range of 0.2 - 1 mM (Nelson & Quayle, 1995). In addition, these channels are inhibited by high

concentrations of tetraethylammonium (TEA^+) and barium (Ba^{2+}) ions, although these agents also inhibit a variety of other K^+ channels (Brayden, 1996). K_V channels open in response to membrane depolarisation. They appear to play a significant role in maintaining the membrane potential of smooth muscle, since inhibition by 4-AP in isolated arterial myocytes causes membrane depolarisation (Yuan, 1995), and in intact preparations causes vasoconstriction (Knot and Nelson, 1995). There are multiple types of Kv subunits in smooth muscle that gives rise to delayed rectifier K^+ channels and rapidly activating and inactivating (transient) K^+ channels. These channels have auxiliary beta subunits that regulate both the activation/inactivation properties of these channels as well as their pharmacology (reviewed in Korovkina *et al.*, 2002). In addition, vasoconstrictors such as histamine may utilise inhibition of Kv channels as a mechanism of contraction in some vascular beds (Ishikawa *et al.*, 1993). These channels probably also contribute to hypoxic pulmonary constriction (Yuan *et al.*, 2003).

(ii) K_{Ca} channels

Ca^{2+}-activated K^+ channels exist in virtually all excitable cells (Nelson & Quayle, 1995). Three main types have been described in endothelial and smooth muscle cells; small (SK_{Ca}), intermediate (IK_{Ca}) and large (BK_{Ca}) conductance K^+ channels with conductances ranging from 10-15 pS, 20-60 pS and 200-300 pS, respectively (Brayden, 1996). The BK_{Ca} channel is the most widely studied K^+ channel. The channel is made up of the alpha subunit that spans the membrane, forms the pore and contains the voltage sensor, while the beta subunit modulates Ca^{2+} sensitivity. BK_{Ca} channels are inhibited by TEA^+, though at a much lower concentration than other K^+ channels, the K_i

being ~200 μM (Langton *et al.*, 1991). It is widely accepted that use of TEA^+ at concentrations of 1 mM or less is considered specific for this channel. Other highly selective blockers include the scorpion toxins iberiotoxin (IbTx) (Giangiacomo *et al.*, 1992) and charybdotoxin (ChTx) (Jones *et al.*, 1990) which inhibit BK_{Ca} channels with a K_i of less than 10 nM.

BK_{Ca} channels are voltage-sensitive channels activated in response to increasing $[Ca^{2+}]_i$ their function is probably geared towards prevent excessive contraction. Indeed, they may be important in regulating arterial myogenic response. In certain small arteries, an increase in intravascular pressure may cause vasoconstriction (myogenic tone), thereby activating the channels, probably to limit excessive depolarisation. IbTx has been demonstrated to depolarise contracted coronary arteries (Brayden & Nelson, 1992 and Nelson & Quayle, 1995). It is not clear whether these channels contribute to the resting membrane potential as inhibitors have had no effect on resting tension (Nelson & Quayle, 1995). However, genetic studies have identified the β1 subunit as a physiological regulator of vascular tone; mice lacking this gene develop systemic hypertension and their arteries show vascular hyper-reactivity to agonists (Brenner *et al.*, 2000 and Pluger *et al.*, 2000). BK_{Ca} channels are the target for vasodilators such as NO (Bolotina *et al.*, 1994 and Mistry & Garland, 1998) and adenosine (Cabell *et al.*, 1994). In addition, inhibition of BK_{Ca} channels may represent a mechanism employed by vasoconstrictors as this will hinder the channel's ability to antagonise further depolarisation. Both angiotensin II and the thromboxane mimetic U-44619 inhibits BK_{Ca} channels in coronary arterial smooth muscle (Toro *et al.*, 1990 and Sornik and

Toro, 1992). Endothelial SK_{Ca} channels have also been shown to have a profound tonic, hyperpolarizing influence on resistance arteries in mice. This suggests that the level of this channel's expression in endothelial cells is a fundamental determinant of vascular tone and blood pressure (Taylor *et al.*, 2003).

(iii) K_{IR} channels

Inward rectifier (K_{IR}) channels pass inward current more readily than outward current. Unlike most other K^+ channels, they are significantly activated by hyperpolarisation negative to E_K. They are found mainly in small resistance arteries, but due to their peculiar current-voltage relationship can be activated by small increases in K^+ ion concentration which, in turn, causes vasodilatation (Quayle, *et al.*, 1996). K^+ ions may be released either from nerve terminals adjacent to smooth muscle cells (McCarron and Halpern, 1990) or from the endothelium (Edwards *et al.*, 1998). K_{IR} channels may be inhibited by low concentrations of Ba^{2+} (half block at -60mV being 2 μM), the inhibition of which is voltage-dependent (Nelson and Quayle, 1995). RT-PCR analysis in rat mesenteric artery suggests that the channel is likely to be made up of Kir2.1 subunits (Bradley *et al.*, 1999). K_{IR} channels are strongly expressed in endothelial cells where they probably regulate resting membrane potential (Nilius & Droogmans, 2001).

(iv) K_{ATP} channel

ATP-sensitive K^+ channels belong to a class of K^+ channels activated by low cytosolic ATP or elevated nucleotide diphosphate (NDP) levels, and inhibited by sulphonylurea

agents such as glibenclamide. K_{ATP} channels can also be activated by potassium channel opening drugs (KCOs), such as levcromakalim and pinacidil. In vascular smooth muscle, KCOs cause membrane hyperpolarisation, increase K^+ efflux and produce smooth muscle relaxation, the effects of which can be inhibited by glibenclamide (Nelson & Quayle, 1995).

The K_{ATP} channel is an octomeric complex consisting of four pore-forming subunits (Kir6.x) and four sulphonylurea receptor (SUR) subunits (Bryan & Aguilar-Bryan, 1999). The pore is thought to confer ATP inhibition and determine conductance, while the SUR is considered the primary target for sulphonylureas, KCOs and NDP. Different combinations of these subunits give rise to K_{ATP} channels with different conductances and channel pharmacology. It is generally accepted that the pancreatic β cell K_{ATP} channel is composed of Kir6.2 and SUR1, and the cardiac type of Kir6.2 and SUR2A. SUR2B together with either Kir6.1 or Kir6.2 are thought to be the K_{ATP} channels within smooth muscle. The former corresponds to the nucleotide-diphosphate regulated K^+ (K_{NDP}) channel described in most vascular smooth muscles, while the latter is the more classical type of K_{ATP} channel. In all tissues the SUR subunit can be inhibited by agents such as glibenclamide, tolbutamide and PNU-99963 (Khan *et al.*, 1997; Bryan & Aguilar-Bryan, 1999 and Cui *et al.*, 2003). The latter is a pinacidil-derived inhibitor which potently blocks relaxation to the K_{ATP} channel opener, pinacidil with an IC_{50} of 18 nM in rat aorta (Khan *et al.*, 1997). Interestingly, the IC_{50} for inhibition of the SUR by glibenclamide is ~10-200 nM (Fujita & Kurachi, 2000) whereas at a 1000-fold higher concentration, glibenclamide appears to interact with the pore directly (Gribble

et al., 1998 and Bryan & Aguilar-Bryan, 1999). The pore-forming subunit can be inhibited by barium chloride ($BaCl_2$) or PNU-37883A, though the latter only appears to inhibit smooth muscle K_{ATP} channels (Nelson & Quayle, 1995; Wellman *et al.*, 1999; Surah-Narwal *et al.*, 1999 and Cui *et al.*, 2003). Both the pore and the SUR contain Ser/Thr phosphorylation sites for regulation by protein kinases. In smooth muscle, vasodilators that increase cAMP and activate protein kinase A (PKA) open the channel, whereas vasoconstrictors that activate protein kinase C (PKC) close it (Quayle *et al.*, 1997).

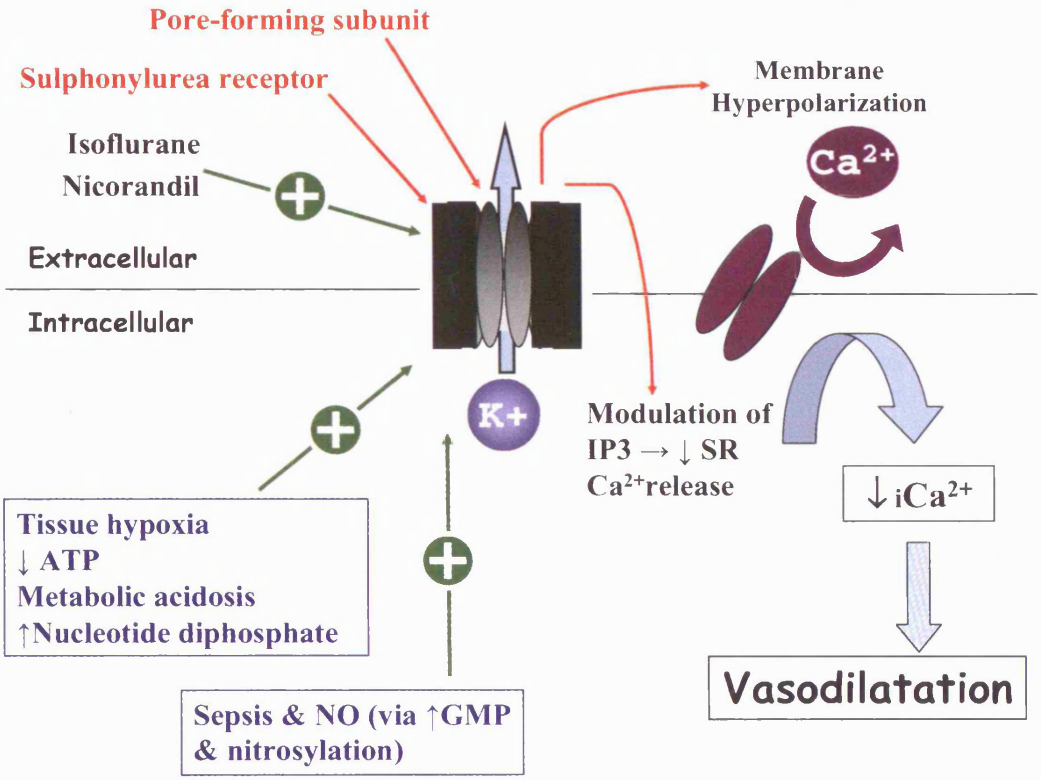


Figure 1.2 Mechanism of activation of the K_{ATP} channel within vascular smooth muscle cells leading to vasodilatation. The channel may be activated pharmacologically or by hypoxia, ↓ATP, lactic / metabolic acidosis & ↑NO – all of which may occur during sepsis.

1.7.2 Sepsis and K^+ channels

K^+ channels, in particular the K_{ATP} and BK_{Ca} channels, can be activated by a variety of endogenous vasodilators such as NO (Bolotina *et al.*, 1994), prostacyclin (PGI_2; Murphy & Brayden 1995 & Clapp *et al.*, 1998) and calcitonin gene-related peptide (CGRP) (Nelson & Quayle, 1995). Since plasma levels of these are all elevated in septic shock (Bernard *et al.*, 1997 and Gomez-Jimenez *et al.*, 1995) it seems highly likely that excessive activation of K^+ channels will occur.

Numerous *in vitro* and *in vivo* laboratory studies support an important role for the K_{ATP} channel in sepsis-induced vascular hyporeactivity and hypotension. In canine, porcine and rat *in vivo* models of LPS-induced shock, the K_{ATP} channel inhibitor glibenclamide restored blood pressure without having any effect in control animals (Landry & Oliver, 1992; Vanelli *et al.*, 1995; Wu *et al.*, 1995; Vanelli *et al.*, 1997; Gardiner *et al.*, 1999; Sorrentino *et al.*, 1999 and Preiser *et al.*, 2003). Glibenclamide also increased the magnitude of vasopressor responses to phenylephrine in LPS-treated rats (Sorrentino *et al.*, 1999) suggesting that K_{ATP} channels mediate both hypotension and vascular hyporeactivity *in vivo*.

Partial reversal of LPS-induced hyperpolarisation with glibenclamide was observed in rat aortic smooth muscle (Chen *et al.*, 2000) and in rat mesenteric resistance arteries (Wu *et al.*, 2004). Yet in all *ex-vivo* organ bath studies to date, glibenclamide had either no effect on vascular hyporeactivity (Wu *et al.*, 1995; Taguchi *et al.*, 1996 and Preiser *et al.*, 2003), or even further reduced contractions (Sorrentino *et al.*, 1999). This apparent paradox may relate to differences in the mechanisms of LPS-induced vascular dysfunction *in vivo* versus *in vitro*, or to the 10-100-fold higher concentrations of glibenclamide used in the *in vivo* studies (Wu *et al.*, 1995). An intriguing possibility to consider is that LPS alters the pharmacology of the K_{ATP} channel such that agents inhibiting the channel *via* the sulphonylurea receptor (SUR) become less effective (Wilson & Clapp, 2002). Metabolic stress in cardiac muscle has previously been reported to render SUR inhibitors ineffectual (Findlay, 1993). In this respect, it is worth noting that the pressor effect of glibenclamide *in vivo* is transient (Landry & Oliver,

1992; Vanelli *et al.*, 1995; Vanelli *et al.*, 1997; Gardiner *et al.*, 1999 and Sorrentino *et al.*, 1999) despite its long half-life in plasma (Wu *et al.*, 1995). Moreover, the use of glibenclamide as a tool with which to probe K_{ATP} channel function is complicated by its known inhibitory action on thromboxane receptors (Delaey & Van de Voorde, 1995). Such a mechanism would be predicted to increase hyporeactivity. Thus it is pertinent to investigate the mechanism of vascular hyporeactivity using more potent and selective K_{ATP} channel inhibitors, as well as agents with a different mode of action (Cui *et al.*, 2003).

Work from this laboratory has shown that hyporeactivity is fully reversed using the non-selective K^+ channel inhibitor, TEA^+ at 10 mM (Hall *et al.*, 1996) and there is evidence demonstrating involvement of BK_{Ca} channels, in addition to K_{ATP} channels. NO produced by vascular expression of iNOS activates BK_{Ca} channels; such a mechanism contributes to impaired vasoconstrictor responses during sepsis (Taguchi *et al.*, 1996). Similarly, Chen and co-workers, using charybdotoxin, concluded that that activation of BK_{Ca} channels and overproduction of NO in the vascular smooth muscle also contribute to vascular hyporeactivity to vasoconstrictor agents in endotoxaemia (Chen *et al.*, 1999). Furthermore, patch clamp studies in vascular smooth muscle cells have shown that LPS (10-100 $\mu g\ ml^{-1}$) can activate BK_{Ca} channels by a mechanism independent of iNOS (Yakubovich *et al.*, 2001).

1.8 Vasopressin (Arginine Vasopressin)

Vasopressin, also known as antidiuretic hormone, is an endogenous hormone produced in the hypothalamus and released from the posterior pituitary gland in response to increased plasma osmolality, hypovolaemia and hypotension. It is a nonapeptide with a disulfide bridge that differs from oxytocin by one amino acid. Despite this difference, oxytocin has clearly defined roles in partuition, lactation and sexual behaviour (reviewed in Holmes *et al.*, 2003A).

1.8.1 Vasopressin Receptors

The actions of vasopressin are mediated by stimulation of tissue-specific G-protein coupled receptors (GPCRs), which belong to a superfamily of receptors with seven transmembrane spanning domains. Vasopressin receptors are classified into V_1 (V_1R), V_2 (V_2R), V_3 (V_3R) and oxytocin (OTR) subtypes. Some actions of vasopressin may also be mediated via P_2 purinergic receptors (P_2R) (reviewed in Holmes *et al.*, 2003A).

V_1Rs are found in high density on vascular smooth muscle but are also located in the renal medulla, myometrium, bladder, adipocytes, hepatocytes, platelets, spleen, cardiac myocytes, brain, and testis (Philips *et al.*, 1990). The V_1R, once stimulated activates Gq/11 and the β isoform of phopholipase C to produce the intracellular messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers release of calcium from intracellular stores, thus initiating contraction of vascular smooth muscle, while a sustained rise in Ca^{2+} by influx of extracellular Ca^{2+} results from activation of both voltage-gated and receptor operated Ca^{2+} channels (Katori *et al.*, 2001). The mechanism

is likely to involve both DAG and also PKC, the latter being activated by DAG and recruited to the membrane (Neves *et al.*, 2002). There is considerable interspecies variation in the V_1R. For example, although rat and human vasopressin are identical, the human V_1R is only 80% homologous with the rat V_1R. This should be borne in mind when interpreting animal studies aimed at interpreting receptor subtypes based on the use of specific receptor inhibitors.

V_2Rs are distributed on the renal collecting tubules and endothelial cells and are coupled to the s subunit of the G protein complex (Barberis *et al.*, 1998). These are responsible for vasopressin's antidiuretic effect via activation of adenylyl cyclase and increased cAMP levels in the kidney. Vasopressin produces vasodilatation in some vascular beds with V_2Rs possibly involved in some instances. Infusion of a selective V_2 agonist in anephric dogs elevated cAMP and this was coincident with a fall in peripheral vascular tone (Liard *et al.*, 1992). Secondly, in healthy human volunteers, high doses of vasopressin will reduce forearm vascular resistance and this appears to be both V_2R and NO dependent (Tagawa *et al.*, 1993). The V_3R is a G-protein-coupled pituitary receptor that causes secretion of adrenocorticotrophic hormone (ACTH) and it is over-expressed in ACTH-secreting tumours. More than one G-protein appears to participate in signal transduction pathways linked to V_3Rs (Holmes *et al.*, 2003A).

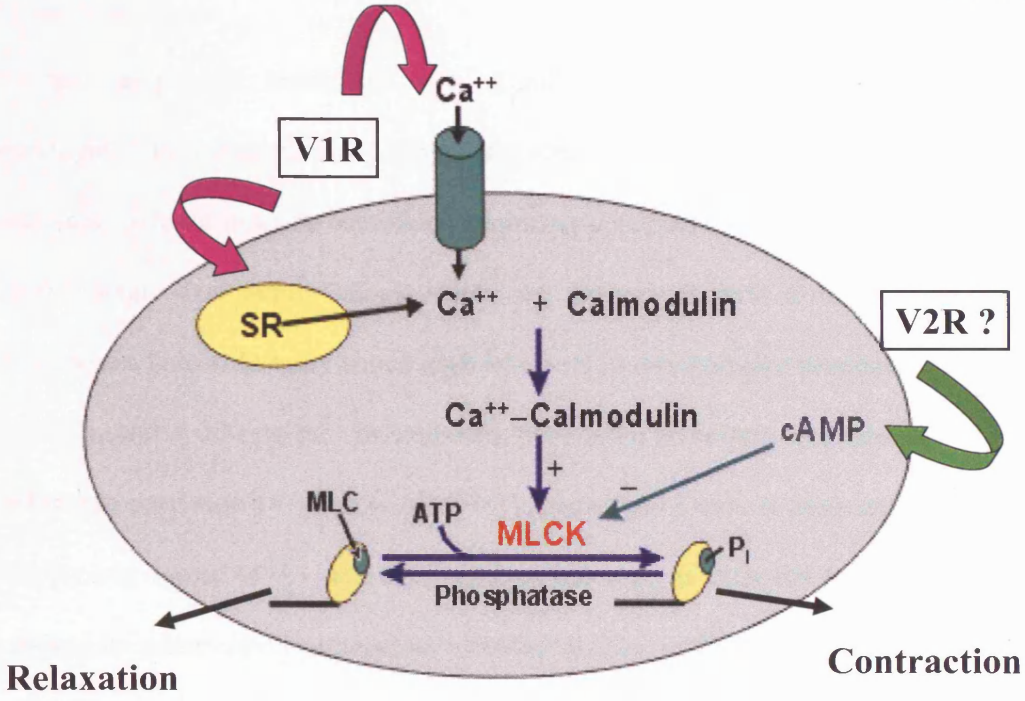


Figure 1.3 Vasopressin: mechanism of action. Activation of vasopressin 1 receptors (V1R) in vascular smooth muscle cells triggers and an increase in $Ca^{2+}{}_i$ from the endoplasmic reticulum and outside the cell causing contraction via activation of myosin light chain kinase (MLCK). Vasopressin 2 receptors (V2R) may cause vasodilatation via activation of cAMP

1.8.2 Endogenous vasopressin response in sepsis and other shock states

The vasopressor effect of pituitary extract was first observed in 1895 (Oliver & Schaefer). It was isolated and synthesised in the 1950s and shown to have antidiuretic and vasopressor effects (Turner *et al.*, 1951 and Du Vigneaud, 1954).

Plasma vasopressin levels are < 4 ρg ml^{-1} in hydrated humans who have fasted overnight. The osmoreceptor-vasopressin renal mechanism is highly sensitive, with a maximal increase in urine osmolality requiring vasopressin levels of ≥ 20 ρg ml^{-1}. Both haemorrhagic and septic shock states are associated with a biphasic response in vasopressin levels. In early shock high levels of vasopressin are produced (> 500 ρg ml^{-1} in dogs and > 300 pg ml^{-1} in baboons), producing profound vasoconstriction to assist end-organ perfusion (Wilson *et al.*, 1981). Prolonged shock is associated with a fall in vasopressin levels to 3.1 ± 1.0 pg ml^{-1} in humans, as opposed to cardiogenic shock patients in whom levels remained elevated at 22.7 ± 2.2 pg ml^{-1} (Landry & Oliver, 1997). Several mechanisms underlying this relative deficiency have been considered. These include depletion of neurohypophyseal stores of vasopressin (Morales *et al.*, 1999) due to excessive baroreceptor firing or autonomic insufficiency, with the lack of baroreceptor mediated bradycardia after vasopressin infusion considered as supporting evidence (Landry *et al.*, 1997). Also, low concentrations of norepinephrine excite central vasopressin neurones whereas elevated norepinephrine levels (that occurs in septic shock either endogenously or exogenously) have a central inhibitory effect upon the release of vasopressin (Day *et al.*, 1985). In addition, increased NO production by vascular endothelium in the posterior pituitary during sepsis may inhibit the production of vasopressin (reviewed in Holmes *et al.*, 2001).

1.8.3 Use of vasopressin in septic shock

Vasopressin has been used for over 50 years to treat diabetes insipidus, but only in recent years has it emerged as a potential treatment for catecholamine-resistant septic

shock (reviewed in Holmes *et al.*, 2001). Paradoxically, very small doses of vasopressin, using infusion rates of 0.01 to 0.04 IU min^{-1}, which have no pressor effect in normal subjects, induce an exaggerated response in septic patients, usually allowing considerable reduction (or even cessation) of the high-dose norepinephrine infusion (Landry *et al.*, 1997B). Cardiac output is generally maintained, pulmonary vascular resistance decreased, and urine output increased (reviewed in Holmes *et al.*, 2001 & 2003B and Landry *et al.*, 1997A & B). Subsequent studies have generally studied small patient numbers, ranging from 10-48 (Landry *et al.*, 1997A; Malay *et al.*, 1999; Holmes *et al.*, 2001; Tsuneyoshi *et al.*, 2001; Patel *et al.*, 2002 and Dunser *et al.*, 2003A). Adverse events are generally seen in those patients receiving higher doses of vasopressin (i.e. >0.04 units min^{-1}). These include arrhythmias, digital and mesenteric ischaemia. At levels as low as 10 pg ml^{-1} it may constrict the mesenteric circulation (Altura *et al.*, 1975). This is mediated by V_1Rs and has been demonstrated *in vitro* and *in vivo* in several animal models (Erwald *et al.*, 1976 and Schrauwen *et al.*, 1982). This effect has been used therapeutically in the treatment of variceal bleeding (Ohnisi *et al.*, 1987). It has also been demonstrated to cause platelet aggregation (Bichet *et al.*, 1988 and Haslam & Rosson, 1972). The V_2 receptor agonist, desmopressin, that causes release of factor VIIIc and von Willebrand factor, has been used extensively to treat bleeding due to dysfunctional platelets (Mannucci *et al.*, 1981). However, low doses of vasopressin are less likely to stimulate this effect. Furthermore, vasopressin infusion in septic patients has been associated with thrombocytopaenia (Dunser *et al.*, 2004A). At lower doses it does not appear to constrict the pulmonary circulation and its selective

constriction of renal efferent over afferent (via V_1R) could prevent renal dysfunction in sepsis.

As studies so far performed have been on small numbers of patients, it is uncertain whether vasopressin has a beneficial effect on mortality or organ failure in sepsis and thus its role in the management of septic shock remains undetermined. At present there is a large multicentre, randomised control study comparing vasopressin and norepinephrine in septic shock ongoing in Canada and Australia (VASST; Cooper *et al.*, 2003).

1.8.4 Mechanisms of action of vasopressin in sepsis

The mechanisms for the enhanced sensitivity to vasopressin in sepsis remain largely unknown. Autonomic insufficiency in septic shock unmasking vasopressin's pressor effect has been suggested. Pressor sensitivity to physiological concentrations of vasopressin was profoundly enhanced following baroreceptor denervation in dogs (Cowley *et al.*, 1974). Moreover, patients with orthostatic hypotension exhibit a 1000-fold sensitivity to physiological concentrations of vasopressin (Schwartz & Reid, 1983).

Vasopressin may restore vascular tone by at least four different mechanisms, namely: activation of V_1Rs; inhibition of K_{ATP} channels; modulation of nitric oxide; and potentiation of adrenergic and other vasoconstrictor agents. Activation of K_{ATP} channels may represent a critical mechanism in the hypotension and vasodilatation of septic shock. Vasopressin inhibits K_{ATP} channels and this may possibly account for its ability

to restore vascular tone in septic shock patients (Wakatsuki *et al.*, 1992). Vasopressin also modulates the actions of NO. It may restore vascular tone by blunting the increase in cGMP induced by NO, and by reducing the synthesis of iNOS stimulated by LPS (Umino *et al.*, 1999). This inhibition is likely to occur via the V_1R since administration of the V1 receptor antagonist but not the oxytocin receptor antagonist reversed these effects (Kusano *et al.*, 1997). However, a vasopressin infusion in septic patients had no effect upon serum concentrations of nitrite/nitrate (Dunser *et al.*, 2004B). Vasopressin also potentiates the vasoconstrictor effects of several agents including angiotensin II (Iverson & Arendshorst, 1998) and norepinephrine (Noguera *et al.*, 1997).

Interestingly, unlike catecholamines used to treat septic shock, vasopressin can dilate many vascular beds, for instance pulmonary (Walker *et al.*, 1989), renal (Rudichenko & Bejerwaltes, 1995 and Tamaki *et al.*, 1996), cerebral (Okamura *et al.*, 1997) and coronary (Okamura *et al.*, 1999). The mechanism of vasodilatation is uncertain. It is likely to be mediated by NO and the effect appears at low concentrations. The V_2R present on the vascular endothelium is likely to be involved. V_2 receptor agonists cause facial flushing in humans (Bichet *et al.*, 1988) and peripheral vasodilatation in dogs (Liard *et al.*, 1992). In addition, activation of endothelial oxytocin receptors (OTRs) which, in turn, trigger activation of endothelial isoforms of NOS, will also mediate vasopressin-induced dilatation (Thibonnier *et al.*, 1999). Whether vasopressin causes vasoconstriction or vasodilatation depends on the vascular bed studied (Garcia-Villalon *et al.*, 1996). This may depend upon the receptor density, the model studied (Holmes *et al.*, 2001), or the concentration of vasopressin used (Liard, 1987).

1.9 Role of the Cyclo-oxygenase (COX) pathway in sepsis

During sepsis vasoactive arachidonic acid metabolites of the cyclo-oxygenase (COX) pathway are released. These include prostacyclin, thromboxane A_2 (TXA_2) and prostaglandin E_2 (Cirino *et al.*, 1996 and Bishop-Bailey *et al.*, 1997). COX exists in two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed, whereas COX-2 is only expressed at low levels in resting cells. Marked upregulation of COX-2 occurs in macrophages and endothelial cells during sepsis. COX-2 expression is induced by LPS, a number of inflammatory mediators including the cytokines TNF and IL-1, mitogens and growth factors. In particular, thromboxane A_2 (TXA_2) and prostacyclin levels are raised in septic shock (Bernard *et al.*, 1991 and Holtzman, 1991). TXA_2 is a potent vasoconstrictor and a platelet stimulator (Hamberg *et al.*, 1975). TXA_2 downregulates iNOS expression in vascular smooth muscle cells stimulated with proinflammatory cytokines and it also inhibited NO production in an *in vivo* inflammatory model (Yamada *et al.*, 2003). This suggests that TXA_2 has a protective role against the development of vascular hyporesponsiveness via its inhibitory action on iNOS and the NO pathway in sepsis. Prostaglandin (PGE_2) is one of the most potent and inducible of the prostanoids produced during inflammation. It is a mediator of sepsis-induced immunosuppression, an inhibitor of proinflammatory cytokine expression from monocytes, and an inducer of IL-10 production (Reddy *et al.*, 2001; Strassmann *et al.*, 1994 and van der Pouw *et al.*, 1995). PGE_2 also mediates deleterious effects of sepsis such as vasodilatation and increased vascular permeability (Portanova *et al.*, 1996). Several studies have shown beneficial effects of non-selective COX inhibitors in endotoxin models (Beamer *et al.*, 1987; Wanecek *et al.*, 1997 McKenna, 1990; Gunnett

et al., 1998 and Strong *et al.*, 2000). However, Reddy and coworkers (2001) evaluated the effect of pre-treatment with NS-398, a highly selective COX-2 inhibitor on survival and inflammatory mediator production in two mouse models of sepsis and found only very modest effects on outcome. Patient studies using the non-selective inhibitors ibuprofen and lornoxicam have shown neither benefit nor any haemodynamic effects (Bernard *et al.*, 1997; Arons *et al.*, 1999 and Memis *et al.*, 2004).

1.9 Summary and Aims

Septic shock still carries a considerable mortality and morbidity. In recent years there has been progress in the management of this condition (reviewed by Hotchkiss and Karl, 2003); however numerous trials of agents that inhibit the inflammatory cascade have proved unsuccessful. The reasons for this are complex and include an over reliance upon animal models not representative of the clinical condition. The aim of this thesis was to investigate mechanisms underlying vascular hyporeactivity and hypotension in septic shock. I have chosen specifically to examine 3 key components – nitric oxide overproduction, activation of K^+ channels (especially K_{ATP} channels) and vasopressin. In order to accomplish this I first sought to develop reproducible *in vitro* and *in vivo* rat models of sepsis, with translational work to patients in the clinical setting.

Chapter 2

Characterisation of *in vitro* organ culture models of endotoxin-induced vascular hyporeactivity

2.1 Introduction

Sepsis is characterised by an exaggerated host systemic inflammatory response characteristically leading to a high cardiac output, low systemic vascular resistance circulation with a low blood pressure (Parillo, 1993). In septic patients, an α_1 adrenergic agonist (usually norepinephrine) is often required to reverse hypotension. However, the hypotension may be resistant to high doses of these vasopressor agents, a state known as *vascular hyporeactivity.* A crucial aim of my thesis was to establish a reproducible model of endotoxin-induced vascular hyporeactivity in rat blood vessels and to study the cellular mechanisms responsible for this.

Administration of endotoxin (lipopolysaccharide; LPS), a cell wall component ubiquitous to Gram-negative bacteria has been used extensively to mimic Gram-negative sepsis (see Thiemermann, 1997, Deitch, 1998 and Bateman *et al.*, 2003) in both animals and human volunteers. Substantial evidence from different species suggests that NO is the main mediator of vascular hyporeactivity in sepsis (reviewed in Vincent *et al.*, 2000; Landry and Oliver, 2001 and Hauser *et al.*, 2004,). Endotoxin is also known to induce the expression of other enzymes COX-2 (Bishop-Bailey *et al.*, 1997A), overproduction of arachidonic acid metabolites (reviewed in Mitchell *et al.*, 1995), and thromboxane A_2 (Yamada *et al.*, 2003) suggesting that additional mechanisms are likely to contribute to endotoxin-induced changes in vascular reactivity.

To date, the majority of *in vitro* investigations into the mechanisms underlying vascular hyporeactivity have used aortic tissues from rodents (Julou-Schaeffer *et al.,* 1990; Scott *et al.*, 1996 and Hall *et al.*, 1996). However, the aorta is a large conduit vessel that only makes a small contribution to systemic vascular resistance. In contrast, inducing hyporeactivity in smaller rodent blood vessels has proved very difficult. For example, hyporeactivity could be demonstrated in small mesenteric and femoral vessels *ex-vivo*, but only if the bathing medium contained L-arginine, the precursor for NO synthesis (Schneider *et al.*, 1992, Schneider *et al.*, 1994 and Mitolo-Chieppa *et al.*, 1996). In addition, the response to LPS has been shown to differ among vascular beds with hypocontractility to methoxamine (an α_1 adrenergic agonist) seen in aorta and mesentery but not renal beds (Farmer *et al.*, 2003) and LPS-dependent vasodilatation seen more in coronary and renal arteries than mesenteric and hepatic (Piepot *et al.*, 2002).

I sought to develop a reproducible *in vitro* model of hyporeactivity in a smaller artery than aorta. I chose to study rat mesenteric artery as the mesenteric circulation is an important contributor to vascular tone, receiving 30-40 % of the total cardiac output. I sought to develop an organ culture method that I could use to investigate the effect of LPS on vascular reactivity to the α_1 agonist, phenylephrine (PE) and to assess the role of the NO pathway in vascular hyporeactivity. I used a variety of methods, including pharmacological inhibitors of iNOS, nitrite assay to assess directly the production of NO, as well as immunohistochemistry to look at protein expression of iNOS. I also

assessed the contribution of inducible COX-2 to vascular hyporeactivity using the relatively selective inhibitor, NS-398.

In order to investigate human vessels *in vitro*, I chose the marginal artery. Only a handful of studies have been able to demonstrate vascular hyporeactivity or induction of nitric oxide synthase in human vessels. These have either used a combination of pro-inflammatory cytokines and LPS (Throin-Trescacses *et al.*, 1995 and Chester *et al.*, 1998) or a continuous infusion of lipoteichoic acid derived from staphylococcus aureus (Tsuneyoshi *et al.*, 1996) to mimic Gram-positive infection. The marginal artery is a third order branch of the superior colonic artery, which is also part of the mesenteric circulation and thus compliments my rat *in vitro* model. I applied the same organ culture model principles as with the rat model but added the pro-inflammatory cytokine IL-1β in combination with LPS in an attempt to produce vascular hyporeactivity. When IL-1β is infused into human cancer patients it has been shown to cause hypotension (Dinarello, 1996). As with the rat model, my aim was to characterise the responses of the vessel to LPS and IL-1β and investigate the contribution of the NO pathway.

2.2 Methods

2.2.1 *In vitro* model of LPS-induced vascular hyporeactivity to phenylephrine

2.2.2 Dissection of rat mesenteric artery

Male Sprague-Dawley rats (250-300 g body weight) were killed *via* cervical dislocation in accordance with schedule 1 of the Animals (Scientific Procedures) Act 1986. This procedure conforms to the *Guide for the Care and Use of Laboratory Animals*, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The rats were bred in house at University College London. The mesenteric artery was dissected out and placed in sterile Hanks Balanced Salts Solution (HBSS). The artery was cleaned of connective tissue and cut into four rings. Rings from 2 animals were used per experiment, i.e. 8 in total.

2.2.3 Incubation with LPS

Rings were incubated in sterile Dulbecco's Modified Eagle's Medium (DMEM) for 6, 20 or 46 h in an atmosphere of 95% air / 5% CO_2 in a sterile 12-well dish. In these experiments (except where stated) the culture medium was supplemented with fetal bovine serum (FBS, 10% v/v) and with penicillin-streptomycin solution (100 units ml^{-1} and 100 $\mu g\ ml^{-1}$, respectively). LPS (*Salmonella typhosa*) was added to the appropriate segments at various doses (10 pg ml^{-1} – 100 $\mu g\ ml^{-1}$). In a few experiments, vessels were incubated within the organ bath with or without 1 $\mu g\ ml^{-1}$ LPS for 6 h in physiological saline solution (PSS) with the following composition (in mM): 112 NaCl,

5 KCl, 25 $NaHCO_3$, 1 $MgCl_2$, 0.5 KH_2PO_4, 0.5 NaH_2PO_4, 10 glucose, 1.8 $CaCl_2$ and 0.03 phenol red) gassed with 95% O_2/ 5% CO_2 at 37°C.

2.2.4 Tension experiments

Following incubation in culture medium, tissues were transferred to 25 ml organ bath chambers containing PSS, except for experiments using fresh controls, in which rings were used immediately after dissection. The rings were suspended between two tungsten wires in a jacketed organ bath (Fig. 2.1). One of the wires was fixed to an external platform; the other was connected to an isometric force displacement transducer (FT-03, Grass Instruments, USA) for measurement of tension. The transducers were coupled to a chart recorder. In all, 8 parallel organ baths were used in the tension experiments. Following mounting, the rings allowed to equilibrate at a tension of 1.25 g for 1 h, during which time tissues were washed at 15 min intervals. The rings were permitted to relax to a resting tension of 0.8 – 1 g. Where appropriate, LPS was added to the organ baths for the duration of the experiment. To assess contractility, rings were twice contracted with the α_1 adrenoreceptor agonist phenylephrine (PE, 1 μM), separated by a 20 min period during which time tissues were washed several times in order to restore tension back to 1 g.

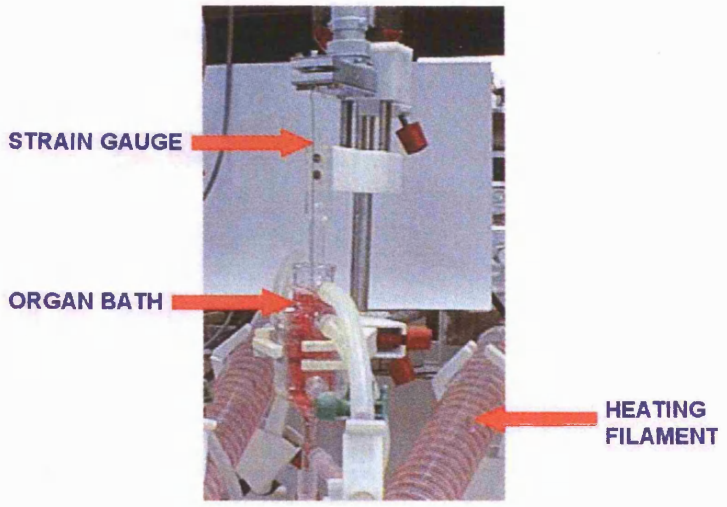


Figure 2.1 The organ bath apparatus.

Endothelial function was assessed by monitoring relaxation to the endothelium-dependent agonist, acetylcholine (Ach, 5 μM) in rings precontracted with 1 μM PE. Endothelium was considered viable if there was greater than 50% relaxation to Ach (Fig. 2.2). Such responses were maintained even after 46 h incubation in culture medium. The magnitude of the relaxation induced by acetylcholine in control tissues did not alter with time in culture medium, being (80.6 ± 0.2%, n=13 at 6 h and 79.0 ± 9.7, n=8, at 20 h). In certain experiments the endothelium was removed by gently passing a small tungsten wire through the lumen of the ring or by rubbing the lumen with some filter paper soaked in PSS, prior to mounting it in the organ bath. The successful removal of the endothelium was confirmed by the lack of response to the ACh.

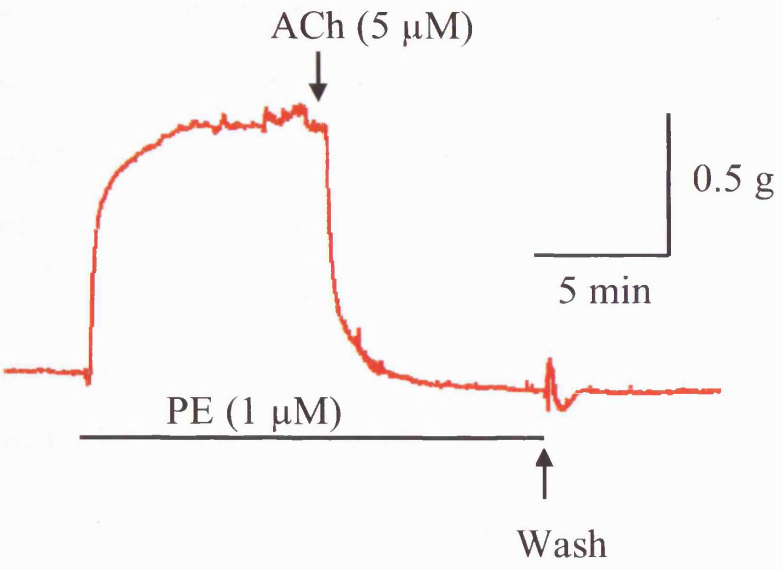


Figure 2.2 Effect of acetylcholine (Ach; 5 μM) on an endothelium-intact rat mesenteric artery ring incubated for 20 h in culture medium. The tissue was pre-contracted with phenylephrine (PE; 1 μM).

2.2.5 Experimental protocols

At least 5 mesenteric rings, taken from a minimum of three animals, were used for each experimental group. Cumulative concentration-response curves were constructed to PE (10^{-9} to 10^{-5} M) with the agonist added at 5 min intervals. The contractile response of the rings to the agonist reached a plateau within 5 min. Except where indicated, all concentration-response curves were constructed using endothelium-intact rings.

I wished to develop a reproducible model of LPS-induced vascular hyporeactivity in rat mesenteric artery. No prior studies had been carried out on this vessel. I initially added LPS to PSS within the organ bath for a 6 h period of incubation, but was unable to

demonstrate hyporeactivity using this method. Thus I developed an organ culture model using culture medium containing fetal bovine serum at 3 different incubation periods – 6 h, 20 h and 46 h. I wished to characterise the vascular response to LPS in rat mesenteric artery and specifically examine the induction of nitric oxide in this response. I thus performed the following experiments.

(i) Concentration-response to LPS

I was interested to see whether pathophysiologically relevant concentrations of LPS measured in humans were sufficient to produce vascular hyporeactivity. I thus used concentrations of LPS ranging from 10 pg ml^{-1} to 100 μg ml^{-1}. Individual concentrations were added to the culture medium and the tissues incubated overnight for 20 h.

(ii) Varying duration of LPS incubation

Tissues were incubated in the presence or absence of LPS (1 μg ml^{-1}) for 6, 20 and 46 h prior to mounting in the organ bath.

(iii) Effect of fetal bovine serum (FBS)

Rings were incubated with and without LPS (1 μg ml^{-1}) for 6, 20 and 46 h in the presence or absence of 10% FBS in the culture medium.

(iv) Role of endothelium

Following incubation with and without LPS (1 μg ml^{-1}) for 20 h some rings were denuded of endothelium. However, for all subsequent experiments I used endothelial-intact rings so as to study the effects of LPS upon the whole blood vessel.

(v) Role of the NO / cGMP pathway in mediating vascular hyporeactivity

(a) Inhibition of nitric oxide synthase (NOS)

Rings were incubated with and without LPS (1 μg ml^{-1}) for 6, 20 and 46 h. The following NOS inhibitors were added to the organ bath 25 min prior to construction of a concentration-response curve to PE: $^{\omega}$-nitro-L-arginine methyl ester (L-NAME; 300 μM), aminoguanidine (400 μM), 1400W (10 μM) and GW273629 (10 μM). The NOS inhibitors were added to LPS treated and non-LPS treated control tissues that had been incubated in culture medium. In a separate series of experiments, aminoguanidine (400 μM) was added to fresh control tissues immediately following harvest from the rat, prior to constructing a concentration-response curve to PE. In a further series of experiments, 1400W (10 μM) was added to the culture medium at the same time as the LPS for incubation periods of 20 and 46 h prior to a concentration-response curve to PE.

(b) Inhibition of soluble guanylyl cyclase

As above, rings were incubated with and without LPS (1 μg ml^{-1}) for 20 and 46 h and the selective soluble guanylyl cyclase inhibitor, 1H-(1,2,4)oxadiazole(4,3-a)quinoxalin-

1-one ODQ (3 μM) was added to the organ bath 25 min prior to the addition of cumulative doses of PE.

(vi) Effect of LPS on responses to the thromboxane A_2 mimetic U46619

I compared the vasoconstrictive properties of the thromboxane A_2 mimetic (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$), U46619 to PE in this model. Rings, either endothelium-denuded or intact, were incubated for 20 h with and without LPS (1 μg ml^{-1}) prior to constructing concentration-response curves to U46619 (10^{-9} to 10^{-6} M).

(vii) Concentration-response curves to the NO donor S–nitroso–N–acetyl–D, L-penicillamine (SNAP)

Concentration-response curves to SNAP (10^{-10}-10^{-5}M) were constructed following precontraction with either 1 μM PE or 0.1 μM U46619 in fresh endothelium-intact tissues. The iNOS inhibitor 1400W (10 μM), and ODQ (1 μM) - were added 25 min prior to the addition of SNAP. Concentrations of SNAP were added in cumulative fashion every 5 min once contraction to PE had plateaued. The tissues had always maximally relaxed to the concentration of SNAP added within 5 min.

(viii) Inhibition of cyclo-oxygenase-2 (COX-2) activity

I investigated the effect of the relatively selective cyclo-oxygenase-2 inhibitor, NS-398 [N-(2-(Cyclohexyloxy)-4-nitrophenyl)- methanesulfonamide] (10 μM) in the *in vitro* model. Rings were incubated with and without LPS (1 μg ml^{-1}) for 20 h and NS-398 was added to the organ bath 25 min prior to addition of PE.

2.2.6 Nitrite determination

Due to the rapid metabolism and breakdown of NO, it is difficult to measure its production directly. However, a stable end product of NO metabolism, nitrite (NO_2^-), is often used as a marker of NO production (Thiemermann, 1997) and it is traditionally measured using the Griess reaction. The Griess reaction is based upon a NO_2^-–dependent diazotisation reaction, which produces a coloured azo compound that can be measured colorimetrically. This reaction can be used to determine NO_2^- levels in cell culture supernatants. NO_2^- reacts with acidified sulphanilamide and *N*-1-napthylenediamine dihydrochloride to produce a coloured azo compound in a concentration-dependent manner. NO_2^- formation can be thus used as a surrogate marker of NO production (Schmidt and Kelm, 1996).

Mesenteric rings were incubated for 20 h in culture medium containing serum, in the absence or presence of LPS 1 μg ml^{-1} ± 1400W (10 μM) or ODQ (3 μM). Following this, 150 μl of the culture medium was pipetted into a 96-well microtitre plate. An equal volume of Griess reagent (1% (w/v) sulphanilamide, 0.01% (w/v) N-1-napthylethylenediamine dihydrochloride in 5% (v/v) phosphoric acid) was added to each well, mixed and left to stand for 15 min. A standard curve (Fig. 2.3) was prepared each time the assay was performed by serially diluting known concentrations of sodium nitrite (0.1 – 30 μM) and mixing with equal volumes of Griess reagent. NO_2^- concentration was determined calorimetrically by subtracting the absorbance at 540 nm from that obtained at 620 nm (620 nm was a reference wavelength only).

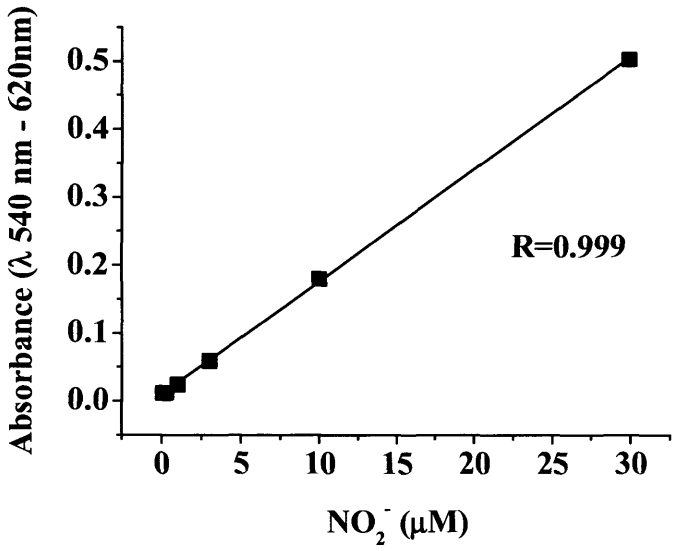


Figure 2.3 Nitrite standard curve were prepared by serially diluting sodium nitrite solution (Sigma, UK) to the desired concentration (n=2).

(i) Protein determination using the Lowry method

To normalise NO_2^- concentration for tissue mass, the protein content of samples was measured following measurements of NO_2^-. Each mesenteric arterial ring was dissolved overnight in 100 μl of 2.5 M NaOH in a sonnicator. The following day samples were diluted in 400 μl H_2O. This was because 0.5 M NaOH was the maximum concentration of alkali acceptable to use in the Biorad DC protein assay (Biorad, U.S.A.), a standard assay based on the Lowry method (Lowry *et al.*, 1951). Protein standards were prepared from bovine serum albumin, which was dissolved in 0.5M NaOH and diluted at a variety of concentrations between 0.2 and 1.4 mg ml^{-1} (Fig. 2.4). 5 μl of standards and samples, 25 μl of an alkaline copper tartrate solution and 200 μl of a dilute Folin reagent were added to each well of a 96 well plate. The solution was mixed for 5 seconds. After 15 min absorbance was read at 750 nm.

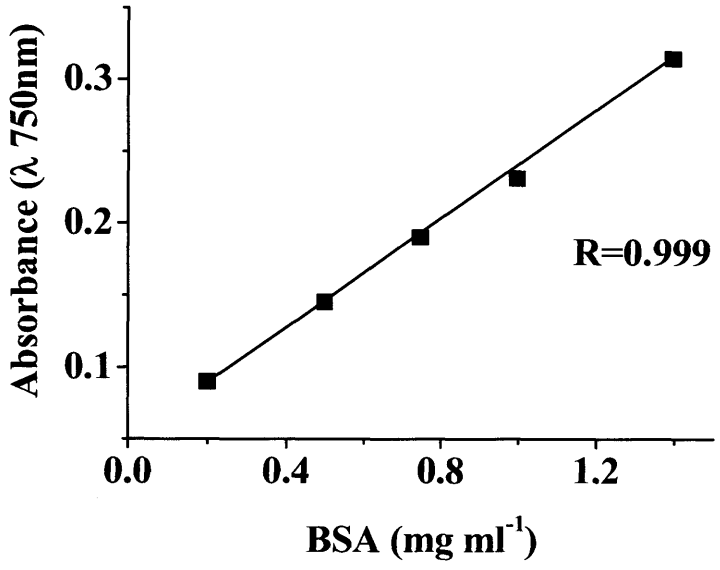


Figure 2.4 An Example of a standard curve using the Biorad DC protein assay.

2.2.7 Immunohistochemical staining of iNOS

Rings of LPS-treated mesenteric arteries and their appropriate controls taken from both *in vitro* (LPS, *Salmonella Typhosa* 1 μg ml^{-1}) or *in vivo* (LPS, 40 mg kg^{-1} *Klebsiella Pneumoniae*; see chapter 3) models of vascular hyporeactivity were rinsed in phosphate buffered saline (PBS), immersed in OCT fixative (Histologicals Ltd, UK) and left on dry ice until frozen. The OCT block was then placed on a cryostat (-20°C) to cut sections. Cryostat sections (7 μM) were brought to room temperature and left to dry for 30 min. The sections were then fixed in acetone (-20°C) for 20 min, allowed to dry and rehydrated in running tap water. Endogenous peroxidase was inhibited with 0.3% H_2O_2 in methanol for 30 min followed by 3 washes in PBS. Non-specific binding of the secondary antibody was blocked by incubating the sections in 3% normal goat serum in PBS. Sections were drained and incubated overnight at 4 °C with the primary antibody -

monoclonal rabbit anti-mouse iNOS (Upstate Biotechnology NY, U.S.A.) at 1:200 dilution. Negative controls were sections incubated overnight without the primary antibody. Sections were washed in PBS three times (5 min) and incubated for 45 min with the biotin conjugated anti-rabbit antibody (1:1000 dilution; Sigma, U.K.) and washed again in PBS 3 times (5 min). Sections were then incubated using the avidin – biotin system for 45 min and washed a further 3 times in PBS (5 min). The antigen was visualised by immersion of the sections in a diaminobenzidine solution. Sections were lightly counterstained with Mayer's haematoxylin and dehydrated through a series of alcohols, cleaned in xylene and permanently mounted in DPX (Merck, U.K.).

2.2.8 *In vitro* model of vascular hyporeactivity in human marginal artery

2.2.9 Dissection of human marginal artery

This artery is formed by the anastomosis of the distal portions of the superior and inferior mesenteric arteries and runs in the wall of the colon. Human colonic marginal artery was collected from patients undergoing large bowel resection for a neoplasm or inflammatory bowel disease. The tissues were provided, with the patients' consent, by the colorectal surgeon Professor Boulos at University College London Hospitals. The artery was dissected clear of the resected bowel, placed in HBSS and cleaned of connective tissue. This procedure took at least one hour as there was a substantial amount of fatty tissue surrounding the vessels. There were often delays in being notified that the operation was complete and thus the specimen may have been left in a bowl for

over 1 hour prior to collection. Once cleaned, the artery was cut into rings, up to 6 depending on the length of tissue obtained.

2.2.10 Incubation with LPS ± interleukin-1 beta (IL-1β)

Tissues were then incubated with LPS (*Salmonella typhosa*) ± IL-1β to induce vascular hyporeactivity. Previous studies have used 100 units ml^{-1} alone to induce hyporeactivity (Thorin Trescases *et al.*, 1995) or COX-2 (Bishop-Bailey *et al.*, 1997 b). A combination of 5 units ml^{-1} of IL-1β with LPS 10 μg ml^{-1} had been used to induce iNOS in human tissue (Chester *et al.*, 1998) and, as cytokines are expensive, I used this concentration together with LPS up to 100 μg ml^{-1}. Rings were incubated in sterile DMEM for 20 or 46 h at 37 °C in an atmosphere of 95% air / 5% CO_2. In all experiments the culture medium was supplemented with 10% FBS.

2.2.11 Tension experiments

Following incubation in culture medium, rings of marginal artery were that had been incubated in culture medium either alone (control), or with IL-1β (10 units ml^{-1}) and / or LPS (10 μg ml^{-1}) were transferred to the organ bath chamber as described previously for the rat mesenteric artery. The tissues were subjected to a tension of 2 g and permitted to relax to a resting tension of 1.5 g and concentration-response curves were constructed to PE (10^{-9} to 10^{-4} M). No previous studies using this vessel had been performed to suggest a resting tension. The human vessels have a much tougher layer of adventitia and I found that whatever tension I used they would relax to 1-1.5 g and thus I used 1.5 g.

2.2.12 Experimental protocols

(i) Dose and duration of incubation of LPS and IL-1β

LPS, at doses of 1, 10 or 100 μg ml^{-1}, IL-1β (10 units ml^{-1}) or a combination (10 μg ml^{-1} LPS + 10 units ml^{-1} IL-1β) were added to the culture medium and tissues incubated for a total of 20 h (3 patients) or 46 h (6 patients). LPS was not added to the organ bath during the experiments.

(ii) Nitric oxide synthase inhibition

As with the rat model, I wished to characterise the vascular responses within this vessel. Since the non-selective NOS inhibitor, L-NMMA reversed hypotension in septic patients (Grover *et al.,* 1999), I investigated the effect of iNOS inhibition in this model. The non-specific NOS inhibitor, L-NAME (300 μM) or the highly specific iNOS inhibitor, 1400W (10 μM) was added to the organ bath of tissues treated with LPS + IL-1β 25 min prior to a cumulative concentration-response curves to PE.

2.3 Statistical analysis

All data are represented as mean ± standard error of the mean (s.e.m.) of *n* observations. Statistical analysis was performed using SigmaStat (Jandel Corporation, Chicago, U.S.A.). To compare differences between two groups, a paired or unpaired Student's t-test was used. For multiple groups where several measurements were made in the same tissue / whole animal over time, where appropriate, two way ANOVA with repeated measures was used and, corrected for multiple comparisons against the control group (Bonferroni) or all groups (Student-Newman Keuls). The concentration of agonist

causing a 50% contraction or relaxation of the maximal response is expressed as the pEC_{50} value and was calculated using the sigmoidal curve fitting routine in Origin 6.0 software (Microcal, Northampton, MA, U.S.A.). Individual pEC_{50} values were obtained from each experiment and the mean values between experimental groups compared using one way ANOVA (with Bonferroni or Student-Newman Keuls correction as appropriate). In all cases, a P value < 0.05 was considered statistically significant.

2.4 Reagents and solutions

Hanks Balanced Salt Solution containing calcium and magnesium (Invitrogen Ltd, Paisley, U.K.) was supplemented with 5 mM HEPES, 2 mM $NaHCO_3$, 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Dulbecco's Modified Eagle's Medium was supplemented with penicillin-streptomycin solution (100 units ml^{-1} and 100 μg ml^{-1}, respectively) and 2 mM L-glutamine (Invitrogen Ltd) and where appropriate fetal bovine serum. L-NAME, aminoguanidine, LPS (*Salmonella typhosa*), acetylcholine, sodium nitrite and phenylephrine were all obtained from Sigma Chemical Company (Poole, Dorset, U.K.). Fetal bovine serum was obtained from Invitrogen Ltd, U46619 from Affiniti-Research, (Exeter, U.K.) and 1400W, ODQ, NS-398 and SNAP from Alexis Corporation (Nottingham, U.K.). GW 273629 was kindly donated by GlaxoSmithKline (Stevenage, U.K.).

2.5 Results

2.5.1 Characterisation of an *in vitro* rat model of sepsis

2.5.1.1 Effect of LPS on contractile responses to PE in mesenteric artery

A typical cumulative concentration-response curve to phenylephrine (PE) in an endothelium-intact rat mesenteric artery (RMA) incubated in culture medium supplemented with 10% fetal bovine serum in the absence (control) and presence of 1 μg ml^{-1} LPS for 6 h and 20 h is shown in Figure 2.5. Unless otherwise stated all results refer to endothelial intact RMA. LPS induced marked hyporeactivity to PE, causing a substantial depression in the maximal response and a shift to the right of the concentration-response curve compared to control tissues. In contrast, no hyporeactivity to PE was observed if tissues were incubated with 1 μg ml^{-1} LPS in PSS for 6 h in the organ bath chamber (data not shown).

Reported levels for biologically active endotoxin in sepsis vary widely, reflecting differences in LPS standards, assays, diseases and models utilized. In humans, maximal reported concentrations of LPS range from as low as 0.05 – 4 ng ml^{-1} (van Deventer *et al.*, 1988 and Parillo *et al.*, 1991) to as high as 10 ng ml^{-1} of lipopolysaccharides in systemic meningococcal disease (Brandtzaeg *et al.*, 1989). It has been shown that concentrations as low as 1 ng ml^{-1} of LPS suppressed contractions to norepinephrine in rat aorta when incubated for 16 h (Mckenna, 1990). Thus I examined the effect of varying the LPS concentration in RMA incubated for 20 h in culture medium

supplemented with serum. At all concentrations LPS (1-100 μg ml^{-1}) induced significant hyporeactivity to PE ($P < 0.001$, two-way ANOVA; Fig. 2.6A) with the maximal response (E_{Max}) in the presence of 1 μg ml^{-1} LPS being suppressed from 1.66 ± 0.27 g to 0.7 ± 0.14 g (n=5-6). In addition, LPS caused a rightward shift of the pEC_{50} compared to controls (n=5-6; $P < 0.001$, one-way ANOVA, Table 2.1A). The degree of hyporeactivity induced by LPS was similar over this concentration range. A further set of experiments using the same organ culture model investigated what threshold concentration of LPS was required to induce significant vascular hyporeactivity. LPS induced significant hyporeactivity at concentrations as low as 0.001 μg ml^{-1} ($P < 0.001$, two-way ANOVA; Fig. 2.6B). In addition, there was a rightward shift of the pEC_{50} from control to LPS 0.01 ng ml^{-1} ($P < 0.05$ one-way ANOVA; Table 2.1B).

Table 2.1 Effect of LPS upon contractile responses to phenylephrine

A. Comparison of pEC_{50} and E_{Max} values for control and LPS treated tissues

Data are expressed as mean ± s.e.m. of > 5 observations

Rat mesenteric artery	**pEC_{50}**	**E_{Max} (g)**	***n***
Control	6.54 ± 0.19	1.55 ± 0.19	5
LPS 1 μg ml^{-1}	5.81 ± 0.05*	0.69 ± 0.11	6
LPS 10 μg ml^{-1}	5.64 ± 0.07*	0.60 ± 0.12	6
LPS 100 μg ml^{-1}	5.61 ± 0.05*	0.79 ± 0.14	5

* P<0.001 compared to control

B. Comparison of pEC_{50} and E_{Max} values for control and LPS treated tissues

Rat mesenteric artery	**pEC_{50}**	**E_{Max} (g)**	***n***
Control	7.17± 0.18	0.86 ± 0.08	6
LPS 0.1 ng ml^{-1}	6.79 ±0.11	0.15 ± 0.06	7
LPS 0.01 ng ml^{-1}	6.33 ± 0.13*	0.21 ± 0.08	6
LPS 10ρg ml^{-1}	7.07 ± 0.29	0.85 ± 0.2	6

*$P<0.05$ compared to control

It was not possible to calculate accurate pEC_{50}s for LPS 1μg ml^{-1} and 1 ng ml^{-1} as the concentration response curves were flat.

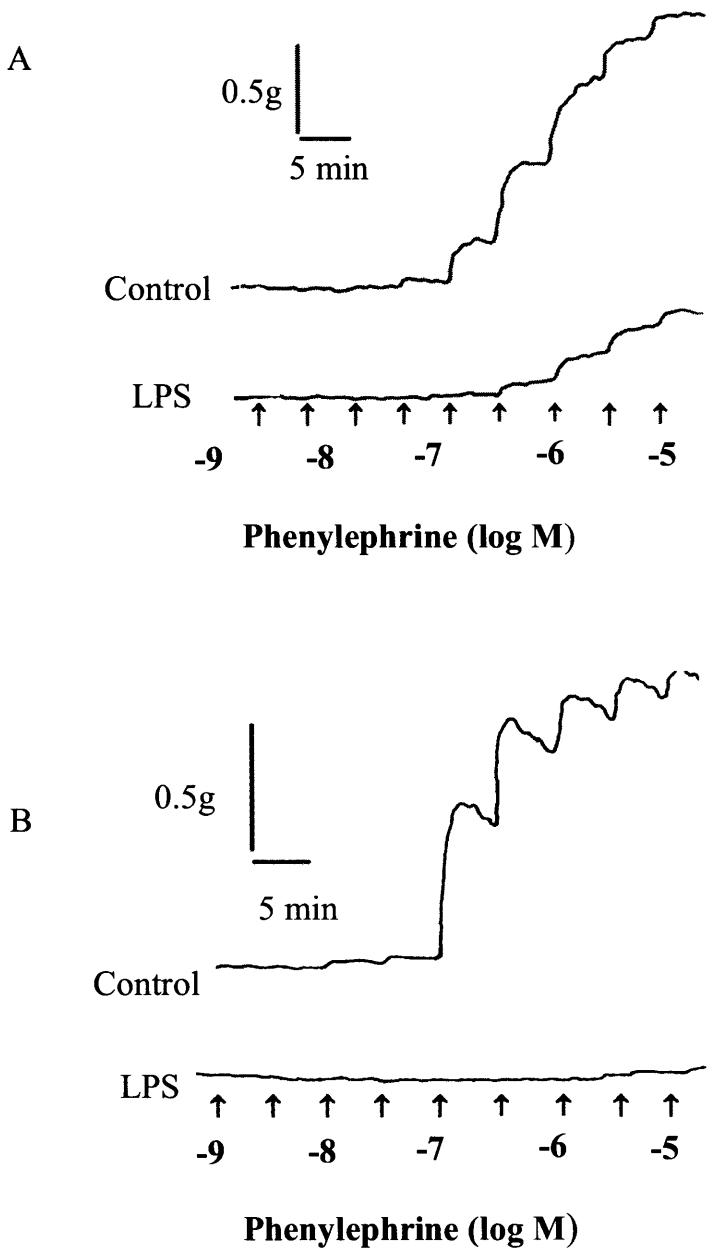


Figure 2.5 Representative traces of contractile responses to phenylephrine for control and LPS (1 μg ml^{-1}) treated rat mesenteric arteries for either A) 6 h or B) 20 h. All tissues were endothelium intact and incubated in culture medium containing serum. Phenylephrine was added at half log units at 5 min intervals as shown by the arrows.

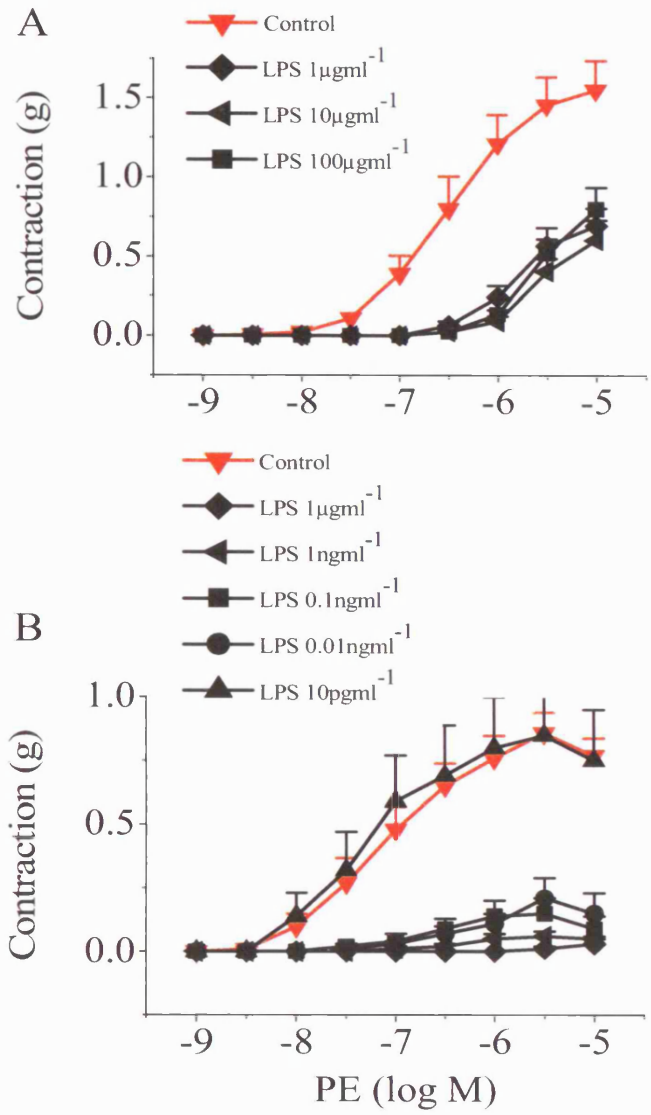


Figure 2.6 Effect of varying concentrations of LPS on contractile responses to PE. Tissues were incubated (with serum) for 20 h with A) LPS 1-100 μg ml^{-1} and B) LPS 10 pg ml^{-1} to 1μg ml^{-1}. LPS induces hyporeactivity at all concentrations aside from 10 pg ml^{-1} (P<0.001, ANOVA). Data are expressed as mean ± s.e.m. of 5-6 observations from 8 animals.

2.5.1.2 Effect of duration of incubation and presence of serum upon response to LPS

In vivo models have shown a difference in the amount of NO generated and haemodynamic response over time in animals given LPS (Lui *et al.*, 1997 and Rees *et al.*, 1998). In addition, LPS-induced expression of mRNA for iNOS has been shown to vary in rat aorta *in vitro* (Mitchell *et al.*, 1997). There had been no such work on rat mesenteric artery and thus I investigated 3 different durations of incubation. In the development of the *in vitro* model, I wished to examine the effect of adding serum to the culture medium. Serum contains LPS binding protein and soluble CD14 which have key roles in recognition of LPS via toll receptors (Knapp *et al.*, 2005). I thus wished to investigate whether culture medium with added serum produced more profound vascular hyporeactivity in this model.

Control tissues were incubated in the presence and absence of fetal bovine serum (FBS; 10% v/v) for 6, 20 or 46 h. Over this time course, peak responses to PE were comparable over the whole concentration range to those observed in fresh controls ($P > 0.05$, two-way ANOVA; Fig. 2.7A and C). However, contractions elicited from tissues incubated for 46 h in culture medium with or without serum were much less sustained than those observed at either 6 or 20 h. For example, at 1 μM PE, peak contractions over a 5 min period declined by 2% at 6 h, 9% at 20 h and 42 % at 46 h. Moreover, when tissues were incubated in the presence of LPS, there appeared to be a differential effect on the contractile responses to PE. In serum-free conditions, substantial hyporeactivity to LPS (1 μg ml^{-1}) was evident at 6 and 20 h time points (E_{Max} 0.47 ±

0.13 g and 0.75 ± 0.09 g, respectively compared to 1.56 ± 0.16 g in controls; n=5-6, $P <$ 0.001, two-way ANOVA), but not at 46 h (E_{Max} 1.55 ± 0.17 g) (Fig. 2.7 D). However, in tissues incubated in serum, LPS induced significant hyporeactivity at all time points including 46 h (maximal response 0.21 ± 0.05 g compared to 1.56 ± 0.14 g in controls; n=7, $P < 0.001$, two-way ANOVA) (Fig. 2.7 B).

When comparing the effect of serum on pEC_{50} values, there was only a difference between the control tissues at 20 h incubation with the concentration-response curve shifting to the right when the tissue was incubated with serum (7.44 ± 0.09 to 6.75 ± 0.11, $P = 0.008$, n=10, ANOVA; Table 2.2).

Table 2.2 Effect of serum on contractile responses in rat mesenteric artery to PE in control or LPS-treated tissues

Comparison of pEC_{50} and E_{Max} values for endothelium-intact LPS-treated and control tissues incubated in culture medium ± serum for 6 – 46 h. Data are expressed as mean ± s.e.m. of > 5-10 observations

Incubation time	**Control pEC_{50}**	**E_{Max} (g)**	**LPS pEC_{50}**	**E_{Max} (g)**
		-Serum		
0 h	6.87±0.09 (*n*=6)	1.54±0.15		
6 h	6.45±0.23 (*n*=8)	1.29±0.06	5.78±0.10 (*n*=5)	0.75±0.09
20 h	7.44±0.09* (*n*=10)	1.31±0.13	6.10±0.16 (*n*=6)	0.4±0.13
46 h	7.11±0.14 (*n*=6)	1.7±0.15	6.78±0.21 (*n*=6)	1.55±0.17
		+ Serum		
6 h	6.48±0.09 (*n*=6)	1.63±0.07	5.79±0.07 (*n*=8)	0.31±0.04
20 h	6.75±0.11* (*n*=10)	1.71±0.1	5.78±0.19 (*n*=8)	0.19±0.04
46 h	6.93±0.13 (*n*=9)	1.57±0.06	5.99±0.57 (*n*=7)	0.23±0.07

* P=0.008 when directly compared

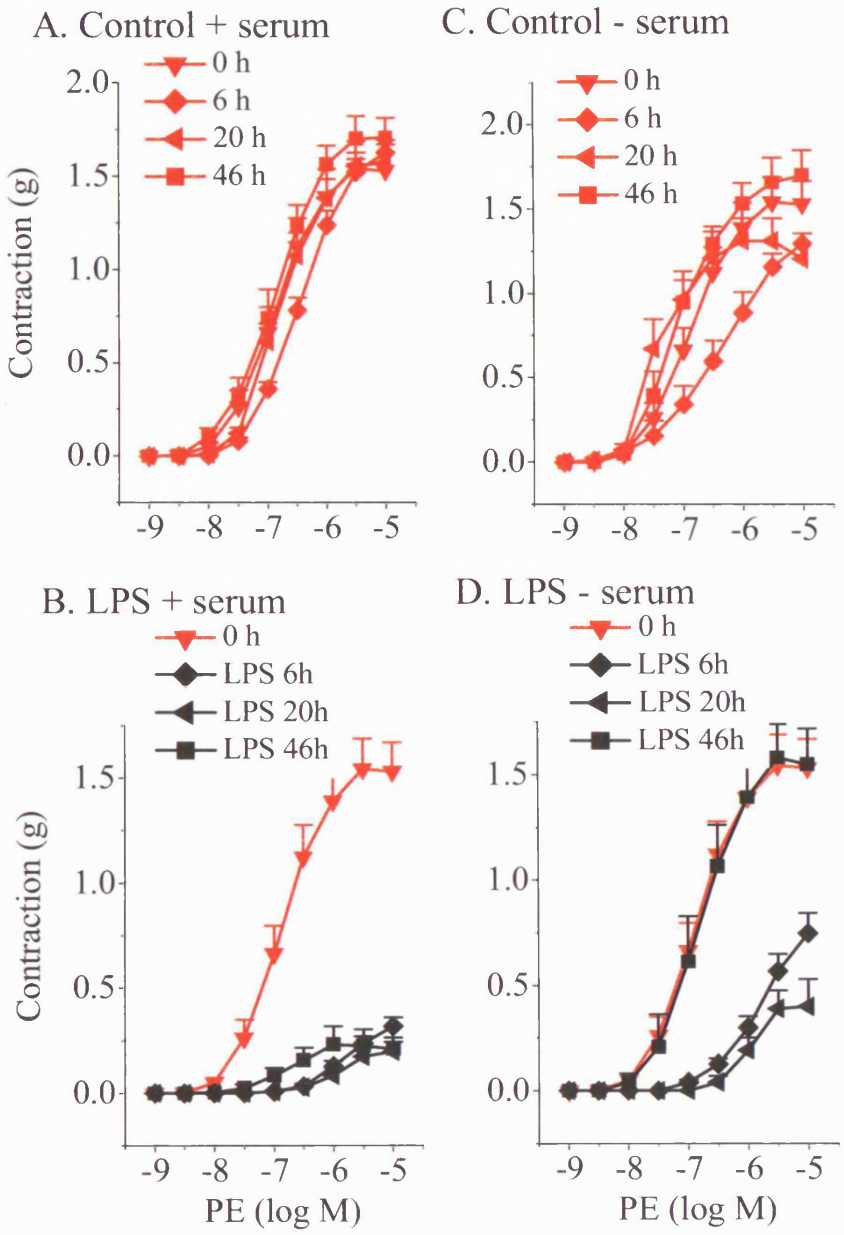


Figure 2.7 Effect of incubation time and serum on LPS-induced hyporeactivity in RMA. Endothelium intact tissues were incubated in the absence (controls) or presence of LPS for 0-46h with (A, B) or without (C, D) serum. LPS induces hyporeactivity at all times ($P<0.001$) except at 46 h in the absence of serum. Data are expressed as mean of 6-10 observations from 8-12 rats.

2.5.1.3 Effect of endothelium on contractile responses to phenylephrine

iNOS can be expressed in all 3 arterial cell layers (the endothelium, smooth muscle and adventitia). The major site of production is however, controversial with earlier studies suggesting the smooth muscle (Knowles *et al.*, 1990) but more recent evidence points towards the adventitia as the primary location of iNOS (Zhang *et al.*, 1999 and Muller *et al.*, 2000). It has been shown that the presence of endothelium appears to enhance the sensitivity to LPS (Baydoun *et al.*, 1993), but also that the magnitude of the hyporeactivity seen is unaffected by endothelial removal (Rees *et al.*, 1990). Thus I investigated the effect of LPS on hyporeactivity in the presence and absence of endothelium.

In the absence of LPS, the presence of endothelium, in either fresh control tissues or tissues incubated in culture medium containing serum for 20 h, produced a rightward shift in the concentration-response to PE, although the maximum contraction observed was similar in both cases (Fig. 2.8A and B). In fresh tissue the pEC_{50} was shifted from 6.87 ± 0.09 in endothelium-intact rings to 8.19 ± 0.27 in endothelium-denuded rings ($P < 0.001$, ANOVA; $n=6$). In tissues incubated at 20 h, the pEC_{50} was shifted from 6.35 ± 0.29 in endothelium-intact rings to 7.73 ± 0.27 in endothelium-denuded rings ($n=6$; $P < 0.01$, one-way ANOVA). However, following a 20 h incubation in LPS (1 μg ml^{-1}), the same level of hyporeactivity was observed regardless of whether the endothelium was present or not (Fig. 2.8B) and there was no difference in the pEC_{50} values (Table 2.3).

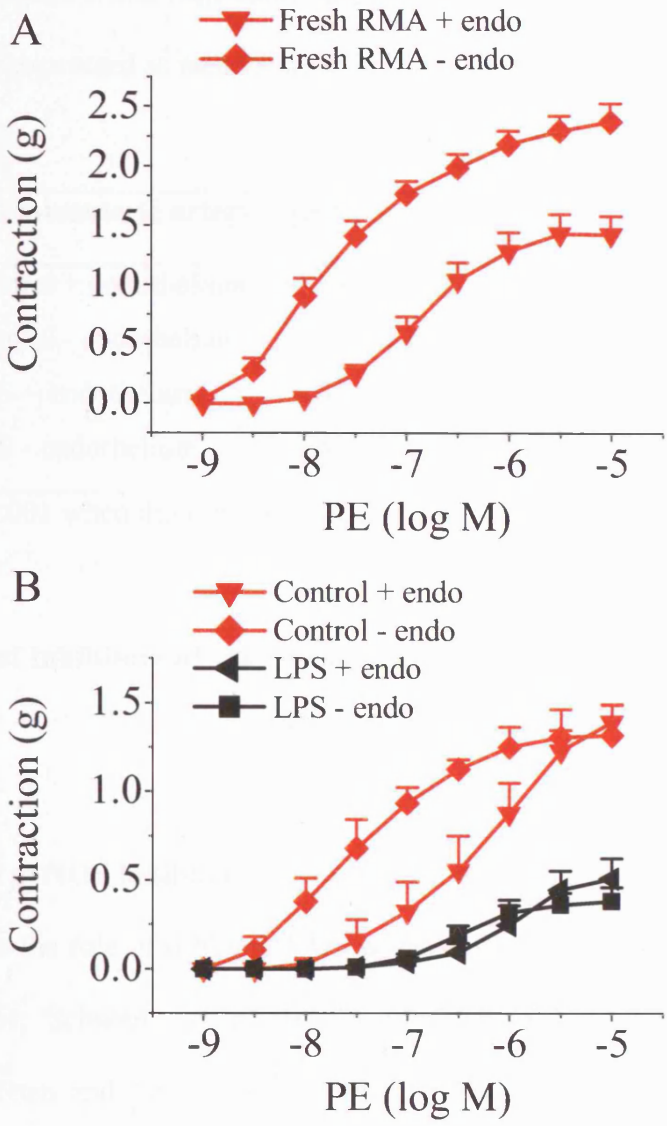


Figure 2.8 Effect of endothelium (endo) on contractile responses to phenylephrine. Tissues were either immediately transferred to the organ bath (A) or incubated with serum in the absence (controls) or presence of 1 μg ml^{-1} LPS for 20 h (B). Removing endo after incubation increased contractile responses in fresh RMA (P<0.001, n=6-7) and controls (P<0.05, n=6); responses in LPS treated RMA were unaffected. Data are expressed as mean of 6-8 observations from 8-10 animals.

Table 2.3 Effect of the endothelium on contractile responses to PE

Comparison of pEC_{50} and E_{Max} values for LPS-treated and control tissues incubated for 20 h. Data are expressed as mean ± s.e.m. of 6-9 observations.

Rat mesenteric artery	pEC_{50}	E_{Max} (g)	*n*
Control + endothelium	6.35 ± 0.30*	1.36 ± 0.09	6
Control - endothelium	7.74 ± 0.27*	1.31 ± 0.17	6
LPS + endothelium	6.05 ± 0.22	0.51 ± 0.11	6
LPS - endothelium	6.41 ± 0.14	0.38 ± 0.8	6

* $P<0.001$ when directly compared

2.5.2 Effect of inhibitors of the NO pathway on LPS responses in rat mesenteric artery

2.5.2.1 Effect of NOS inhibition on contractile responses to PE

To investigate the role of different NOS isoforms enzymes, L-NAME (a non-selective NOS inhibitor; Schwartz *et al.*, 1997), aminoguanidine (a more selective iNOS inhibitor; Ruetten and Thiemermann, 1996), and the highly selective iNOS inhibitor 1400W (Garvey *et al.*, 1997) were used. At 6 h after incubation, the addition of either aminoguanidine (400 μM) or L-NAME (300 μM) to the organ bath fully reversed LPS-induced hyporeactivity (n=7-9) (Fig. 2.9 A). There was no difference in pEC_{50} values between controls and both these iNOS inhibitors (Table 2.4 A).

Surprisingly, at 20 h no reversal was seen with L-NAME (Fig. 2.9 B and Table 2.4 B). However, partial reversal was achieved with either aminoguanidine or 1400W, the latter being significantly more effective above 0.3 μM PE ($P<0.01$, two-way ANOVA) (Fig. 2.9 B). At 46 h partial reversal of hyporeactivity was seen with 1400W, whereas aminoguanidine and L-NAME had no effect on PE contractions (Fig. 2.9 C). There was little difference between the pEC_{50} values for PE using the 3 inhibitors (Table 2.4 C). The differential and time-dependent effects of these NOS inhibitors prompted further investigations using GW273629, another specific iNOS inhibitor (Young *et al.*, 2000; Alderton *et al.*, 2005). Similar effects to 1400W were seen at 20 h and 46 h with 10 μM of this agent (Fig. 2.10 A & B and Table 2.4 B & C). In addition, when tissues were preincubated with both LPS and 1400W i.e. the inhibitor was added to the culture medium at the same time as LPS, hyporeactivity was fully reversed at 20 h and 46 h. Thus, the effect was superior to that seen when the inhibitor was added to the organ bath 25 min prior to contraction with PE (Fig. 2.11 A & B and Table 2.4 B & C).

In control tissues, small but significant ($P < 0.05$, two-way ANOVA; n=6-10) increases in the contractile responses to PE were seen in the presence of 1400W and aminoguanidine at all time points (Fig. 2.12 B). The maximal response was increased by 14 %, 26 % and 19 % to 1.75 ± 0.16 g, 1.75 ± 0.22 g and 2.06 g ± 0.12 g at 6, 20 and 46 h, respectively; although there was no significant effect on pEC_{50} values (Table 2.4 D). However, since aminoguanidine did not potentiate PE contractions in fresh control tissues (Fig. 2.12 A) this suggests some induction of iNOS when tissues were incubated in culture medium containing serum.

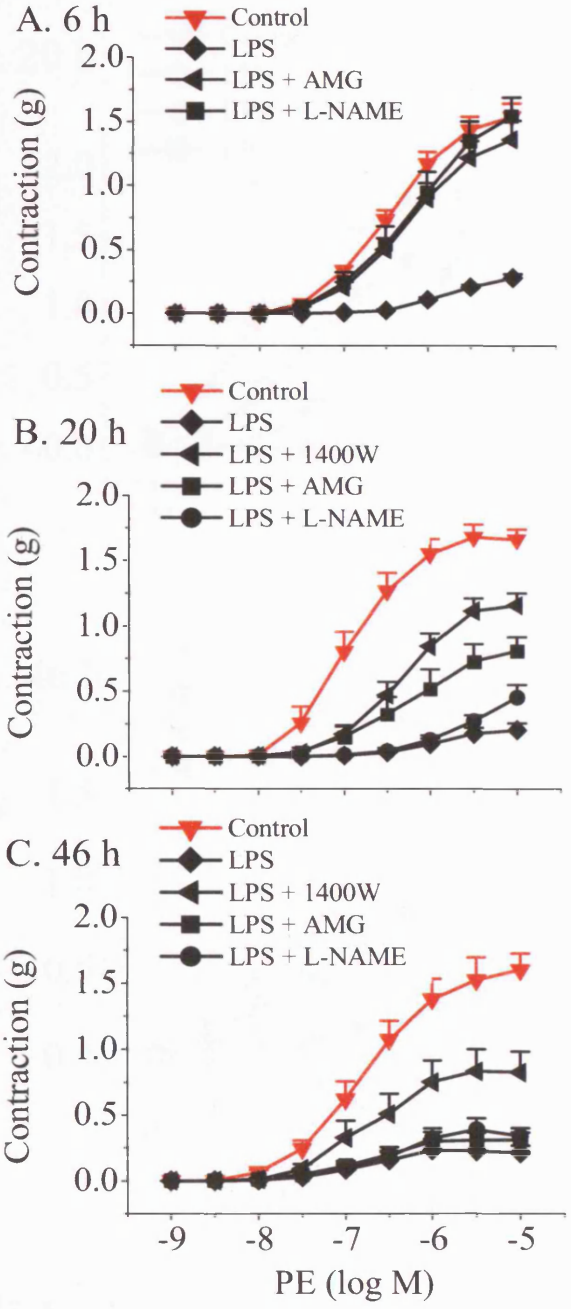


Figure 2.9 Effect of NOS inhibitors, aminoguanidine (300μM), L-NAME (300μM) & 1400W (10μM) on LPS-induced hyporeactivity. RMA was incubated ± LPS for (A) 6, (B) 20 & (C) 46 h. All completely reversed hyporeactivity at 6h; AMG & 1400W caused partial reversal at 20h (P<0.05 c.f. LPS) whereas 1400W alone caused partial reversal at 46h (P<0.05 c.f. LPS). Data is expressed as mean ± s.e.m. of 7-11 observations from 12-14 rats.

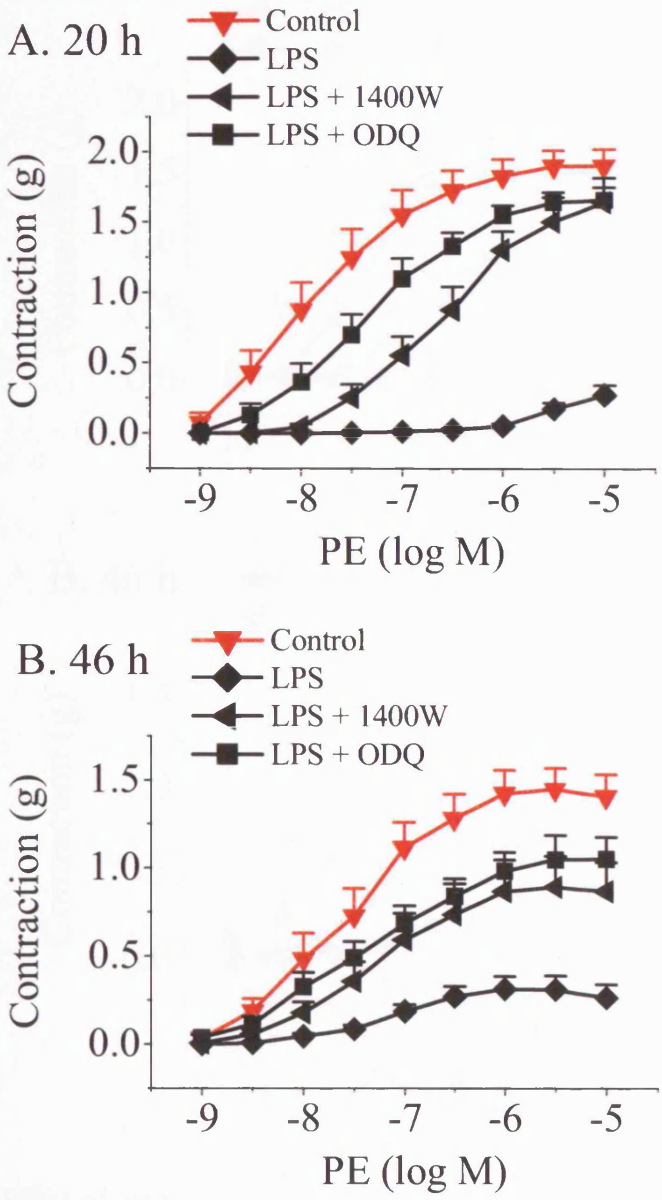


Figure 2.10 A comparison of the specific iNOS inhibitors, 1400W (10μM) and GW273629 (10μM). Endothelium-intact rings were incubated for (A) 20 h and (B) 46 h in the absence or presence of 1 μg ml^{-1} LPS ± iNOS inhibitor. Both inhibitors had a similar effect and partially reversed LPS-induced hyporeactivity at 20 & 46 h (P<0.001 c.f. LPS). Data are expressed as mean ± s.e.m. of 7-11 observations from 14-16 animals.

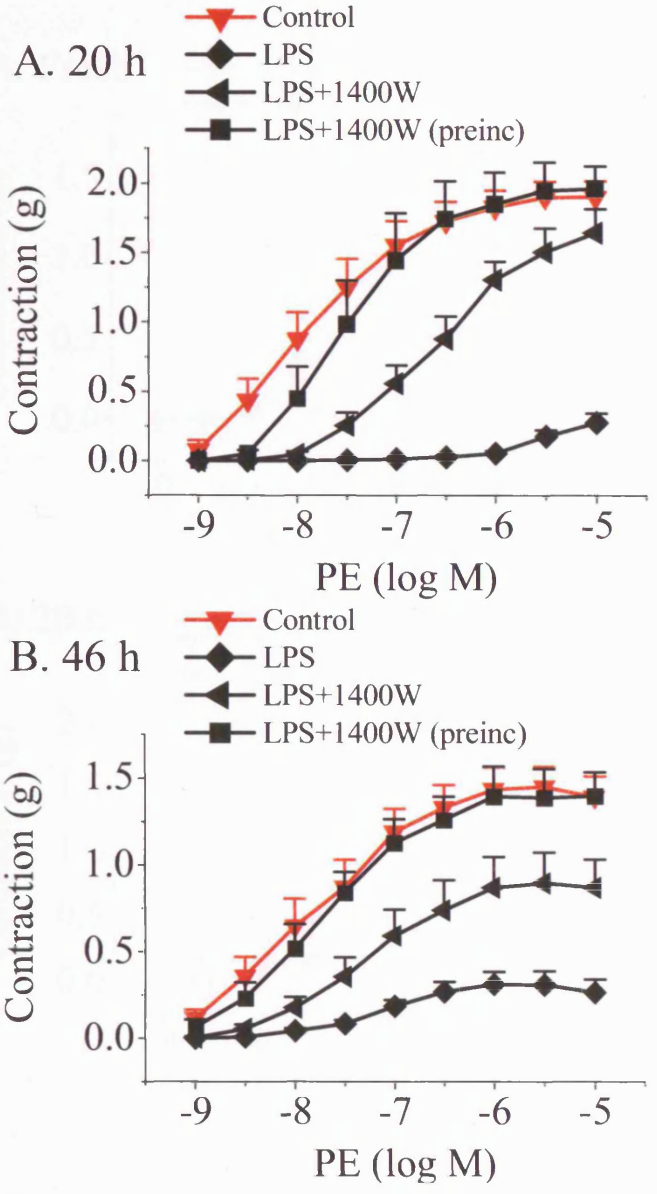


Figure 2.11 Effect of preincubation (adding to culture medium) with 1400W (10 μM) for (A) 20 or (B) 46 h compared to adding 1400W to the organ bath 25 min prior to concentration-response curve. Preincubation with 1400W fully reversed LPS-induced hyporeactivity at 20 & 46 h (P<0.001 compared to adding 1400W to the organ bath). Data are expressed as mean ± s.e.m. of 7-11 observations from 10-12 animals.

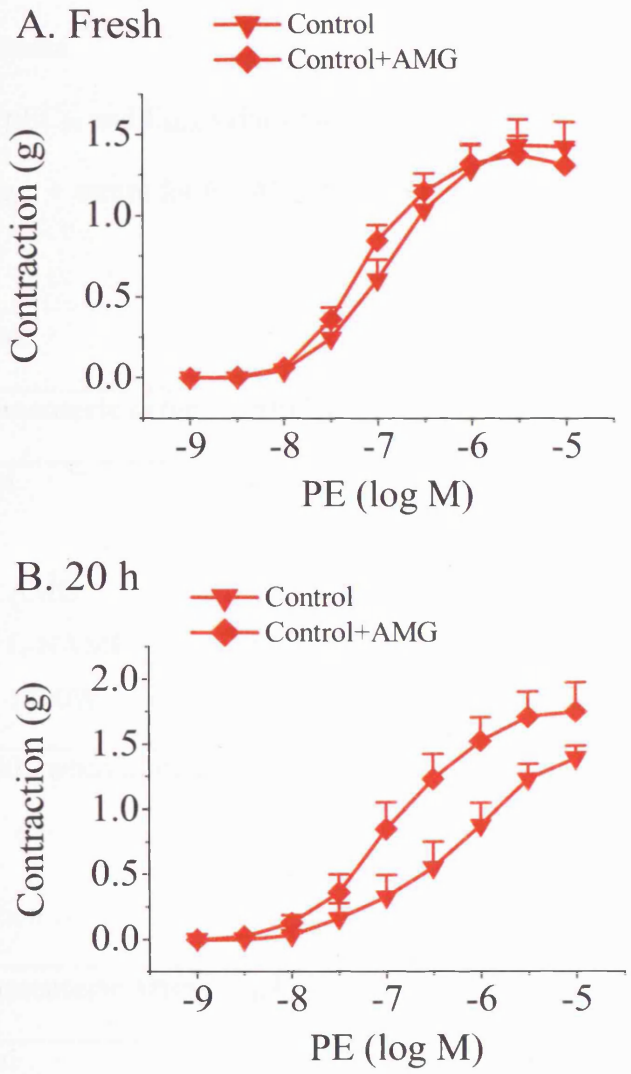


Figure 2.12 Effect of AMG (300μM) on endothelium intact fresh control tissues compared with rings incubated for 20 h. AMG increased contractile responses in RMA incubated for 20 h compared to fresh tissue ($P<0.05$). Data are expressed as mean ± s.e.m of 6 observations from 6 animals.

Table 2.4 Effect of NOS inhibition on contractile responses to PE in control and LPS-treated tissues

Comparison of pEC_{50} and E_{Max} values for LPS-treated and control tissues incubated in culture medium + serum for 6 – 46 h. Data are expressed as mean ± s.e.m. of > 8 observations.

A. 6 h incubation

Rat mesenteric artery	**pEC_{50}**	**E_{Max} (g)**	***n***
Control	6.44 ± 0.07 *	1.54 ± 0.1	7
LPS	5.77 ± 0.08 *	0.28 ± 0.03	7
LPS + AMG	6.27 ± 0.10	1.36 ± 0.15	9
LPS + L-NAME	6.15 ± 0.11	1.54 ± 0.15	8
LPS + 1400W	6.75 ± 0.14	1.69 ± 0.09	7

* $P<0.001$ when directly compared

B. 20 h incubation

Rat mesenteric artery	**pEC_{50}**	**E_{Max} (g)**	***n***
Control	7.97 ± 0.27	1. 9 ± 0.11	10
LPS	5.78 ± 0.22*	0.28 ± 0.07	7
LPS + AMG	6.20 ± 0.20*	0.81 ± 0.11	8
LPS + L-NAME	5.55 ± 0.24*	0.45 ± 0.11	8
LPS + 1400W	6.35 ± 0.09*	1.16 ± 0.1	10
LPS + GW273629	6.84 ± 0.29#	1.44 ± 0.13	7
LPS + ODQ	7.86 ± 0.53	1.66 ± 0.09	8

*$P<0.001$ and $P<0.05$ compared to control

C. 46 h incubation

Rat mesenteric artery	**pEC_{50}**	**E_{Max} (g)**	***n***
Control	7.06 ± 0.16*	1.45 ± 0.11	10
LPS	5.99 ± 0.57*	0.31 ± 0.08	7
LPS + AMG	6.51 ± 0.14	0.31 ± 0.06	10
LPS + L-NAME	6.45 ± 0.15	0.34 ± 0.06	10
LPS + 1400W	6.40 ± 0.33	0.89 ± 0.18	7
LPS + GW273629	7.11 ± 0.19	0.8 ± 0.09	11
LPS + ODQ	7.55 ± 0.20	1.05 ± 0.12	11

*$P<0.05$ when directly compared

D. Effect upon control tissues

Rat mesenteric artery	**pEC_{50}**	**E_{Max} (g)**	***n***
Fresh	6.87 ± 0.09	1.44 ± 0.16	6
Fresh + AMG	7.18 ± 0.06	1.38 ± 0.12	6
Control (6 h)	6.77 ± 0.14	1.32 ± 0.09	8
Control (6 h) + 1400W	7.22 ± 0.21	1.7 ± 0.07	6
Control (20 h)	6.91 ± 0.10	1.67 ± 0.08	10
Control (20 h) + 1400W	7.13 ± 0.09	1.85 ± 0.1	7

2.5.2.2 Effect of guanylyl cyclase inhibition on vascular hyporeactivity

Once formed, NO diffuses to adjacent target cells where it activates soluble guanylyl cyclase by binding to the iron located in the active site of this enzyme. The resulting formation of cGMP, in turn, is considered to mediate many, though not all of the biological effects of NO (reviewed by Carvajal *et al.*, 2000). In view of the substantial inhibition of vascular hyporeactivity by NOS inhibitors, I wished to test whether cGMP

was also responsible for the relaxant effects of LPS. I used the highly selective soluble guanylyl cyclase inhibitor, 1H-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one, ODQ (Schmidt *et al.*, 2003). ODQ (3μM) produced similar reversibility of LPS-induced hyporeactivity to 1400W. In tissues incubated with LPS for 20 h and 46 h, ODQ, like 1400W, only produced partial recovery of PE contractions when added to the organ bath (Fig. 2.13 A & B and Table 2.4 B & C).

2.5.3 Comparison of the contractile responses to U46619 and phenylephrine

Thromboxane A_2 is a potent vasoconstrictor of many vascular beds (reviewed by Samuelsson *et al.*, 1975). It has been suggested that its production may act as a compensatory mechanism to LPS induced hypocontractility (Cirino *et al.*, 1996). Interestingly, there was no significant hyporeactivity to U46619 following 20 h incubation with LPS in endothelium-intact tissue despite LPS inducing marked hyporeactivity to PE in tissues taken from the same animal. In endothelium-denuded tissue however, some hyporeactivity was observed (Fig. 2.14 B). When compared to PE, the maximum response obtained to U46619 in LPS-treated tissues was significantly greater in either the presence (1.31 ± 0.13 g *vs* 0.51 ± 0.11 g; Fig. 2.14 A) or absence (1.01 ± 0.07 g *vs* 0.38 ± 0.08 g; Fig. 2.14 B) of endothelium (n=6 - 10; $P < 0.001$, one-way ANOVA). In addition the concentration-response curves to U-46619 were shifted to the left compared to PE (Table 2.5).

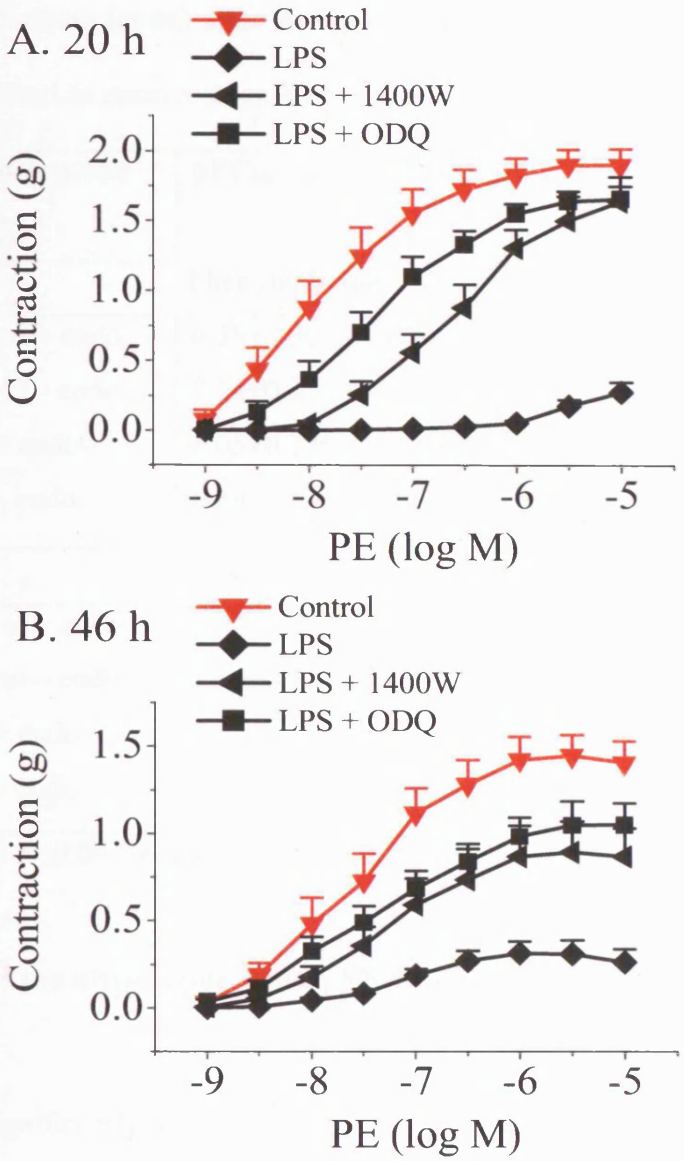


Figure 2.13 Effect of the soluble guanylyl cyclase inhibitor, ODQ (3 μM), on LPS-induced hyporeactivity; comparison with 1400W (10 μM). Rings were incubated for 20 h (A) or 46 h (B) in the absence or presence of 1 mg ml^{-1} LPS. There was no difference between the 2 agents – both partially reversing hyporeactivity (P<0.001 c.f. LPS). Data are expressed as mean ± s.e.m. of 8-10 observations from 8 animals.

Table 2.5 Comparison of U-46619 vs phenylephrine in control or LPS-treated tissue ± endothelium (endo) after 20 h incubation with serum.

Data are expressed as mean ± s.e.m. of ≥ 5 observations

Rat mesenteric artery	pEC_{50}	E_{Max} (g)	*n*
Phenylephrine			
Control + endo	6.35±0.30	1.4±0.1	6
Control – endo	7.74±0.27	1.32±0.18	6
LPS + endo	6.05±0.22*	0.51±0.11	6
LPS – endo	6.39±0.13#	0.38±0.78	9
U-46619			
Control + endo	6.65±0.07	1.88±0.26	5
Control – endo	7.52±0.13	1.46±0.12	10
LPS + endo	7.46±0.18*	1.3±0.13	7
LPS – endo	7.04±0.11#	1.01±0.17	8

* and # $P \leq 0.002$ when directly compared to each other

2.5.4 Effect of the nitric oxide donor, SNAP upon contractile responses to PE and U-46619

LPS caused significantly less hyporeactivity to U46619 than PE. In order to determine whether the differential effect of LPS on responses to PE and U46619 relate to differences in the ability of NO to inhibit contraction to these agonists, I examined the effect of the NO donor, SNAP. SNAP is considered to cause vasorelaxation by releasing NO from within the smooth muscle cell (Lopez-Lopez *et al.*, 2001). Endothelium-intact tissues were precontracted with either 1 μM PE (mean contraction 1.09 ± 0.05 g; n=6) or 0.1 μM U46619 (mean contraction 1.33 ± 0.08 g; n=8). Figure

2.15A shows that SNAP relaxed PE and U46619 induced contractions with a similar potency, the log pEC_{50} values being 6.41 ± 0.13 and 6.32 ± 0.19 for U46619 and PE, respectively (n=6). The concentration-relaxation curves to SNAP following precontraction with 1 μM PE were significantly inhibited by the guanylyl cyclase inhibitor, ODQ ($P < 0.001$). The highly specific iNOS inhibitor 1400W had no effect (Fig. 2.15 B).

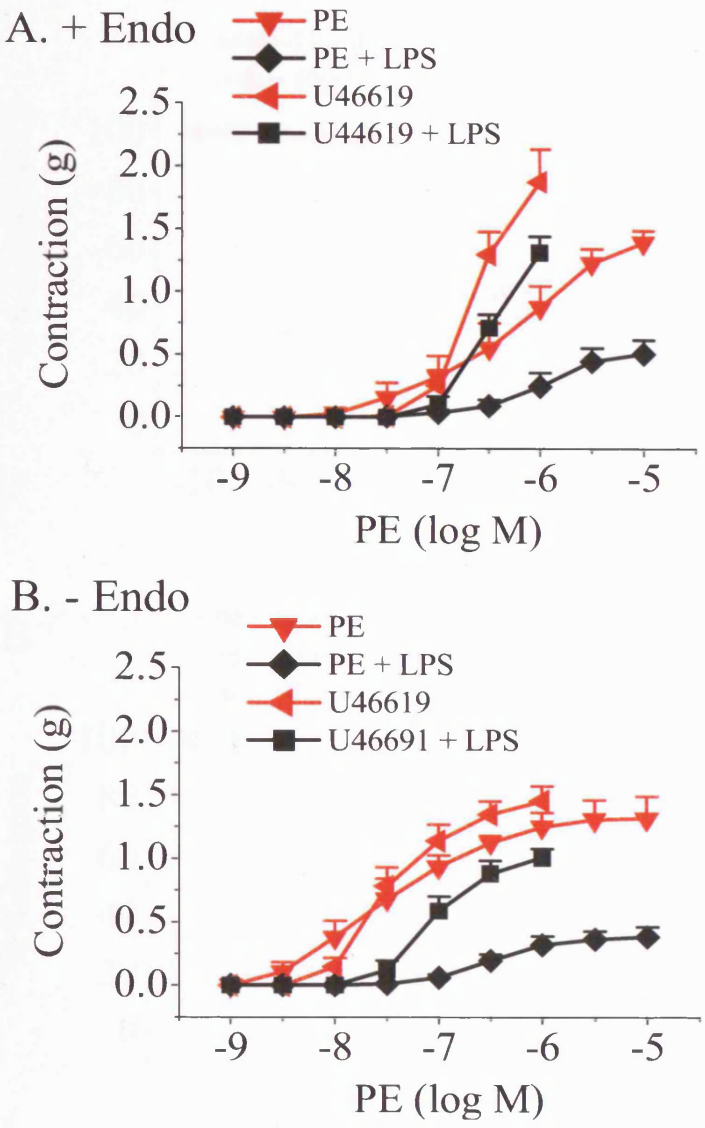


Figure 2.14 Effect of LPS (20h) on PE or U46619 induced contractions in RMA in the presence (A) or absence (B) of endothelium (endothelium removed after incubation in culture medium). LPS did not induce significant hyporeactivity to U46619 with 20 h incubation in endothelium-intact RMA, some hyporeactivity was induced by LPS in endothelium-denuded RMA ($P<0.05$). Data are expressed as the mean ± s.e.m. of 8-10 observations from 12 animals.

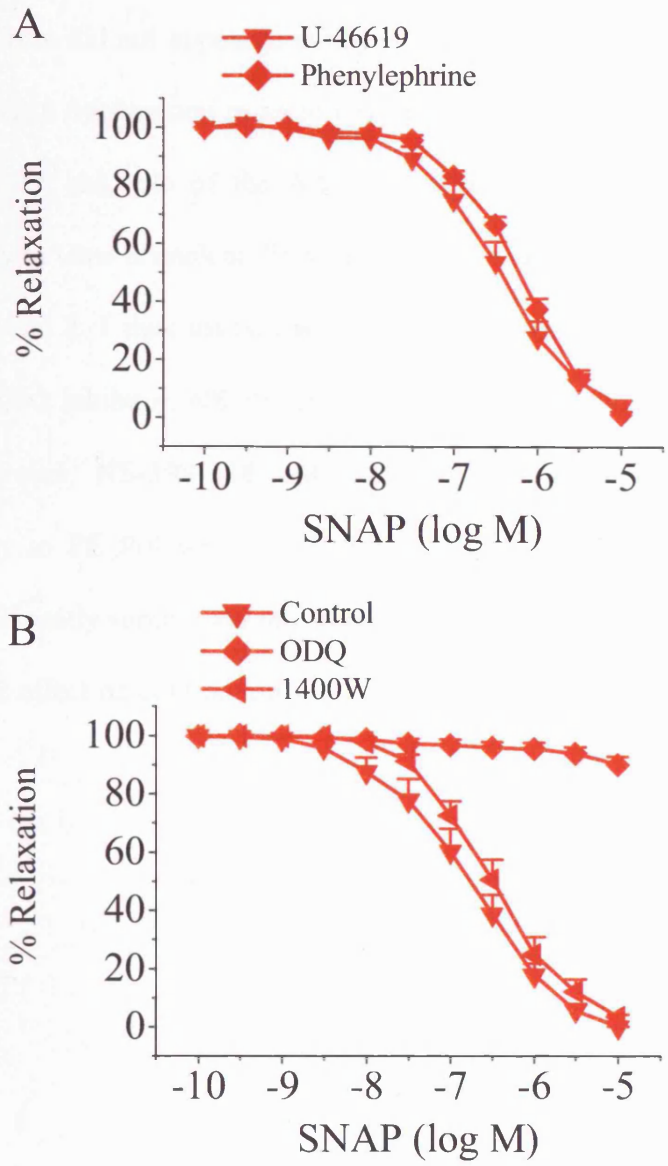


Figure 2.15 (A) Effect of SNAP following precontraction with PE and U-46619. (B) Effect of ODQ (3 μM) and 1400W (10 μM) on concentration-relaxation curves to SNAP following contraction with PE in fresh RMA. SNAP relaxed PE and U46619 induced contractions with a similar potency. Concentration-relaxation curves to SNAP were significantly inhibited by ODQ ($P < 0.001$); 1400W had no effect. Data are expressed as mean ± s.e.m.

2.5.5 Effect COX-2 inhibition on contractile responses to PE

COX-2 inhibition did not appear to contribute to the development of circulatory failure or multiple organ dysfunction in an *in vivo* model of rat endotoxic shock (Leach *et al.*, 1998). However, the role of the AA metabolites generated by COX-2 in vascular hyporeactivity *in vitro* is unclear. Previous studies tend to use indomethacin that inhibits both COX 1 and 2. I thus investigated manipulation of the cyclo-oxygenase pathway using the COX-2 inhibitor, NS-398 (Futaki *et al.*, 1994) a relative selective inhibitor of COX-2. However, NS-398 (10 μM) had no effect upon the degree of vascular hyporeactivity to PE following incubation with LPS for 20 h (Fig. 2.16). In fact, it actually significantly suppressed responses to PE in control tissues, possibly suggesting a non-specific effect on contraction.

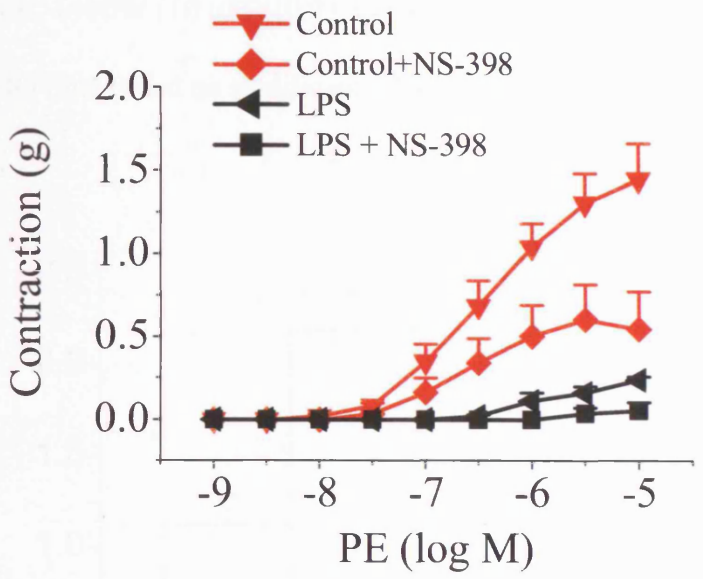


Figure 2.16 Effect of the COX-2 inhibitor, NS-398 on LPS-induced hyporeactivity. RMA was incubated for 20 h in the presence/absence of 1 μg ml^{-1} LPS ± NS-398 (10 μM). NS-398 had no effect upon vascular hyporeactivity in LPS treated tissues and indeed significantly suppressed responses to PE in control tissues ($P<0.05$). Data are expressed as the mean ± s.e.m of 5-6 observations from 5 animals.

2.5.6 Nitrite production in organ culture model

Having established an organ culture model of LPS induced vascular hyporeactivity that is reversible by inhibitors of iNOS, I was interested to determine whether NO was being released from the RMA rings. Measuring NO_2^- is the standard way to assay NO production. Incubating RMA with LPS (1 μg ml^{-1}) for 20 h doubled the amount of nitrite that could be detected in the culture media being 1.13 ± 0.15 and 2.19 ± 0.21 μM mg^{-1} protein in control and LPS-treated tissues respectively ($P<0.001$; Fig. 2.17). This

increase in nitrite accumulation induced by LPS could be fully inhibited by the specific iNOS inhibitor, 1400W (10 μM) (0.93 ± 0.9, $P<0.05$; Fig. 2.17). The guanylyl cyclase inhibitor, ODQ (3μM) had no significant effect.

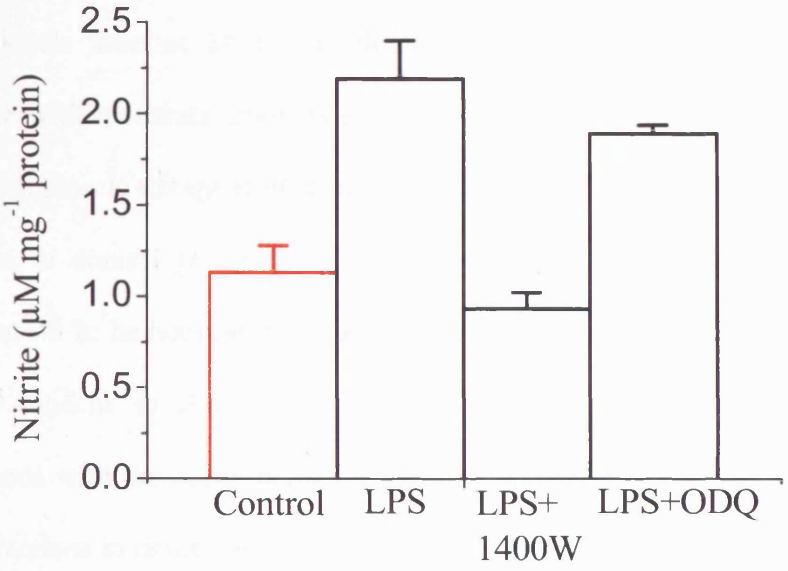


Figure 2.17 Effect of 1400W and ODQ on accumulation of nitrite within culture media induced by LPS. Rings were treated for 20 h in the absence (control) or presence of 1 μg ml^{-1} LPS ± 1400W (10 μM) or ODQ (3 μM). LPS doubled the amount of nitrite produced within the culture media compared to controls ($P<0.001$). This increase was inhibited by 1400W ($P<0.001$) but ODQ had no effect. Data are expressed as the mean ± s.e.m of 6 observations from 5 animals.

2.5.7 iNOS immunoreactivity

I have established that enhanced production of NO corresponded with LPS induced vascular hyporeactivity and that specific inhibitors of iNOS could reverse both. I thus

used an immunohistochemical staining technique to visualise iNOS within the RMA following incubation with and without LPS in the presence of serum.

iNOS protein was seen in all cell layers following incubation with LPS (1 μg ml^{-1}) though expression levels varied between the different layers (n=3; Fig. 2.18 & 2.19 and Table 2.6 and see Fig 3.3 for 4 h data). Staining was time-dependent with maximum protein levels seen at 20 h. The heaviest staining was consistently found in the adventitia with moderate amounts seen in the endothelium and only small amounts in the smooth muscle except at 46 h in LPS (Table 2.6). As expected, iNOS induction was also seen in control tissues incubated in culture medium, with significant staining evident at 46 h; heaviest staining again being in the adventitial layer with only small amounts evident in the smooth muscle layer. iNOS staining in control tissues corresponds with the organ bath experiments, in which 1400W significantly increased PE contractions in tissues incubated in culture medium in the absence of LPS.

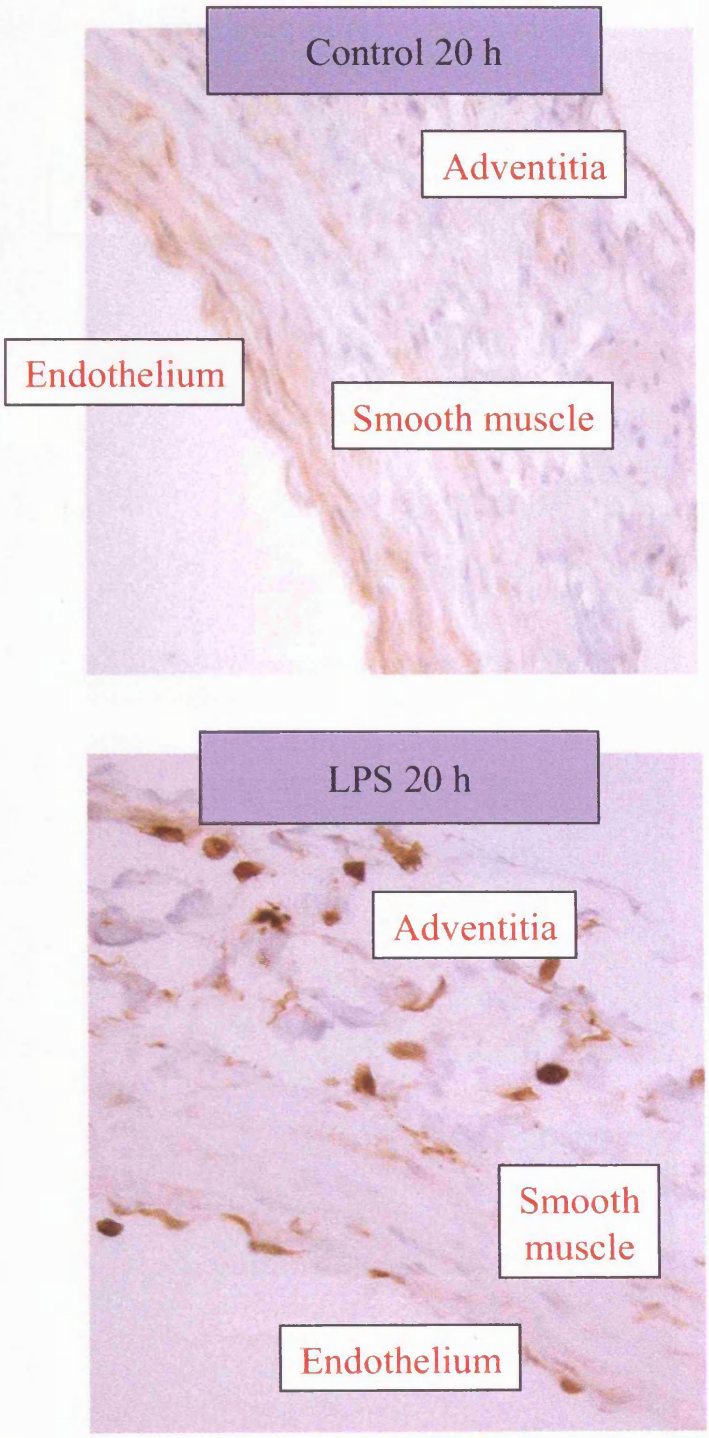


Figure 2.18 Distribution of iNOS within RMA in control and LPS (1µg ml^{-1}) treated RMA following 20 h incubation in culture medium + serum. Dense brown staining represents iNOS immunoreactivity (n=3).

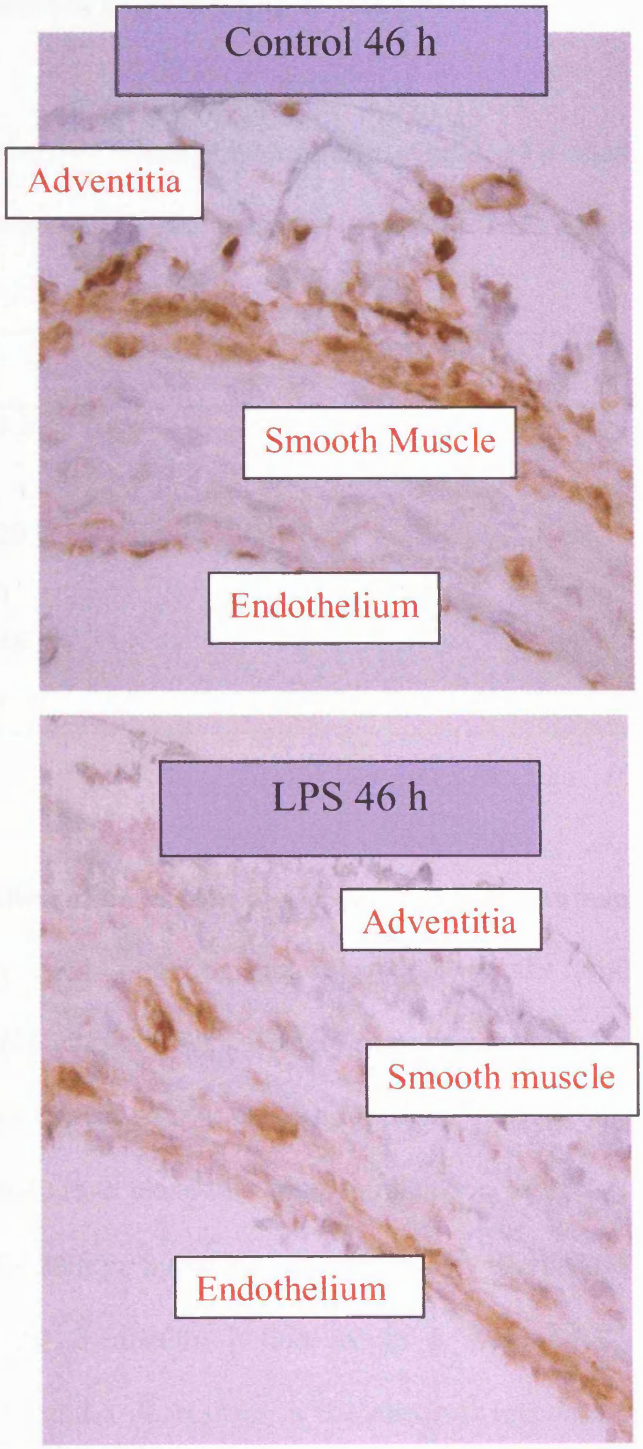


Figure 2.19 Distribution of iNOS within RMA in control and LPS (1µg ml^{-1}) treated RMA following 46 h incubation in culture medium. Dense brown staining represents iNOS immunoreactivity (n=3).

Table 2.6 Distribution of iNOS staining in RMA incubated in culture media ± LPS 1μg ml-1

Symbols: - = no iNOS; -/+ = barely detectable; + = mild; ++ = moderate; +++ = heavy iNOS staining. Data compiled from a number of sections from 3 animals.

	Endothelium	Smooth Muscle	Adventitia
Control 4 h	-	+/-	-
LPS 4 h	+	+	-
Control 20 h	+/-	+/-	+/-
LPS 20 h	++	+	+++
Control 46 h	++	+	+++
LPS 46 h	++	++	+

2.5.8 Characterisation of an *in vitro* model of sepsis using human vessels

Interestingly, there was little or no relaxation to the endothelium-dependent vasorelaxant, ACh (10 μM) in marginal artery rings precontracted with 2 μM PE at time 0 (immediately after dissection), 20 or 46 h (approx. 5-10%). When rings of marginal artery were incubated either alone or with a combination of LPS (1-100 μg ml^{-1}) ± IL-1β (10 units ml^{-1}) for 20 h no hyporeactivity to PE was observed (n=4, data not shown). I thus prolonged the incubation period to 46 h which did produce significant hyporeactivity to PE and a depression in the maximal response compared to control tissues (P<0.001, n=6-10; Fig. 2.20A). The hyporeactivity was observed with either incubation of LPS (100 μg ml^{-1}; n=9) or IL-1β alone (10 units ml^{-1}, n=10) or in combination (n=8). With the combination of LPS and IL-1β, the maximal contraction

was reduced by 68 % from 3.81 ± 0.50g to 1.26 ± 0.16g). Despite a depression in E_{Max}, there was no difference between the pEC_{50} values (control 5.59 ± 0.08 vs. LPS + IL-1β 5.58 ± 0.08). However, neither the non-specific NOS inhibitor, L-NAME (300 μM; n=4) nor the specific iNOS inhibitor 1400W (10 μM) (n=2) increased contractions to PE at 46 h (Fig. 2.20B).

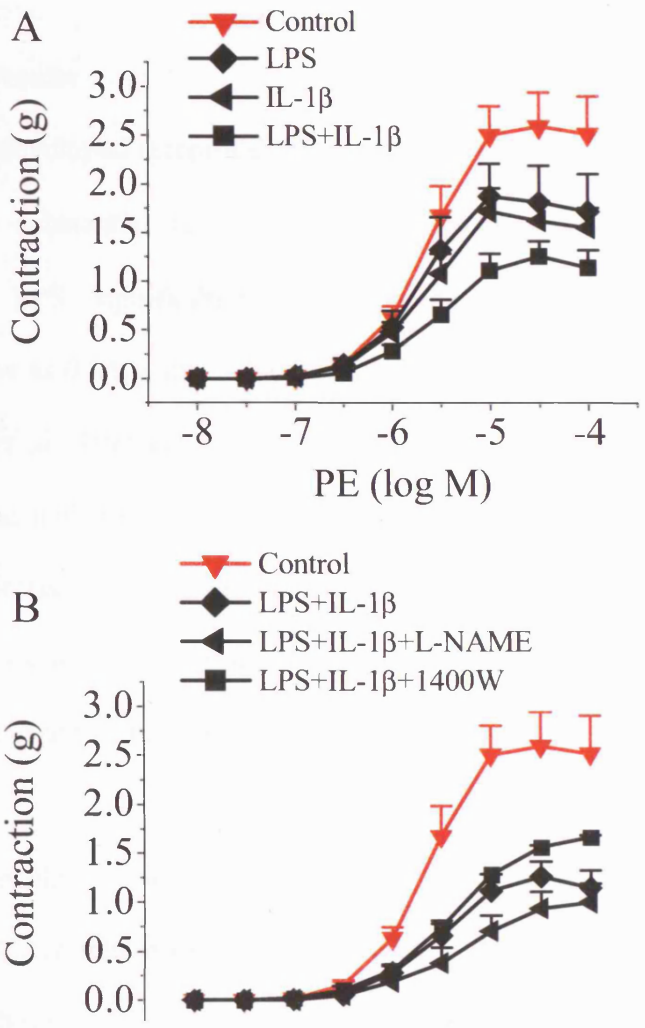


Figure 2.20 (A) Effect of LPS ± IL-1 β on contractile responses to PE in human marginal artery (HMA). There was hyporeactivity to PE incubated for 46 h with LPS (100μg ml^{-1}), IL1-β (5 units ml^{-1}) and LPS (10μg ml^{-1}) + IL-1 β (5 units ml^{-1}) $P<0.001$. (B) NOS inhibition had no effect on contractile responses to PE in HMA treated with LPS+ IL-1β. Data are expressed as mean ± s.e.m. of 6-9 observations from tissue derived from 6 patients (n=4 for L-NAME and n=2 for 1400W).

2.6 Discussion

2.6.1 Summary of results

I have successfully developed a reproducible *in vitro* organ culture model of sepsis in which LPS induces substantial vascular hyporeactivity to the α_1-agonist, PE in rat mesenteric artery. LPS significantly depressed contractile response to PE at concentrations as low as 0.01ng ml^{-1} which is akin to endotoxin levels found in human sepsis (Brandtzaeg *et al.*, 1988 & Danner *et al.,* 1991). The responses were dependent upon incubation time with LPS. Serum prolonged the effects of LPS causing marked depression of the contractile response even at 46 h, whereas without serum, responses had fully recovered by this time. In addition, hyporeactivity did not depend upon the presence of an intact endothelial layer.

I investigated the contribution of iNOS using inhibitors with varying selectivity towards this isoform. While these inhibitors fully reversed the LPS-induced hyporeactivity at 6 h, the effects of these agents varied thereafter. Pre-incubation (adding the iNOS inhibitor to the culture medium at the same time as LPS) provided what appears to be complete reversal of vascular hyporeactivity compared to just adding the inhibitor 25 min prior to the PE concentration-response curve. Consistent with this observation, ODQ reversed (≈75%) the hyporeactivity at 20 and 46 h, confirming involvement of the NO/cGMP pathway in mediating hyporeactivity. I also demonstrated iNOS staining and increased production of nitrite in the culture medium of RMA rings treated with LPS. The latter was inhibited by the iNOS inhibitor 1400W, confirming that NO had been

generated by this NOS isoform. An increase in contractile response to PE following addition of iNOS inhibitors in control tissues incubated in culture medium containing serum is consistent with the observation of iNOS staining in these tissues, which was particularly evident at 46 h. Nonetheless, a greater amount of iNOS staining was found in the RMA incubated with LPS compared to controls. The adventitial layer appeared to produce significantly more iNOS than in the endothelial layer, with only small amounts seen in the smooth muscle layer.

In contrast to PE, contractions to the thromboxane mimetic, U46619 were only weakly affected by LPS. The differential effects of LPS upon PE and U-46619 did not appear to be a NO-mediated phenomenon, as the NO donor SNAP caused an equipotent relaxation of contractions induced by either agonist. Inhibition of the COX-2 pathway had no effect on hyporeactivity. My attempt to transfer the rat model to human vessels was successful in that I managed to produce depression of contractile responses to PE in tissues incubated with a combination of LPS and the pro-inflammatory cytokine IL-1β at the 46 but not the 24 h time point, though neither L-NAME nor 1400W appeared to reverse this hyporeactivity.

2.6.2 Nitric oxide and sepsis

Substantial evidence across a variety of different species suggests that NO is the main mediator of vascular hyporeactivity in sepsis (reviewed in Thiemermann, 1994, 1997 and Vallance and Charles, 1998). In septic patients, elevated levels of the stable breakdown products of NO (nitrate/nitrite) have been measured (Ochoa *et al.,* 1991)

while administration of the non-selective NOS inhibitor, L-NMMA produced significant elevations in blood pressure and a 60-80% reduction in vasopressor requirements (Grover *et al.,* 1999). Similar findings have also been reported in rat and mouse models (reviewed by Rees *et al.,* 1998 and Vincent *et al.*, 2000). Enhanced NO production may result from activation of eNOS in the early stages of the septic insult (<1 h) followed by expression of iNOS commencing after 2-3 h (Thiemermann, 1997). Consistent with a major role for iNOS, vascular hyporeactivity in arteries removed from animals treated with LPS for 12 h was only observed in wild-type but not iNOS-deficient mice (Gunnett *et al.,* 1998). Moreover, long-term exposure to LPS results in changes in the expression of iNOS but not eNOS such that after 24 h in culture with LPS there was no increase in eNOS expression in rat aorta (Bishop-Bailey *et al.,* 1997).

An important new finding in my *in vitro* model was the variable time and individual agent response to NO inhibition. While complete reversal with NO blockade was achieved at 6 h, sensitivity to the NOS inhibitors decreased over time when they were added directly to the organ bath. L-NAME completely lost its effect at 20 h, though 1400W and aminoguanidine still remained partially effective, whereas only 1400W increased PE contractions at 46 h. As 'pre-incubation' with 1400 W for 20 or 46 h appeared to fully restore hyporeactivity this could imply that prolonged activation of iNOS may have irreversible effects on the contractile machinery, either through NO itself or from the down-stream activation of other pathways by iNOS. Whether reversibility was indeed really complete remains undetermined since I did not pre-incubated the control tissues with 1400W. Based on my results in which I showed that

iNOS was produced in control tissues, NO^{2-} was detected in the culture media of controls and that addition of other NOS inhibitors increased contractile responses to PE in control tissues, I would predict 1400W to have effects in control tissues. An alternative explanation to the above is that incomplete NO blockade is occurring in the organ both or that NO-independent mechanisms do indeed produce hyporeactivity at 20 and 46 h. Incomplete NO blockade may be due either to inadequate NOS inhibition, NOS-independent generation of NO, or release of NO from nitrosylated thiols (*e.g.* albumin). An inadequate dose of AMG or L-NAME is a possibility, although I did observe a full reversal with both these agents at 6 h. In addition, the doses I used are well above the reported IC_{50} values for inhibition of NOS, being 30 μM and 11 μM, respectively for aminoguanidine and L-NAME against iNOS and 0.6 μM for L-NAME against eNOS (Yen *et al.,* 1995). However, it has been shown that a combination of NOS inhibitor and NO scavenger was required to inhibit NO production in rat mesenteric and hepatic arteries (Chauhan *et al.,* 2000). Furthermore, NO release induced by LPS in rat aortic segments after 48 h in culture medium was found to be only partially inhibited by 1 mM L-NAME, though fully by a protein synthesis inhibitor (Bishop-Bailey *et al.,* 1997). While this latter observation suggests that NO production was probably occurring through iNOS, continued production of NO and/or its metabolites may lead to the formation of NO stores within the blood vessel.

Little is known regarding the ability of iNOS-derived NO to interact with physiological substrates such as thiols to yield biologically active S-nitrosothiols during endotoxemia. It has been suggested that these relatively stable, vaso-active compounds may act as a

storage system for NO and thus play an important role NO mediated effects. Indeed, endotoxemia has been shown to enhance circulating S-nitrosothiol formation considerably (Jourd'heuil *et al.*, 2000 and Ottesen *et al.*, 2001). In addition mice with a targeted gene deletion of S-nitrosoglutathione reductase exhibited substantial increases in whole-cell S-nitrosylation, tissue damage, and mortality following endotoxic or bacterial challenge (Liu *et al.*, 2004). The generation of nitrated/nitrosylated compounds by reaction of NO or its metabolites with either components of the tissue bathing medium or complexes in blood vessels has been well recognised (Dowell & Martin, 1998; Muller *et al.*, 1998; Stubauer *et al.*, 1999 and Amirmansour *et al.*, 1999). Cellular degradation and subsequent release of NO may also explain at least in part, the ineffectiveness of aminoguanidine and L-NAME at reversing vascular hyporeactivity at 46 h. It may also be responsible for the greater hyporeactivity seen when serum was added to the incubation medium. Such a mechanism has been proposed to explain L-NAME-resistant vascular hyporeactivity (Muller *et al.*, 1998).

I was surprised to find an effect, albeit partial, of 1400W at 46 h, but no reversal at all from either AMG or L-NAME. This could simply reflect the greater potency (nanomolar range) of this agent or its apparent irreversibility as an inhibitor of iNOS (Garvey, *et al.*, 1997). Alternatively, 1400W may have actions unrelated to iNOS inhibition that have not been demonstrated thus far. It is not likely to be due to an effect of 1400W on COX-2 activity, since I was unable to show any reversal of hyporeactivity with the selective COX-2 inhibitor, NS-398 after 20 h in LPS. The production of prostaglandins E_2 and $F_{2\alpha}$ has been demonstrated *in vitro* in LPS-treated rat aortic

tissues, and this was successfully blocked by NS-398 (Bishop-Bailey, 1997). Although blockade of the inducible and/or constitutive COX pathways rarely reverses hypotension (Bernard *et al.*, 1997 and Leach *et al.*, 1998), it has been shown to partially reverse LPS-induced hyporeactivity (McKenna, 1990 and Gunnett *et al.*, 1998).

To compliment the finding that overproduction of NO via iNOS was the major factor in the development of vascular hyporeactivity to LPS, I measured nitrite levels in the culture medium supernatant and examined the rat mesenteric artery for iNOS expression at different time points. Compared to control supernatant, NO_2^- levels doubled in the presence of LPS (1 μg ml^{-1}) after 20 h incubation and this rise was completely inhibited by the specific iNOS inhibitor, 1400W (10 μM). In addition, I also demonstrated that LPS increased production of iNOS within the vessel itself via immunohistochemistry. In the presence of LPS NO_2^- levels peaked at 20 h but significant amounts were seen in non-LPS incubated (control) tissues which peaked at 46 h. Complimentary to these results was the observation that significant iNOS activity was seen in the control tissues at 20 and 46 h.

2.6.3 LPS and vascular hyporeactivity

The continued presence of LPS within the incubation medium is likely to have prolonged the duration of vascular hyporeactivity. *In vivo* animal models given a bolus of LPS have demonstrated shorter-lived effects. In mice given *i.v.* LPS, maximum haemodynamic effects were seen at 12 h, which corresponded with peak iNOS activity (Rees *et al.*, 1998). Surviving animals showed some recovery by 18 h, this

corresponded with a fall in iNOS activity. In rats receiving intraperitoneal LPS, levels of mRNA for iNOS peaked at 4-8 h, decreasing markedly thereafter (Lui *et al.*, 1997). In a similar model, Mitchell and co-workers (1993) showed maximum induction of NOS at 6 h which returned to control levels by 24 h. However, arterial and venous mesenteric vascular beds removed from these animals at 6 h were not hyporeactive to PE, U44619, endothelin-1 or 5-HT. In contrast, using an *in vitro* organ culture model in rat aorta, the expression of iNOS and COX-2 remained elevated over the entire 10-day duration in the presence of LPS (Bishop-Bailey *et al*, 1997). Although vascular hyporeactivity was not assessed, nitrate/nitrite levels peaked on day 2 and 9. There was hyporeactivity at 46 h in my model when LPS was added to culture medium containing serum. I thus speculate that continued presence of LPS in the incubation media prolongs the period of iNOS induction in blood vessels compared to a bolus dose administered to rodents. I have been unable to find organ bath experiments utilising vessels incubated in LPS or harvested from endotoxaemic rats that have been extended beyond 24 h.

Previous results from this laboratory demonstrated that a 6 h incubation of aortic rings with LPS in the organ bath is sufficient to produce substantial hyporeactivity to PE (Hall *et al.*, 1996). However, I was unable to reproduce this effect in mesenteric rings unless tissues were first incubated in culture medium. This represents the first *in vitro* model of hyporeactivity using mesenteric vessels. Subsequently LPS-induced hyporeactivity in mesenteric arteries has been demonstrated after 5-6 h incubation (Mitsumizo *et al.*, 2004 and Hernanz *et al.*, 2004). Studies have demonstrated hyporeactivity *ex-vivo,* albeit inconsistently, where the mesenteric artery is harvested 4-

6 h from rats treated with LPS (e.g. Schneider *et al.,* 1992 and Mitchell *et al.*, 1993 demonstrated hyporeactivity but Buyukafsar *et al.*, 2004 did not).

The addition of serum to the incubation medium enhanced and prolonged the effects of LPS. This most likely reflects the fact that culture medium contains soluble CD 14 and LPS-binding factor that are involved in recognition of LPS by toll receptors causing subsequent cellular activation and production of pro-inflammatory cytokines and transcription nuclear factor NFκ-B activation of cells within the blood vessel (reviewed by Heumann and Roger, 2002). However, membrane bound CD14 also contributes to this process accounting for the profound hyporeactivity observed with LPS in the absence of serum.

I found the lowest concentration of LPS required to produce significant hyporeactivity was 0.01 ng ml^{-1}. Most other *in vitro* studies use LPS concentrations of 1-10 μg ml^{-1} (Glembot *et al.*, 1995; Scott *et al.,* 1966 and Muller *et al.,* 1998). In septic humans endotoxin concentrations measure a mean peak of 0.5 ng ml^{-1} (Danner *et al.,* 1991), although levels can rise up to 100 ng ml^{-1} in septic patients with meningitis (Brandtzaeg *et al.*, 1988). There has been one report of concentration-dependent depression of vascular contractility at much lower concentrations of LPS (1-100 ng ml^{-1}) in aortic rings incubated for 16 h (McKenna, 1990). Perhaps, in retrospect it would have been more relevant if experiments had been performed using LPS at a more 'physiological' concentration. Unfortunately, having managed to succeed with an organ culture model of vascular hyporeactivity using a concentration of 1 μg ml^{-1} of LPS, I continued with

this and only performed the LPS threshold experiments towards the end of my time in the laboratory.

2.6.4 The role of the endothelium, vascular smooth muscle and adventitia

I observed that the concentration-response curve to PE in control tissues shifted to the left at 20 h incubation in culture medium compared to that observed with only 6 h incubation. While I have no precise explanation for this, the removal of the endothelium did increase the responsiveness to PE in my experiments, suggesting that loss of endothelial function may be occurring over time. Against this, I found that endothelium-dependent relaxation to acetylcholine was similar at 6 & 20 h, suggesting that alterations in the release of endothelial-derived relaxing factors *per se* cannot account for these changes. Alternatively, increased basal production of contracting factors may be occurring over time that, in turn, might enhance sensitivity to PE. In rat mesenteric artery, organ culture increases the potency of ET-1 and this is associated with the appearance of contractile ET_B receptors after 24 h (Adner *et al.*, 1998).

Denuding vessels of endothelium following incubation with LPS and serum did not affect the level of vascular hyporeactivity observed with LPS. This would suggest that the medial and/or adventitial layers are principally responsible for continued hyporeactivity once the vessel has been transferred to the organ bath. This is consistent with previous studies showing that an intact endothelium is not necessary for endotoxin-mediated vascular suppression (McKenna 1990; Julou-Schaeffer *et al.*, 1990 and Hall *et al.*, 1996). However, to have proved this hypothesis, I should have denuded the

endothelium prior to incubation with LPS and serum. Interestingly, data in rat aorta suggest that the adventitia is responsible for the majority of iNOS expression, NO production and medial hyporeactivity following exposure to LPS (Kleschyov *et al.*, 1998 and 2000; Zhang *et al.*, 1999 and reviewed in Muller *et al.*, 2000). In my studies, I found that the major site of iNOS expression in mesenteric artery was also the adventitia with lesser amounts in the endothelium and only small amounts in the media. Adventitial derived NO can exert immediate down-regulatory effects on smooth muscle contraction via activation of cyclic GMP pathway. However, it may also initiate longer lasting effects through the formation of NO stores within the medial layer. One possible candidate is dinitrosyl non-heme iron complexes (Muller *et al.*, 2000). Recently, in a model of LPS-induced hyporeactivity in rat mesenteric arteries, Hernanz and co-workers (2004) concluded that hydrogen peroxide generated from superoxide dimutase and release of NO from N-acetylcysteine-sensitive stores both contributed to the vascular hyporeactivity.

2.6.5 A human model of sepsis

Naturally, I wished to transfer my findings to human blood vessels. However, it proved far more difficult to develop a reproducible *in vitro* model and required a higher concentration of LPS or the pro-inflammatory cytokine IL-1β as well as longer incubation times. Previous studies have used *ex-vivo* vessels from septic patients (Stoclet *et al.*, 1999), 'normal' coronary arteries (Chester *et al.*, 1998) or saphenous veins (Thorin-Trescases *et al.*, 1995) harvested from patients undergoing cardiac surgery. A continuous perfusion set-up using lipoteichoic acid over vessels has also

been employed rather than an organ culture model (Tsuneyoshi *et al.*, 1996). I chose the marginal artery to compliment my rat model adapting the organ culture model with the addition of the pro-inflammatory cytokine interleukin 1L-β. Other groups have found cytokines necessary for iNOS induction (Thorin-Trescases *et al.*, 1995 and Chester *et al.*, 1998). I was able to achieve significant hyporeactivity to PE, although 1400 W was not able to reverse it. This result may not be surprising given the rat data, where at the longer time points of LPS incubation, reversibility was only achieved by pre-incubation with the iNOS inhibitor. My experiences mirror existing data, in that overwhelming evidence exists for the role of iNOS in experimental sepsis in animal studies yet relatively little exists in humans. The reasons why *in vitro* expression of iNOS has been so difficult in human tissue are unclear. Several studies have shown that administration of a non-selective NOS inhibitor to humans with septic shock will raise blood pressure (Grover *et al.*, 1999); so perhaps eNOS is more important in humans than iNOS. Certainly eNOS not iNOS has been shown to mediate cytokine-induced venodilatation in humans (Bhagat *et al.*, 1999). In contrast, both iNOS and eNOS mRNA were found to be down regulated in mesenteric arterial smooth muscle cells and peripheral blood mononuclear cells obtained from patients with early septic shock compared to control patients. In the same study inducible hemoxygenase mRNA was found to be increased, suggesting that CO might contribute to vasodilation in sepsis (Reade *et al.*, 2005). However, iNOS activity has been found in muscle and fat in patients with septic shock from cellulitis (Annane *et al.*, 2000), in rectus abdominis muscle obtained during surgical procedure in 16 septic patients (Lanone *et al.*, 2000) and in omental arteries from patients with peritonitis (Stoclet *et al.*, 1999). Interestingly in an *in vitro* model of

LPS, endotoxin had no direct vasodilatory effect on the arterioles of human skeletal muscle, but, when endotoxin was allowed to pass over an upstream conduit vessel, the arteriole showed marked dilation. The authors proposed that release of an endothelial factor from the upstream conduit vessels produced the loss of tone in the microvasculature (Campbell and Britt, 2004). Clearly this remains a complex area that is yet to be fully understood.

2.6.6 The role of thromboxane

As observed previously in pig mesenteric and pulmonary artery (Perez-Vizcaino *et al.*, 1996), the thromboxane A_2 mimetic, U46619 was substantially less affected by LPS compared to PE in our study. In an attempt to explain these differences, I thought that contractions to U46619 might be significantly less sensitive to the relaxing actions of NO. This was not the case, as the NO donor, SNAP was equipotent against PE and U46619. Thromboxane A_2 has been shown to regulate vascular tone via an inhibitory effect upon the expression of iNOS (Yamada *et al.*, 2003) and this could explain the results seen here. Consistent with this notion, increased sensitivity to U46619 was observed in the presence of L-NAME in the perfused mesenteric bed taken from rats treated with LPS (Mitchell *et al.*, 1993). In addition PE contractions are worsened by thromboxane antagonists (Cirino *et al.*, 1996). Increased production and/or sensitivity of thromboxane A_2 may account for the vasoconstriction seen in some vascular beds, following endotoxin treatment and for the development of pulmonary hypertension in early septic shock (Hales *et al.*, 1981).

In summary, I have developed a reproducible model using rat mesenteric artery that demonstrates *in vitro* vascular hyporeactivity to LPS. My results highlight the importance of both incubation time and serum on the degree of hyporeactivity. Using assays for nitrite and iNOS, I have demonstrated that nitric oxide via the induction of iNOS is extremely likely to be responsible for this hyporeactivity. Further strong evidence to this effect is that the hyporeactivity was reversed by inhibitors of the NO pathway. Perhaps hyporeactivity at later time points (46 h) is determined by additional pathways to the upregulation of iNOS. I was very keen to further investigate mechanisms in human vessels; unfortunately, although I managed to develop a hyporeactive model, I was unable to obtain enough tissue to ascertain the role of NO.

Chapter 3

Characterisation of an *in vivo* fluid resuscitated model of endotoxin-induced shock

Characterisation of an in vivo fluid resuscitated model of LPS-induced shock

3.1 Introduction

It should be recognised that animal models are not necessarily representative of the clinical condition of septic shock (reviewed in Deitch, 1998). For example, the duration of the study may be short, and blood flow, when measured, is often reduced, whereas a hyperdynamic response is commonplace in the critically ill patient (Krishnagopalan *et al.*, 2002). In addition, most animal models look at blood pressure response alone to an agent, and do not assess concurrent regional blood flow. The flow may be compromised if the blood pressure rise is at the expense of profound vasoconstriction in certain vascular beds. Patients usually receive aggressive fluid resuscitation as first line therapy, yet most animal models neglect this.

I sought to characterise a fluid-resuscitated short-term *in vivo* model of hyperdynamic sepsis secondary to endotoxin in order to assess vascular hyporeactivity and responsiveness to various agents. Our laboratory has previously described a hyperdynamic model of endotoxin-induced sepsis in an anaesthetised, spontaneously breathing rat (Rosser *et al.*, 1995 and 1996). I adapted this model, using a different fluid resuscitation regimen of gelatin rather than normal saline, a different type of endotoxin, and I measured hepatic microvascular oxygen tension rather than bladder oxygen tension as a marker of organ perfusion. As inhibition of inducible nitric oxide synthase appears to be a successful treatment in animal models, yet not in humans, I investigated the effects of the highly specific iNOS inhibitor GW273629 (Young *et al.*, 2000 and Alderton *et al.*, 2005). I examined not only the response of mean arterial blood pressure to this agent but also the consequences on macrocirculatory (aortic and renal artery

blood flow measured by ultrasonic flow probes) and hepatic microvascular oxygen tension using the technique of palladium porphyrin phosphorescence. This technique is based on the phosphorescence decay lifetime of an injected palladium-porphyirn complex illuminated by a phosphorimeter which is inversely related to the oxygen tension of the interrogated vasculature. The relationship between phosphorescence lifetime and oxygen pressure is quantitatively described by the stern-volmer equation (reviewed by Wilson, 1992). The oxygen measurements are weighted toward detection of longer phosphorescence lifetimes; thus the measured values are obtained primarily from blood within capillaries and venules. For the Pd-meso-tetra(4-carboxyphenyl) porphyrin used in these experiments, pH dependence is neglible between pH 7.2 and 7.8 and temperature changes are also too small to be considered significant for *in vivo* experiments. As the porphyrin is bound to albumin, the complex remains in the vascular compartment, so measurement is restricted to the microvasculature. The presence of any significant capillary leak of the porphyrin-albumin complex would be readily identifiable as an increase in delay time (this was not observed during the experiments). Sinaasappel & Ince (1996) first validated a phosphorimeter calibration of Pd-porphyrin phosphorescence for oxygen concentration measurements *in vivo*. We have used this technique successfully in our laboratory (Davies *et al.*, 2003 & 2005).

The microcirculation is a complex and integrated system that supplies and distributes oxygen throughout the tissues. Sepsis affects almost every cellular component – endothelial cells, smooth muscle cells, leucocytes, erythrocytes and tissue cells. Microcirculatory distress not corrected for 24 h was found to be the single independent

factor most predictive of subsequent patient outcome (Sakr *et al.*, 2004). NO is a central component in the autoregulatory control of microvascular patency. The overproduction of NO in different organ beds results in pathological shunting of flow (reviewed by Ince, 2005). Fluid resuscitation alone has been shown to restore systemic haemodynamics but left 'weak' microcirculatory areas hypoxaemic (Ince and Sinaasappel, 1999 and Golmann *et al.*, 2004). In septic pigs the combination of fluid and iNOS inhibition but not fluid alone was successful in recruiting vulnerable microcirculatory beds within the intestine (Pittner *et al.*, 2003 and Siegmund *et al.*, 2005). I was thus interested in comparing the effects of LPS and iNOS inhibition upon macro- and micro-circulatory blood flow within my rat model of sepsis.

I also took advantage of this model to examine the presence and distribution of iNOS within the rat mesenteric artery at the end of the 4-hour experiment using immunohistochemical analysis as before (see chapter 2). Following LPS treatment iNOS mRNA had been detected in rat kidney by 60 min after intravenous endotoxin; levels had peaked at 2-4 h and declined by 16 h (Sade *et al.*, 1999). In another sepsis model, nNOS mRNA increased by 2 h and iNOS mRNA by 3 h in rat brain, with both isoforms returning to baseline by 12 h (Harada *et al.*, 1999). Similarly, iNOS mRNA was detected at 1 h in the small intestine peaked at 4 h and was barely detected by 24 h (Chen *et al.*, 1996). Infusion of Staphylococcus aureus cell wall components caused progressive increases in iNOS activity in thoracic aorta, lung and liver and plasma NO_2^- / NO_3^- from 2-6 h (Kengartharan *et al.*, 1996). No such time profiles have been

determined in humans although there is evidence for elevated both NO_2^- / NO_3^- in plasma (Evans *et al.*, 1993) and skeletal muscle (Brealey *et al.*, 2002).

3.2 Methods

3.2.1 Animals and anaesthesia

In vivo procedures were performed under Home Office approval according to the Animals (Scientific Procedures) Act (1986). Experiments were performed on anaesthetised, spontaneously breathing, male Wistar rats (280-310 g) given free access to food and water until surgery. The animals were bought from Charles River and given a few days to settle prior to the experiments. The animals were placed in a large glass container, and anaesthesia was induced using isoflurane in air *via* a simple air pump driving a Tec 4 vaporiser (Abbott, Maidenhead, UK). A heated operating table was used to maintain rectal temperature at 37°C.

3.2.2 Surgery and monitoring

The left carotid artery was cannulated for monitoring of mean arterial pressure (MAP) (pressure transducer, and monitor, Powerlab, ADInstruments, Castle Hill, NSW, Australia) and intermittent sampling for blood gas analysis (165 µl heparinised capillary tube samples, processed by an ABL300 blood gas analyser (Radiometer, Copenhagen, Denmark). A cannula was inserted into the right internal jugular vein and connected to an infusion pump for fluid resuscitation. A midline laparotomy was performed to allow placement of transit time flow probes around the left renal artery (1mm, J reflector, 1RB) and infrarenal aorta (2mm, J reflector with sliding gate, 2SB, Transonic Systems, Ithaca, N.Y., U.S.A.). These probes were connected to a monitor displaying both blood flows continuously (monitor T206, Transonic Systems). Pd-meso-tetra-(4-Carboxyphenyl)porphyrin (protoporphyrin) (0.9 ml kg^{-1}) dissolved in 5% albumin was

injected intravenously via the internal jugular catheter. A fibreoptic cable from a phosphorimeter (OxySpot, Digitimer, Hertfordshire, U.K.) was held on a manipulating arm such that the tip could be maintained at 1 mm above the surface of the left lobe of the liver in order to measure hepatic microvascular PO_2. The machine was set to emit five flashes over 1 s with delay times set at 40 μs to eliminate flash artefact. These were repeated at 30 s intervals to minimize the risk of tissue bleaching, with online measurements directly recorede onto computer. Through the avascular dome of the bladder a drainage cannula (1.57 mm outside diameter polythene tubing) was sited to drain urine. After instrumentation, the animals were left to stabilise for 30-60 min until three consistent haemodynamic measurements had been obtained at 5 min intervals.

3.2.3 Experimental protocols

Three experimental groups were used:

(i) Group A (n=6) was given 40 mg kg^{-1} LPS (*Klebsiella Pneumoniae*) in 0.9 % saline by infusion over 30 min at a rate of 20 ml kg^{-1} h^{-1}. Ideally, I would have liked to have used *Salmonella Typhosa* as for my *in vitro* model in chapter 2; however, previous work within the laboratory had found that *Klebsiella Pneumoniae* gave the best haemodynamic response for this model. For the remainder of the experiment, all groups were fluid resuscitated with 25 ml kg^{-1} h^{-1} of the synthetic gelatin colloid, Gelofusin®. This was supplemented with 1 ml 10% glucose solution per 25 ml to keep blood sugar levels above 5 mM. I used Gelofusin® as this was the fluid most commonly used for resuscitation in sepsis in the intensive care unit at University College London Hospitals. Rosser's previous model had used 0.9 % saline at 20 ml kg^{-1} h^{-1}.

(ii) Group B (n=6) received isotonic saline rather than LPS over the first 30 min (sham-operated controls).

(iii) Groups C comprised either LPS-treated rats (n=6) or sham-operated controls (n=3). These rats received the specific iNOS inhibitor, GW273629, a 50 mg kg^{-1} bolus (approx. 0.7ml volume) followed by a 50 mg kg^{-1} h^{-1} infusion. This dose was suggested by Richard Knowles, a senior pharmacologist at GlaxoSmithKline, whose group had used GW273629 previously.

Continuous recordings were made of mean arterial pressure (MAP), aortic blood flow (ABF), renal blood flow (RBF), hepatic microvascular oxygenation ($HmvO_2$) and rectal temperature. Arterial blood gas analysis (for measurement of arterial pO_2, pCO_2, pH and standard base excess) was performed at 0, 120, 180 and 240 min after injection of LPS (or saline in sham-operated animals). After the last recordings were made, the animals were killed via a rapid intravenous injection of pentobarbitone sodium, 200mg kg^{-1}.

3.2.4 Analysis of iNOS via immunohistochemistry

The mesenteric artery was immediately removed and stored in HBSS. Immunohistochemical analysis was performed as described in chapter 2.

3.3 Statistical analysis

As chapter 2. Where appropriate, Student's t-test was used to compare two groups

3.4 Reagents and solutions

Immunohistochemistry - as chapter 2. Pd-meso-tetra-(4-Carboxyphenyl) Porphyrin (Protoporphyrin) was supplied by Digimeter Ltd (Herts, U.K.). LPS (*K. Pneumoniae*) was obtained from Sigma Chemical Company (Poole, Dorset, U.K.). GW273629 was kindly donated by Glaxo SmithKline (Stevenage, U.K.). Pentobarbitone sodium was obtained from Rhone Merieux Ltd, (Harlow, U.K.).

3.5 Results

3.5.1 Effect of endotoxin (LPS) upon MAP, aortic and renal blood flow and microvascular oxygen tension in an *in vivo* model of rat sepsis

In sham animals MAP, RBF, hepatic microvascular oxygenation ($HmvO_2$) and arterial base deficit did not change significantly throughout the experiment (see Fig. 3.1A-D). However, there was a tendency for MAP and RBF to increase, while ABF and $HmvO_2$ initially rose then fell. These changes, particularly in ABF, were probably related to the fluid resuscitation.

Following infusion of LPS, there was a rapid fall in all measured variables within 0-30 min (see Fig. 3.1A-D). Recovery in MAP was observed within 30 min (before fluid resuscitation was commenced), although aortic and renal blood flow took 2-3 h to return to baseline values. However, $HmvO_2$ failed to show any significant recovery after the initial abrupt fall. After 120 min the MAP fell significantly in LPS treated rats ($P<0.001$, $n=6$, ANOVA), I thus chose this timepoint to administer GW273629.

3.5.2 Effect of treatment with the selective iNOS inhibitor GW273629

Injection of the iNOS inhibitor at 120 min after injection of LPS (or saline control) resulted in a marked pressor response in the LPS-treated rats compared to shams (Fig. 3.2A). MAP was maintained at this elevated level until termination of the experiment at 240 min ($P=0.03$, $n=6$, ANOVA). However, in the LPS group no differences were seen in ABF, RBF, $HmvO_2$ or arterial base deficit between GW273629 treated and non-

treated animals (Fig. 3.2B-D). In sham animals treated with or without the iNOS inhibitor, RBF or arterial base deficit did not change however ABF and $HmvO_2$ fell significantly with GW273629 treatment ($P = 0.042$, n=6, ANOVA; Fig. 3.2 B-D).

3.5.3 iNOS immunoreactivity

Immunohistochemistry revealed that iNOS induction was seen in the smooth muscle and endothelium of the mesenteric artery but not in the adventitial layer 4 h following the bolus dose of LPS (40 mg kg^{-1} over 30 min) (n=1; Table 3.1). Only a small amount of iNOS was seen in the endothelium and smooth muscle of sham-operated rats (Fig. 3.3 and Table 3.1). This was similar to the distribution observed after 4 h incubation in the *in vitro* model. GW273629 completely inhibited the LPS-induced production of iNOS within the mesenteric artery removed from the animal at the end of the experiment (Fig. 3.4 and Table 3.1).

Table 3.1 Distribution of iNOS seen in RMA at 4 h following a bolus dose of LPS (40 mg kg^{-1} over 30 min)

Symbols: - = no iNOS; + = mild / ++ = moderate staining for iNOS (n=1)

	Endothelium	**Smooth Muscle**	**Adventitia**
Sham	+	+	-
LPS	++	++	-
LPS + GW273629	-	-	-

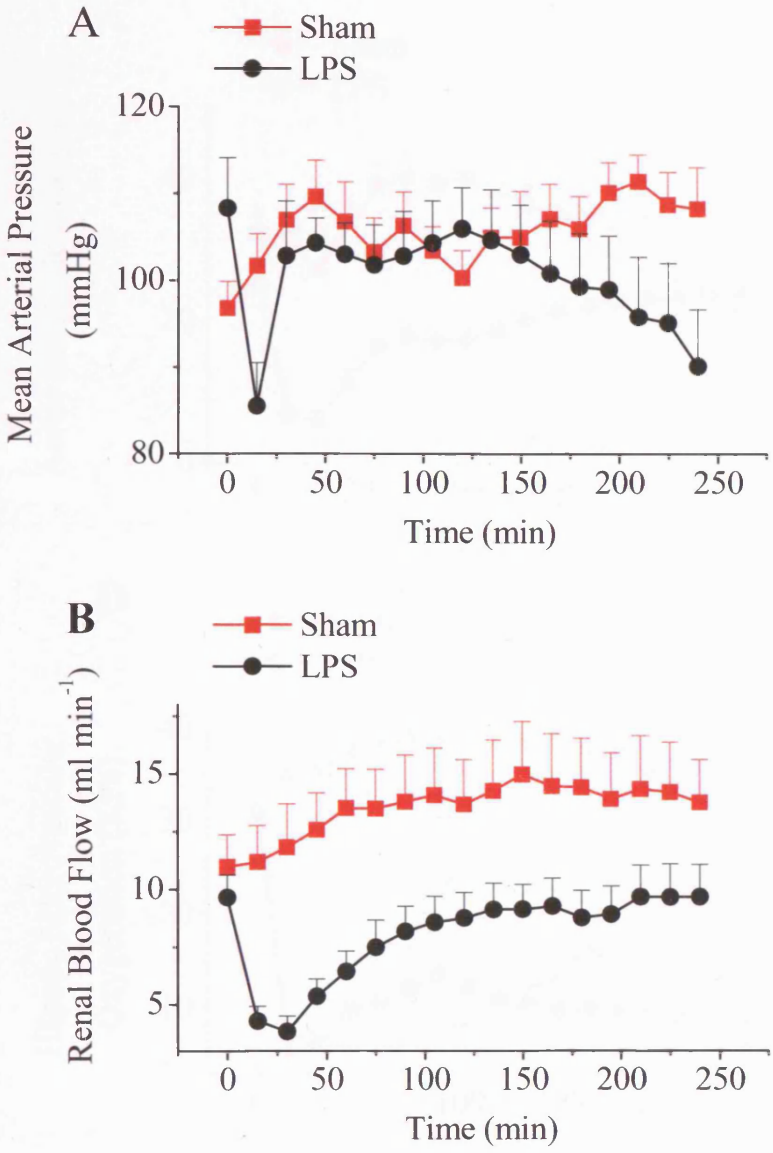


Figure 3.1 Effect of LPS (*K. Pneumoniae* 40 mg kg^{-1}) on (A) mean arterial blood pressure and (B) renal blood flow. MAP fell after 120 min (P<0.001, ANOVA) and RBF immediately following LPS (P=0.028). LPS was infused over 30 min and then Gelofusin® at 25 ml kg^{-1} hr^{-1}. Data are expressed as mean ± s.e.m. for 6 rats per group.

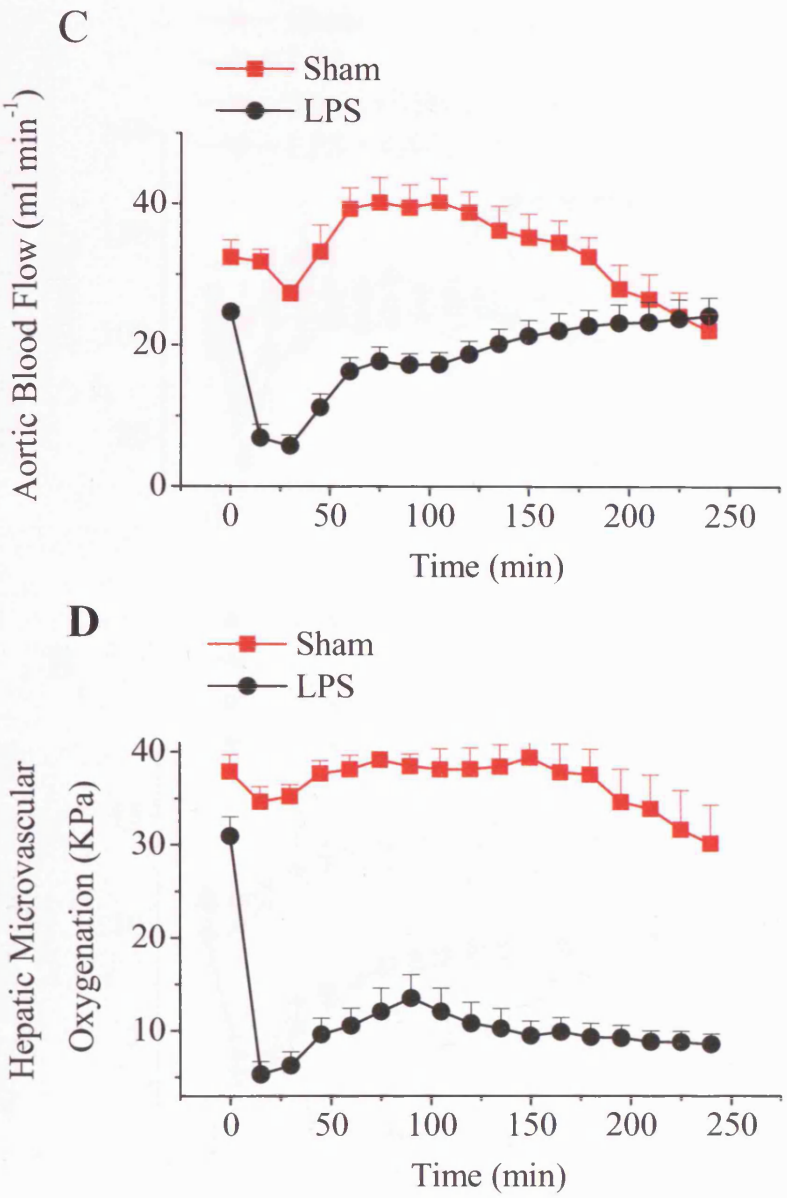


Figure 3.1 Effect of LPS (*K. Pneumoniae* 40 mg kg^{-1}) on (C) aortic blood flow and (D) hepatic microvascular oxygenation. LPS reduced both significantly ($P<0.001$, ANOVA). LPS was infused over 30 min and then Gelofusin® at 25 ml kg^{-1} hr^{-1}. Data are expressed as mean ± s.e.m. for 6 rats per group.

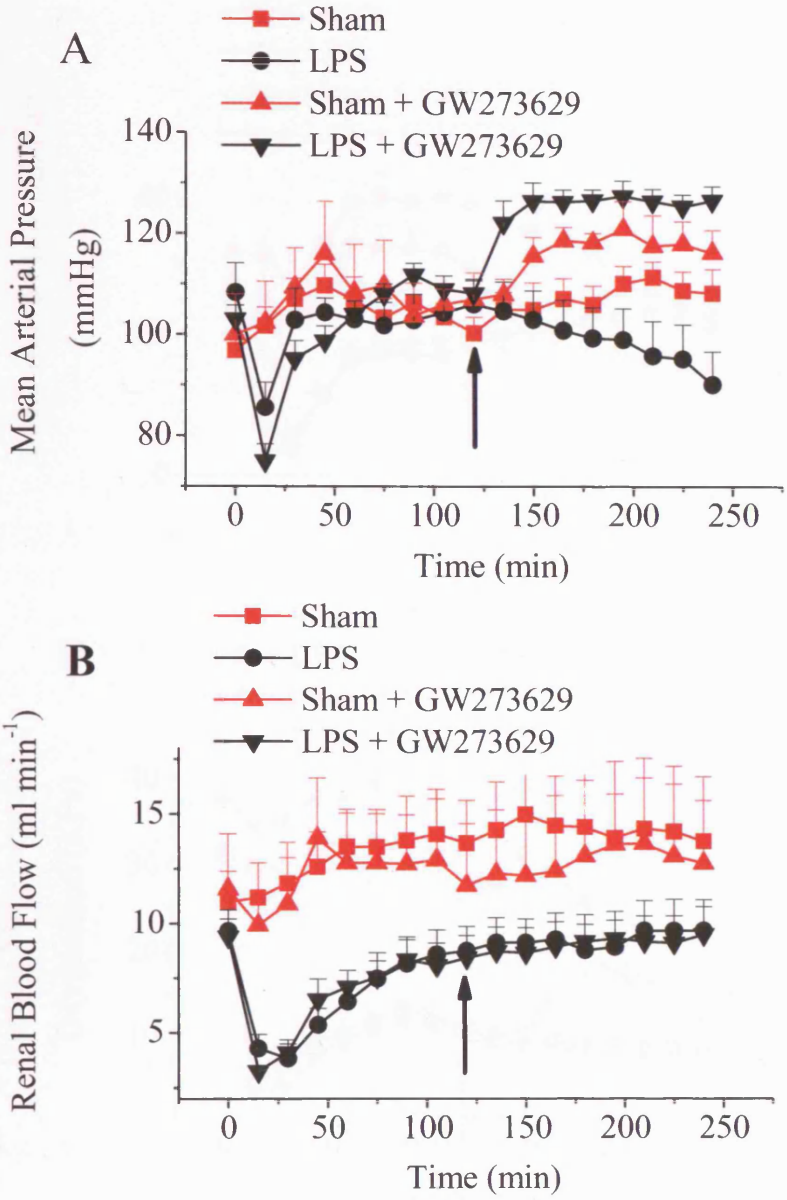


Figure 3.2 Effect of the highly specific iNOS inhibitor GW273629 (50 mg kg^{-1} bolus + 50 mg kg^{-1} h^{-1} infusion) on (A) mean arterial blood pressure and (B) renal blood flow in sham and LPS-treated rats. GW273629 raised MAP in LPS-treated rats (P =0.003, ANOVA) but non-significantly in shams. There was no effect upon RBF in either group. GW273629 was given at 120 min (see arrow). Data are expressed as mean ± s.e.m for 3-6 rats per group.

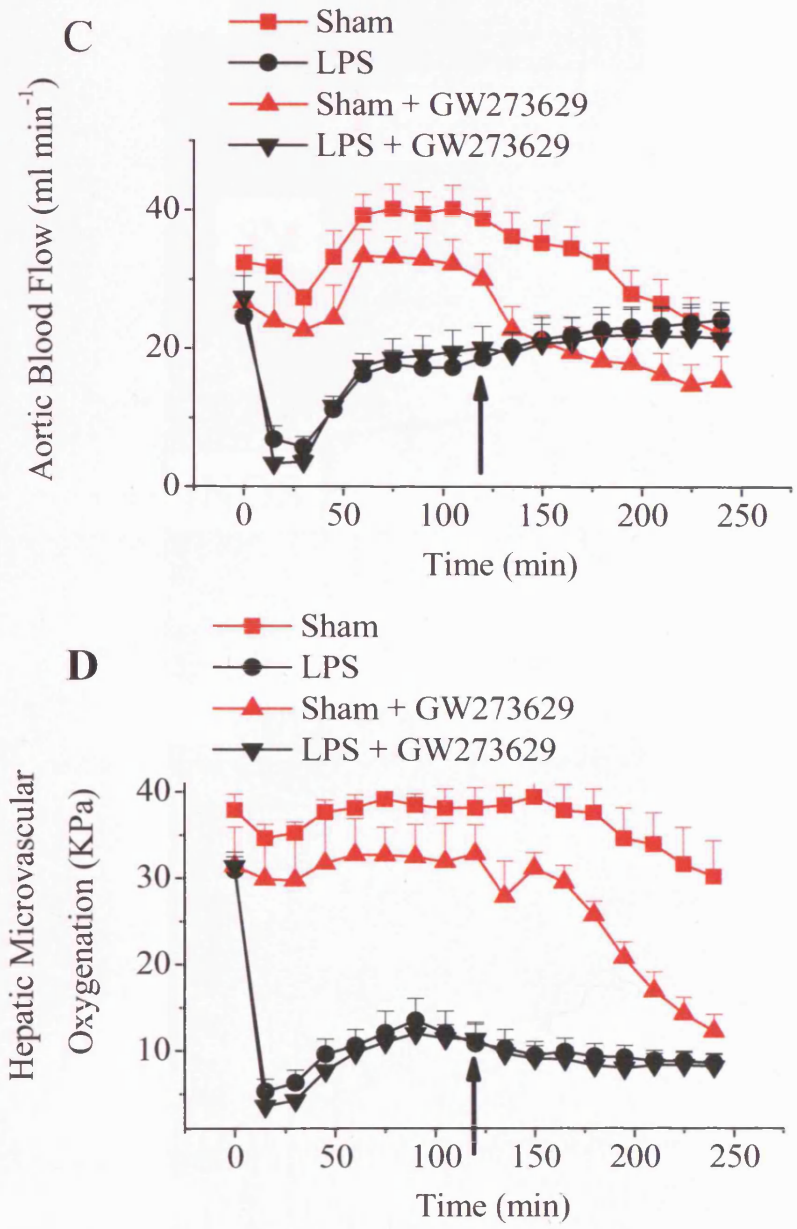


Figure 3.2 Effect of the highly specific iNOS inhibitor GW273629 given at 120 min – see arrow (50 mg kg^{-1} bolus + 50 mg kg^{-1} h^{-1} infusion) on (C) aortic blood flow and (D) microvascular oxygenation in sham and LPS-treated rats. GW273629 had no effect upon ABF or $HmvO_2$ in LPS-treated rats. In shams GW273629 reduces both ABF and HmvO2 (P=0.042). Data are expressed as mean ± s.e.m. for 3-6 rats per group.

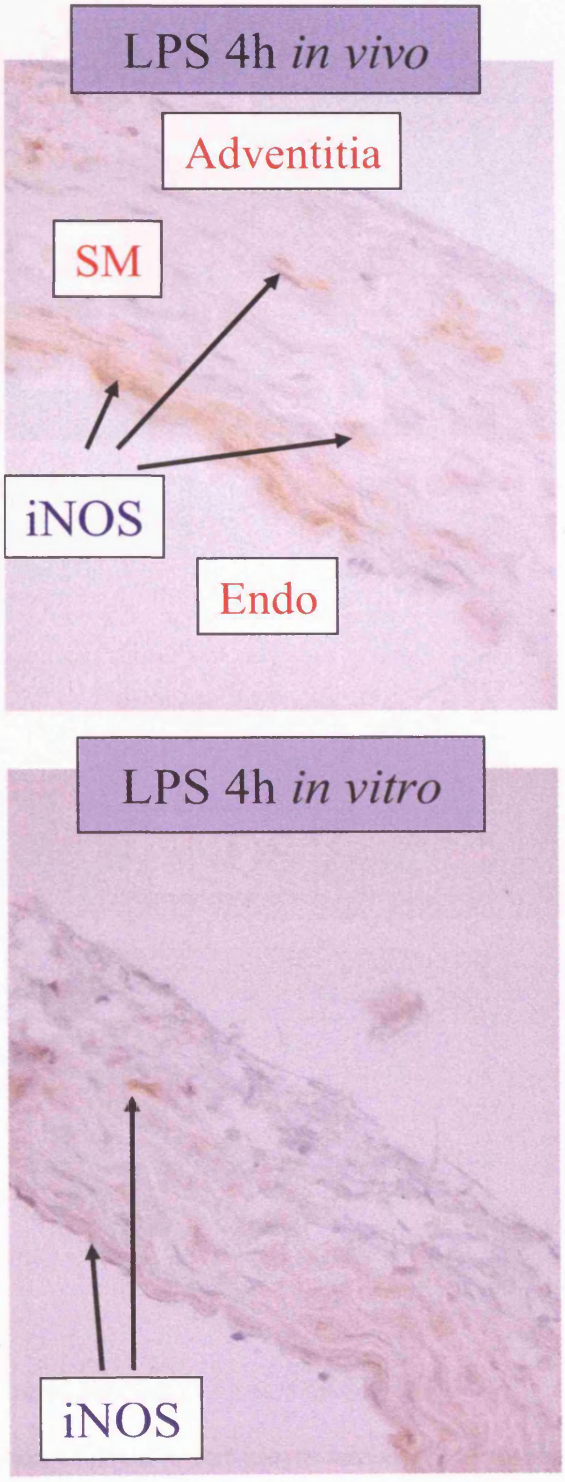


Figure 3.3 Comparison of the distribution of iNOS within RMA following either *in vitro* incubation with LPS for 4 h or 4 h following a bolus dose *in vivo* (n=3 for *in vitro* and 1 for *in vivo*). (Endo – endothelium; SM – smooth muscle).

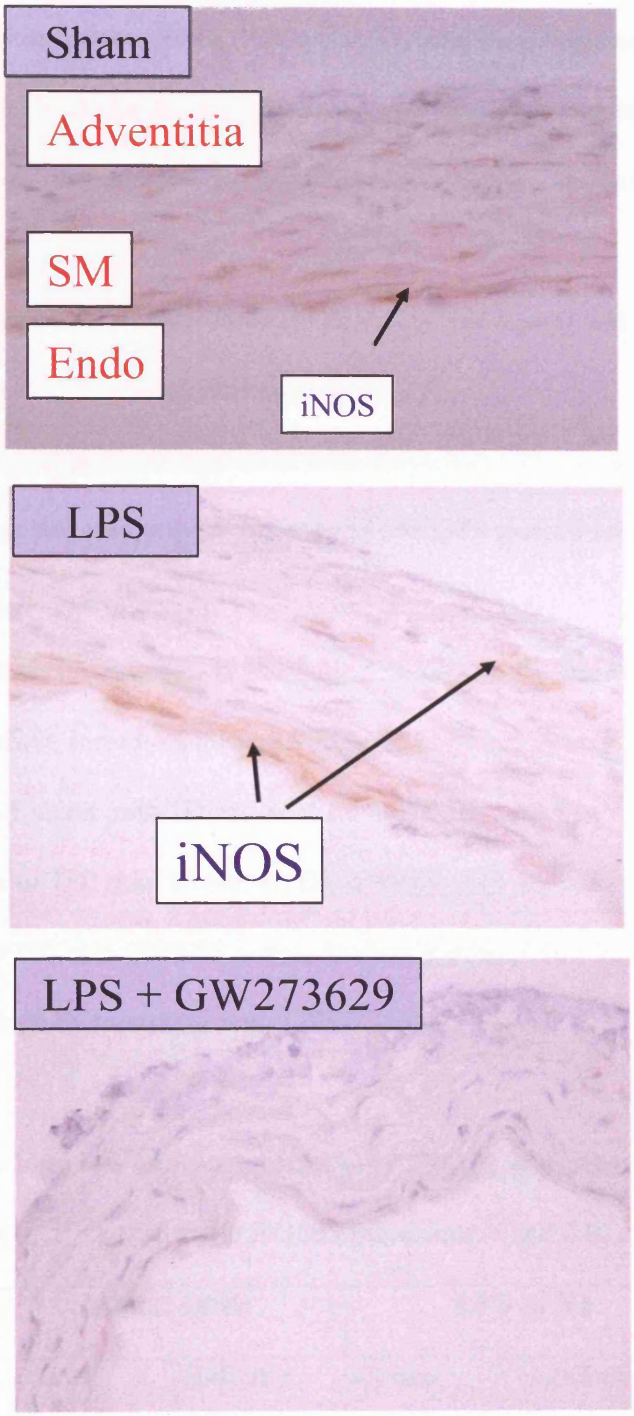


Figure 3.4 Distribution of iNOS within mesenteric artery removed after the *in vivo* experiment at 4 h from sham and LPS-treated animals ± the iNOS inhibitor GW273629 (n=1 for each). (Endo - endothelium ; SM - smooth muscle).

3.5.4 Effect of LPS upon haemoglobin, PaO_2, $PaCO_2$ and metabolic acidosis

(i) Haemoglobin: In both sham and LPS treated rats the haemoglobin dropped significantly over the course of the experiment (P <0.001, Student's t-test, n=6; Table 3.2).

(ii) PaO_2: There was no difference between arterial pO_2 at time 0 and 240 min for either the sham or LPS-treated animals (Table 3.2).

(iii) $PaCO_2$: The arterial pCO_2 decreased in both sham and LPS treated rats over the study period, but this was only significant for the LPS treated animals (P<0.001, Student's t-test, n=6; Table 3.2).

(iv) Metabolic acidosis (base excess): The degree of acidaemia, as measured by arterial base deficit, increased throughout the experiment in both the LPS-treated rats and sham rats. There was no difference between sham and LPS values at 0 min or 180 min. However, the acidosis was worse in the LPS-treated rats at 120 and 240 min (P<0.05, t-test, Student's n=6; Table 3.2). This increase was not attenuated by treatment with GW2733629.

Table 3.2 Differences between haemoglobin, PaO_2, $PaCO_2$ and arterial base excess at time 0 and 240 min (* P <0.001 and # P<0.05 when time 0 and 240 min compared)

	Sham (n=6)		**LPS (n=6)**	
	0 min	240 min	0 min	240 min
Haemoglobin (g dl^{-1})	15.9±0.41	13±0.28	16.47±0.77	11.25±0.22
PaO_2 (kPa)	11.26±0.29	12.17±0.78	10.96±0.37	12.24±0.99
$PaCO_2$ (kPa)	5.27±0.09	4.77±0.25	5.41±0.1*	4.03±0.22*
Base Excess	-4.43±0.94	-6.73±1.13	-3.35±0.45#	-11±1.45#

3.6 Discussion

I have developed a reproducible short-term *in vivo* rat model of septic shock with monitoring of both macro- and microcirculation by adapting the model used previously by our group (Rosser *et al.*, 1995). I have used this model to investigate the effect of iNOS inhibition upon mean arterial blood pressure and regional blood flow. A true infectious insult such as caecal ligation and puncture would better represent sepsis seen in humans rather than a 30 min bolus of LPS. Certainly, iNOS protein and activity is detected in such rats at later time points than in LPS models; for instance Sheih and co-workers found that thoracic aorta and lung iNOS protein increased by 6 h and remained elevated for 48 h (Sheih *et al.*, 2000). I also did not administer antibiotics, which is an integral part of management of sepsis in humans. However, this model did generate a critically ill animal with highly reproducible hypotension, disruption of both macro and micro-circulatory blood flow and metabolic acidosis. Thus I believe the model has merit and was highly suitable to investigate the effects of iNOS inhibition *in vivo*.

LPS infusion induced the characteristic early response, presumably related to myocardial depression, of a fall in blood pressure that spontaneously recovers. Although in my model MAP was restored within 15 minutes, aortic blood flow took much longer to recover, requiring major intravascular volume resuscitation with a colloid solution. Despite the fluid infusion, renal blood flow never regained baseline values. The hepatic microvascular circulation, as assessed by $HmvO_2$, an indicator of microvascular perfusion, failed to show significant improvement despite fluid resuscitation. This indicates persistence of microcirculatory defects, possibly related to local shunting.

Local tissue hypoxia, metabolic alterations such as increased Na/K ATPase activity (Levy *et al.*, 2005) and, possibly, increased anaerobic respiration are likely to account for the increased metabolic acidosis seen in the LPS-treated rats compared to the shams. Immunohistochemistry revealed a greater production of iNOS in the LPS-treated rat compared to the sham animal in the mesenteric artery removed at the end of the experiment. The amount and distribution of iNOS detected was similar to that seen *in vitro* following 4 h incubation with LPS. At this early time point the endothelial and smooth muscle layer produce iNOS rather than the adventitial layer which becomes the major source of iNOS *in vitro* which occurs after 20 h (see chapter 2).

Administration at 120 min of the iNOS inhibitor GW273629 succeeded in elevating blood pressure in the LPS and sham-treated rats, but was more pronounced in LPS-treated rats. Despite the improvement in blood pressure, there was no improvement in the macro or microcirculatory blood flow and no attenuation of the metabolic acidosis. Immunohistochemistry revealed that GW273629 completely attenuated the production of iNOS in RMA, an unexpected finding for an iNOS inhibitor.

As described previously, overproduction of nitric oxide is considered to play a key role in the pathogenesis of septic shock. Although an upregulation of constitutive NOS has been demonstrated in early sepsis (Salvemini *et al.*, 1990), the major route of NO production in sepsis is considered to be via induction of iNOS by specific pro-inflammatory stimulants including endotoxin, tumour necrosis factor, interferon-gamma and interleukin-1. The mechanisms by which NO causes vasodilatation, hypotension

and vascular hyporeactivity in sepsis are not fully elucidated but are thought to primarily involve activation of soluble guanylyl cyclase and increased production of guanosine 3'5'-cyclic monophosphate (cGMP). Overall benefits have been observed with inhibitors selective for iNOS or with low doses of non-selective inhibitors, whereas high doses of non-selective NOS inhibitors have been detrimental (reviewed in Vallance and Charles, 1998 and Vincent *et al.*, 2000). We chose to use GW273629, which is considered highly selective for iNOS (Young *et al.*, 2000 and Alderton *et al.*, 2005). This produced a marked rise in blood pressure when given 2 h following LPS and the response was significantly greater than that seen in sham rats. The lack of any decrease in aortic blood flow implies improved ventricular contractility to compensate for the increase in afterload. Indeed NO-induced myocardial depression has been previously described (Kumar *et al.*, 1999). However, despite this rise in blood pressure, there was no improvement in renal blood flow, hepatic microvascular oxygenation nor metabolic acidosis. This study clearly offers further evidence for the role of iNOS in the hypotension induced by sepsis, though iNOS inhibition does not appear to improve the abnormalities seen in regional blood flow.

Although treatment with GW273629 had no effect upon renal blood flow, renal effects of NO inhibition extend beyond its effect upon arterial pressure and renal perfusion pressure. Selective NO inhibition increases arterial pressure without affecting renal function (Schwartz *et al.*, 1997). It has been demonstrated that high levels of iNOS-generated NO could lead to renal vasoconstriction and a reduction in glomerular filtration rate (GFR) by inhibiting endothelial NO synthase activity (Schwartz *et al.*,

2001). In addition, NO scavenging inhibits the increase in medullary perfusion, thus maintaining glomerular hydrostatic pressure and the GFR (Millar and Thiemermann, 2002).

The microcirculation consists of the smallest blood vessels – arterioles, capillaries and venules (<100 μM diameter) in which oxygen release to tissues takes place. In sepsis, the heterogenous expression of iNOS in different areas of the same organ beds results in pathological shunting of flow (Revelly *et al.*, 1996 and Morin *et al.*, 1998). Furthermore, the smooth muscle cells lining the arterioles lose their adrenergic sensitivity and tone (Baker and Willmoth, 1984). Thus tissue oxygenation is severely impaired which is a major contributor to the development of multi-organ dysfunction. Unlike the studies of Siegemund or Pittner, I could not demonstrate any improvement in microcirculatory flow with fluid resuscitation ± iNOS inhibition (Pittner *et al.*, 2003 and Siegemund *et al.*, 2005). Perhaps the dose of LPS used is too 'toxic' to the rat; had I used a smaller dose an improvement may have been observed. Certainly, the discrepancy between flow and pressure is well illustrated in my studies.

Immunohistochemistry confirmed the presence of iNOS in both LPS and shams, with a greater production clearly seen following LPS. In addition, no iNOS was seen in the LPS-treated rat mesenteric artery following administration of GW273629. Interestingly, this would suggest that the drug not only inhibits iNOS but also inhibits its production. This is in contrast to studies using other iNOS inhibitors where iNOS protein was increased (Aulak *et al.*, 2004 and Frost *et al.*, 2005). This may reflect an effect of

GW273629 on the stain itself rather than the agent inhibiting iNOS protein production. As I only performed the staining on 1 occasion it is difficult to make an accurate comment. iNOS seen in the shams probably reflected induction of iNOS following instrumentation and this is supported by the small increase in blood pressure seen in shams following administration of GW273629.

A lesser finding was that the haemoglobin fell to a greater degree in the LPS-treated rats compared to the shams. Clearly there is a dilutional effect related to the large volume of fluid infused as resuscitation. The greater difference in the LPS animals may possibly be related a vasodilatation-related increase in intravascular volume and a decreased renal excretion ability that is not offset by any capillary leak.

My results suggest that overproduction of nitric oxide is heavily implicated in the development of hypotension in septic shock. Inhibiting iNOS reversed the hypotension very effectively but there was a lack of any corresponding improvement in organ perfusion or its microcirculation. Interestingly, in a porcine model of sepsis, NO donors in combination with fluid resuscitation improved gut microcirculatory oxygenation and corrected gastric pCO_2 whereas fluid alone did not (Siegemund *et al.*, 2000). Perhaps future therapies will have multiple sites of action. For instance, combining an NO donor (to open the microcirculation and perfuse weak circulatory units) with an iNOS inhibitor (to reduce pathological shunting and redirect blood flow to recruit weak circulatory units) may prove beneficial. Activated protein C prevented LPS-induced hypotension in rats by inhibiting excessive production of NO (Isobe *et al.*, 2001). In

addition, it reduced leukocyte activation and release of reactive oxygen species (Yamaji *et al.*, 2005). These actions have also been shown to improve the microcirculation in septic animals (Iba *et al.*, 2005).

In summary, I have developed a model of sepsis that is reproducible and allows monitoring of macro and microcirculatory changes. I have demonstrated the induction of iNOS and its role in the development of LPS-induced hypotension. In later studies I investigated K_{ATP} channel inhibition and terlipressin using this model.

Chapter 4

Is the potassium channel an important mediator of endotoxin-induced vascular hyporeactivity or hypotension?

4.1 Introduction

Having developed *in vitro* and *in vivo* models of rat sepsis (see chapters 2 & 3), I proceeded to investigate the role of K^+ channels in mediating vascular hyporeactivity and hypotension with specific reference to the K_{ATP} channel.

The organ culture model of LPS-induced vascular hyporeactivity demonstrated that the degree of reversal with NOS inhibitors was time-dependent (chapter 2 & O'Brien *et al.*, 2001). Thus, NO derived directly from NOS activity appeared to fully account for hyporeactivity in the short term (6 h), but only partially in the long term (>20 h), suggesting involvement of additional mechanisms at the later time points. The full reversal of LPS-induced hyporeactivity seen with 'pre-treatment' of the iNOS inhibitor 1400W suggests a more complex picture possibly involving the formation of NO stores and/or irreversible damage due to prolonged iNOS activation. Using this model, I specifically investigated whether agents acting on the K_{ATP} channel could reverse the effects of LPS. I examined the effects of inhibitors of the sulphonylurea receptor (SUR) – glibenclamide, tolbutamide and PNU-9963, and inhibitors of the pore-forming subunit – $BaCl_2$ and PNU-37883A. There is a variation in arterial responses to glibenclamide in proximal or distal sections of rat mesenteric artery *in vitro* (Tolvanen *et al.*, 1998) so I took care to consider this.

To compliment my *in vitro* work I also investigated the effects of PNU-37883A and glibenclamide *in vivo*. The effects on hypotension, renal and aortic blood flow and microvascular oxygen tension were monitored as per chapter 3. Glibenclamide is

usually diluted in the solvent dimethyl sulfoxide (DMSO) but this has been shown to inhibit LPS-induced NO synthesis (Liang and Akaike, 1997). I thus managed to obtain a lyophilised preparation of glibenclamide, kindly donated by Hoechst. I used two different concentrations, 1mg kg^{-1} and 10mg kg^{-1} followed by a 5mg kg^{-1} per hour infusion for the duration of the experiment. I used an infusion and bolus for the higher concentration as the pressor effect of glibenclamide is transient *in vivo* when administered as a bolus (Landry & Oliver, 1992; Vanelli *et al.*, 1995; Vanelli *et al.*, 1997; Gardiner *et al.*, 1999 and Sorrentino *et al.*, 1999) despite its long half-life in plasma (Wu *et al.*, 1995).

BK_{Ca} channels are also activated by LPS (Taguchi et al., 1996; Chen *et al.*, 1999 and Yakubovich *et al.*, 2001) and this laboratory has previously shown that hyporeactivity is reversed using the non-selective K^+ channel inhibitor tetraethylammonium chloride (TEA^+) (Hall *et al.*, 1996). I thus compared the effects of TEA^+ with the K_{ATP} channel inhibitors.

4.2 Methods

4.2.1 *In vitro* model of LPS-induced vascular hyporeactivity

4.2.1.1 Tension experiments

The methods for the organ bath experiments are fully described in chapter 2.

I assessed endothelial function by monitoring relaxation to acetylcholine (5 μM) in rings precontracted with 1 μM PE. Cumulative concentration-response curves were constructed to PE (10^{-9} to 10^{-5} M) with increasing doses added at 5 min intervals at which time the contraction had reached a plateau. At the end of some experiments in which tissues had been incubated with LPS for 20 h, I added the thromboxane-A_2 mimetic, U44619 (9, 11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$; 10^{-7} M).

4.2.1.2 Experimental protocols

At least six mesenteric rings, taken from a minimum of three animals, were used in each experimental group.

(i) Effect of K_{ATP} channel inhibitors

Rings were incubated with and without LPS (1 μg ml^{-1}) for either 6 or 20 h. Various inhibitors of the K_{ATP} channel were added 25 min prior to the addition of the lowest dose of PE. The sulphonylurea receptor inhibitors, glibenclamide (10 μM), tolbutamide (1 mM) and PNU-99963 (1 μM; Cui *et al.*, 2003), or the pore-forming subunit inhibitors $BaCl_2$ (300 μM) and PNU-37883A (1 μM; Surah-Narwal *et al.*, 1999) were added to

separate organ baths containing tissues previously treated with LPS. In separate studies I used $BaCl_2$ 30 μM – a concentration that selectively inhibits inward rectifier K^+ channels (K_{IR}; IC_{50} 2 μM; Quayle & Nelson, 1995). As tolbutamide and PNU-99963 were dissolved in 100% dimethyl sulphoxide (DMSO), a potent free radical scavenger, and glibenclamide in 50% DMSO and 50% polyethylene glycol, the solvent mixture was added to control tissues (0.1%) to ascertain its effect on contractile function. In a separate series of experiments, $BaCl_2$ (300 μM) and PNU-37883A (1 μM) were added to fresh control tissues.

(ii) Effect of the K^+ channel inhibitor, TEA^+

Rings were incubated with and without LPS (1 μg ml^{-1}) for either 6 or 20 h. 25 min prior to addition of PE the K^+ channel inhibitor, TEA^+ (1 or 10 mM) was added to those organ baths containing LPS-treated tissues. At a concentration of 1 mM, TEA^+ is considered to be a relatively specific inhibitor of BK_{Ca}, channels, while at 10 mM, it blocks other K^+ channels including the K_{ATP} channel, for which it has an IC_{50} of 7 mM (Nelson & Quayle, 1995).

(iii) Effect of a combination of K^+ channel inhibitors

Rings were incubated with and without LPS (1 μg ml^{-1}) for 20 h. 25 min prior to addition of PE, TEA^+ (1 mM) and $BaCl_2$ (300 μM) were added in combination to the organ baths containing LPS-treated tissues.

(iv) Temporal effect of LPS on contractile responses to KCl

Is the K^+ channel an important mediator of LPS-induced vascular hyporeactivity or hypotension

Rings were incubated with or without LPS (1 μg ml^{-1}) for either 6 or 20 h and contracted with PSS containing 60 mM K^+ was added to the organ baths. In this solution KCl replaced NaCl in PSS with the concentration of all other salts remaining the same.

(v) Addition of the thromboxane A_2 mimetic U44619

At the end of some experiments following a 20 h incubation with LPS, the thromboxane-A_2 mimetic, U44619 (10^{-7} M) was added to those tissues previously treated with K_{ATP} inhibitors. Glibenclamide and tolbutamide are known to have antagonist effects at thromboxane receptors (Delaey & Van de Voorde, 1995) which would be predicted to worsen hyporeactivity. I wished to investigate whether PNU-99963 affected thromboxane-induced contractions.

(vi) Concentration-response curves to the NO donor, SNAP in fresh control tissue

Cumulative concentration-relaxation curves to S–nitroso–N–acetyl–D, L-penicillamine (SNAP; 10^{-10} to 10^{-5} M) were constructed in fresh tissues following precontraction with 1 μM PE. 25 min prior to addition of PE, the K^+ channel inhibitors - TEA^+ (1 and 10 mM), $BaCl_2$ (300 μM) and PNU-37738A (1 μM) were added to separate rings. Once contractions to PE had reached a plateau, concentrations of SNAP were added in cumulative fashion every 5 min beforehand.

4.2.1.3 Nitrite production in organ culture model

The method is as described in chapter 2. Tissues were incubated for 20 h in the presence or absence of LPS, with or without the addition of either (i) the sulphonylurea K_{ATP} receptor inhibitors, glibenclamide (10 μM), tolbutamide (1 mM) and PNU-99963 (1 μM) or (ii) the pore-forming subunit inhibitors $BaCl_2$ (300 μM) and PNU-37883A (1 μM). Nitrite was measured as previously described in chapter 2.

4.2.2 *In vivo* fluid resuscitated rat model of endotoxin induced shock

The method and model are described in detail in chapter 3.

Five experimental groups were used:

(a) Group A (n=6) was given 40 mg kg^{-1} LPS *(Klebsiella Pneumoniae)* administered as 0.9% saline infused over a 30 min period at a rate of 20 ml kg^{-1} h^{-1}. Throughout the experiment, animals were fluid resuscitated with 25 ml kg^{-1} h^{-1} of the synthetic gelatin colloid, Gelofusin®. This was supplemented with 1 ml 10% glucose solution per 25 ml to keep blood sugars > 5 mM.

(b) Group B (n=6) received isotonic saline rather than LPS over the first 30 min (sham-operated controls).

(c) Group C comprised either LPS-treated rats (n=6) or sham-operated controls (n = 3). These rats were given glibenclamide as a 1 mg kg^{-1} bolus (low dose glibenclamide), while Group D (n=6) received a 10 mg kg^{-1} bolus of glibenclamide followed by an

infusion of 5 mg $kg^{-1}h^{-1}$ for the duration of the experiment (high dose glibenclamide). Glibenclamide was administered 120 min after the LPS infusion was initiated.

(d) Group E comprised either LPS-treated rats (n=6) or sham-operated controls (n=3). These rats were given the pore-forming subunit K_{ATP} channel inhibitor, PNU 37883A as a 1.5 mg kg^{-1} bolus followed by a 1 mg kg^{-1} h^{-1} infusion for the duration of the experiment. No previous data existed for the use of this agent in septic models. Thus the optimum dose for PNU 37883A was determined by giving 0.5 – 5 mg kg^{-1} boluses to a sham animal that had received the K_{ATP} channel opener levcromakalim (D'Alonzo *et al.*, 1995). A previous *in vivo* study in rabbit had used 3 mg kg^{-1} to inhibit levcromakalim responses (Meisheri *et al.*, 1993). I found that 1.5 mg kg^{-1} had the optimum effect *i.e.* full reversal of hypotension induced by levcromakalim. PNU 37883A was administered 120 min after the LPS infusion was initiated.

4.2.3 Case Report: Effect of glibenclamide in norepinephrine-resistant septic shock in a single patient on an intensive care unit

I gave a single dose of 1 mg kg^{-1} of intravenous glibenclamide over 5 min to a critically ill patient in severe septic shock not responding to high doses of the catecholamine vasopressor norepinephrine. Furthermore the hypotension remained despite treatment with corticosteroids or methylene blue (guanylate cyclase inhibitor). Prior to the study approval had been obtained from the University College London Hospitals (UCLH) Ethics Committee and agreement sought from the patient's next-of-kin. The patient was intubated and ventilated. Cardiac output and fluid loading were assessed by

transoesophageal Doppler ultrasound. Mean arterial blood pressure (MAP), norepinephrine requirements, central venous pressure, oxygen saturations, heart rate, urine output and cardiac output were monitored continuously and arterial blood gases were sampled.

4.3 Statistical analysis

As indicated in chapter 2.

4.4 Reagents and solutions

Aventis Pharma (Kent, U.K.) kindly donated the glibenclamide used in the in vivo model and in the human patient. Glibenclamide (for *in vitro* experiments), tetraethyl ammonium chloride, barium chloride and tolbutamide were all obtained from Sigma Chemical Company (Poole, Dorset, U.K.). PNU 99963 and PNU 37883A were kindly donated by Pharmacia-Upjohn (Kalamazoo, U.S.A.). In later studies PNU 37738A was obtained from Affiniti-Research, (Exeter, U.K.). Glibenclamide, tolbutamide, PNU-99963 and PNU-37883A were dissolved in dimethylsulphoxide and diluted into PSS. The highest final concentration of DMSO used in experiments was 0.1%. The other reagents are described in chapter 2.

4.5 Results

4.5.1 Effect of K^+ channel inhibition in an *in vitro* rat model of sepsis

4.5.1.1 Effect of endotoxin on the contractile responses to phenylephrine

Cumulative concentration-response curves to PE in rat mesenteric arterial rings incubated in the absence and presence of 1 μg ml^{-1} LPS are shown in Figure 4.1. In the presence of LPS, marked hyporeactivity to PE was observed at 6 h ($P < 0.001$, n=14) and 20 h ($P < 0.001$, n=17). In addition, LPS caused a rightward shift of the pEC_{50} value at 6 h which was associated with a marked reduction in the maximal contraction (E_{Max}) to 10^{-5} M PE ($P < 0.001$; Table 4.1). An accurate pEC_{50} value could not be calculated at 20 h as the contractile responses to PE in the presence of LPS were too small. The magnitude of the relaxation induced by acetylcholine in control tissues did not alter with time in culture medium, being 80.6 ± 0.2%, n=13 *versus* 79.0 ± 9.7, n=8, at 6 & 20 h, respectively.

4.5.1.2 Effect of K_{ATP} channel inhibitors on LPS-induced vascular hyporeactivity

The SUR inhibitors, glibenclamide (10 μM), tolbutamide (1 mM) and PNU-99963 (1 μM) all failed to reverse LPS-induced vascular hyporeactivity either at 6 or 20 h (Fig. 4.1 and Table 4.1). Indeed, glibenclamide further reduced PE contractions at 6 h (P = 0.002 compared to LPS alone). Lack of reversal of PE contraction with the SUR inhibitors could not be attributable to solvent effects since DMSO (0.1%) failed to significantly alter hyporeactivity to PE induced by LPS at 20 h (P = 0.34). The

maximum contraction in the presence of DMSO was 0.23 ± 0.01 g, n=4 compared to 0.13 ± 0.05, n=17 with LPS alone.

To investigate the effect of inhibiting the pore-forming subunit of the K_{ATP} channel, we used and PNU-37883A (1 μM). At 6 h, $BaCl_2$ fully reversed LPS-induced hyporeactivity while PNU-37883A produced a 75% reversal (P = 0.035; n=8) (Fig. 4.2A & Table 4.1). In contrast, no reversal was seen with 30 μM $BaCl_2$ (Fig. 4.2A), a concentration sufficient to inhibit strong inwardly rectifying K^+ (Kir) channels (Nelson & Quayle, 1995), suggesting these channels do not contribute to LPS-induced hyporeactivity. At 20 h incubation with LPS, less reversal was achieved with either $BaCl_2$ or PNU-37883A with both inhibitors only producing a 50 % reversal (Fig. 4.2B). To rule out the possibility that pore-inhibitors blocked basal K_{ATP} channel activity, thereby increasing the sensitivity to PE independently of LPS, concentration-response curves to PE were performed in control tissues that had previously been incubated in culture medium for 6 h. In the absence and presence of PNU-37883A, dose-response curves for PE were essentially super imposable, with pEC_{50} values being 7.03 ± 0.07 and 7.22 ± 0.03, respectively (P = 0.11; n=7). Similarly, $BaCl_2$ did not affect E_{Max} (P = 0.71) although it caused a small, but significant ($P < 0.05$) increase in the pEC_{50} value (7.34 ± 0.13; n=4) compared to control.

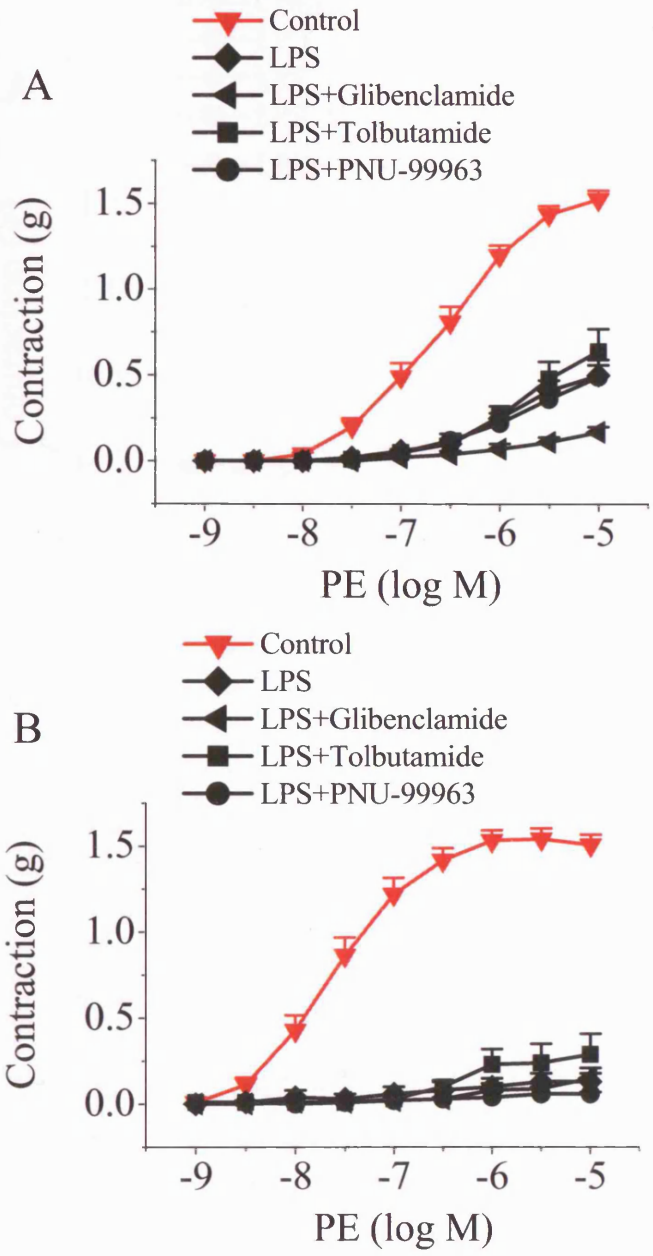


Figure 4.1 Effect of the SUR K_{ATP} inhibitors glibenclamide (10 μM), tolbutamide (1 mM) and PNU-99963 (1 μM) upon LPS-induced hyporeactivity at (A) 6 & (B) 20 h. All failed to reverse hyporeactivity, indeed, glibenclamide further reduced PE contractions at 6 h (*P* = 0.002 compared to LPS alone). Data are expressed as the mean ± s.e.m. of 8-10 observations from 10-12 rats.

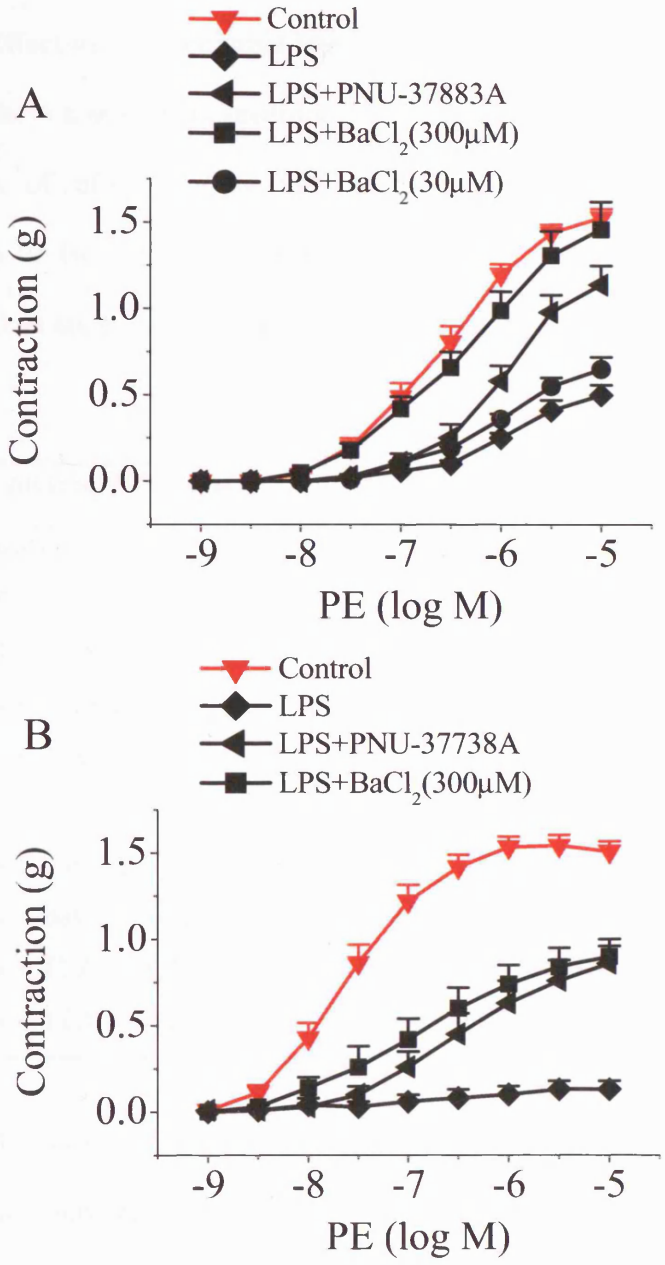


Figure 4.2 Effect of the pore-forming subunit K_{ATP} channel inhibitors, $BaCl_2$ (300 μM) and PNU-37883A (1 μM) upon LPS-induced hyporeactivity at 6 (A) & (B) 20 h. At 6h, $BaCl_2$ fully reversed hyporeactivity and PNU-37883A produced 75% reversal ($P = 0.035$). At 20h both produced partial reversal ($P<0.05$). $BaCl_2$ (30 μM) had no effect. Data are expressed as the mean ± s.e.m. of 8-10 observations from 10-12 rats.

Table 4.1 Effect of K_{ATP} channel blockers on phenylephrine contractions in tissues incubated for 6 h in culture medium ± LPS

Comparison of pEC_{50} values and maximal contraction (E_{Max}) obtained against contractions to 10^{-5} M PE in the absence (control) and presence of LPS ± channel inhibitors. Data are expressed as mean ± s.e.m. of n observations.

Rat mesenteric artery	pEC_{50}	E_{Max} (g)	*n*
Control	6.53 ± 0.10	1.53 ± 0.05	18
LPS	5.71 ± 0.18**	0.50 ± 0.05**	14
LPS + Glibenclamide (10 µM)	5.16 ± 0.38**	0.17 ± 0.03** #	10
LPS + Tolbutamide (1 mM)	5.99 ± 0.08	0.64 ± 0.13**	8
LPS + PNU-99963 (1 µM)	5.80 ± 0.13*	0.49 ± 0.10**	6
LPS + PNU-37883A (1 µM)	6.01 ± 0.12	1.14 ± 0.11* #	6
LPS + $BaCl_2$ (30 µM)	6.03 ± 0.05	0.65 ± 0.07**	6
LPS + $BaCl_2$ (300 µM)	6.41 ± 0.07	1.46 ± 0.16#	8
LPS + TEA^+ (1mM)	6.13 ± 0.21	0.94 ± 0.08* #	8
LPS + TEA^+ (10mM)	6.43 ± 0.22	1.36 ± 0.21	6

* = P <0.05, ** = P< 0.001 when compared to control, # = P < 0.05 when compared to LPS alone

4.5.1.3 Effect of tetraethylammonium chloride

I investigated the effects of TEA^+ - a relatively selective inhibitor of BK_{Ca} channels at 1mM and a non-selective inhibitor of K^+ channels at 10 mM (Nelson and Quayle, 1995). At 6 h, TEA^+ 1 mM produced a partial reversal of LPS-induced hyporeactivity reducing (P=0.029) E_{Max} to 0.94 ± 0.08g (n=8) from -1.53 ± 0.05g (n=18) (Fig. 4.4A &

Table 4.1). Increasing the concentration of TEA^+ to 10 mM produced almost complete reversal of LPS-induced hyporeactivity (Fig. 4.5.2.C) ($P<0.001$, $n=8$).

At 20 h, TEA^+ at 1mM caused a partial reversal of LPS-induced hyporeactivity but only when concentration-response curves of the peak contractions were compared (Figs 4.3 & 4.4B) ($P<0.05$, max contractions 0.81 ± 0.23g, $n=8$ c.f. control tissues max contractions 1.53 ± 0.05, $n=18$). In the presence of TEA, contractions were transient, unlike the contractions obtained in the presence of either PNU-37738A (Fig 4.3B) or $BaCl_2$ which were sustained.

4.5.1.4 Effect of inhibiting K^+ channels using a combination of $BaCl_2$ and TEA^+

The combination of $BaCl_2$ (300 μM) and TEA^+ (1mM) produced no greater peak contractions to PE (Fig 4.4B and Table 4.1). However, the pEC_{50} was significantly (P = 0.034) shifted to the left (6.9 ± 0.18 with $BaCl_2$ + TEA^+, $n=8$ compared to 5.79 ± 0.48 for $BaCl_2$ alone, $n=6$).

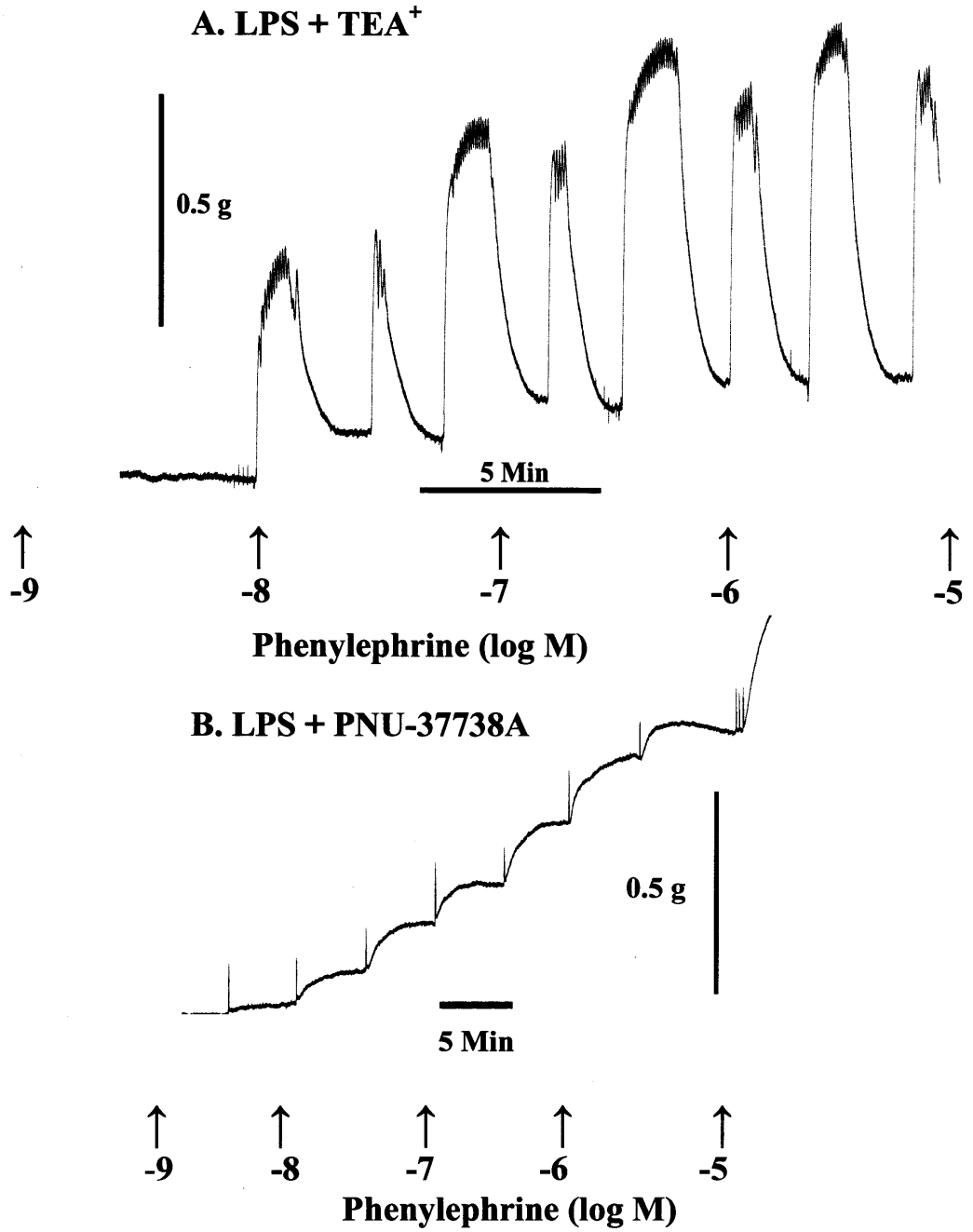


Figure 4.3 A comparison of concentration response traces obtained to PE in LPS tissues (incubated for 20 h) treated with (A) TEA^+ (1 mM) and (B) PNU-37883A (1 μM). The contractions seen in the presence of TEA^+ are transient compared to those in the PNU-37883A tissues.

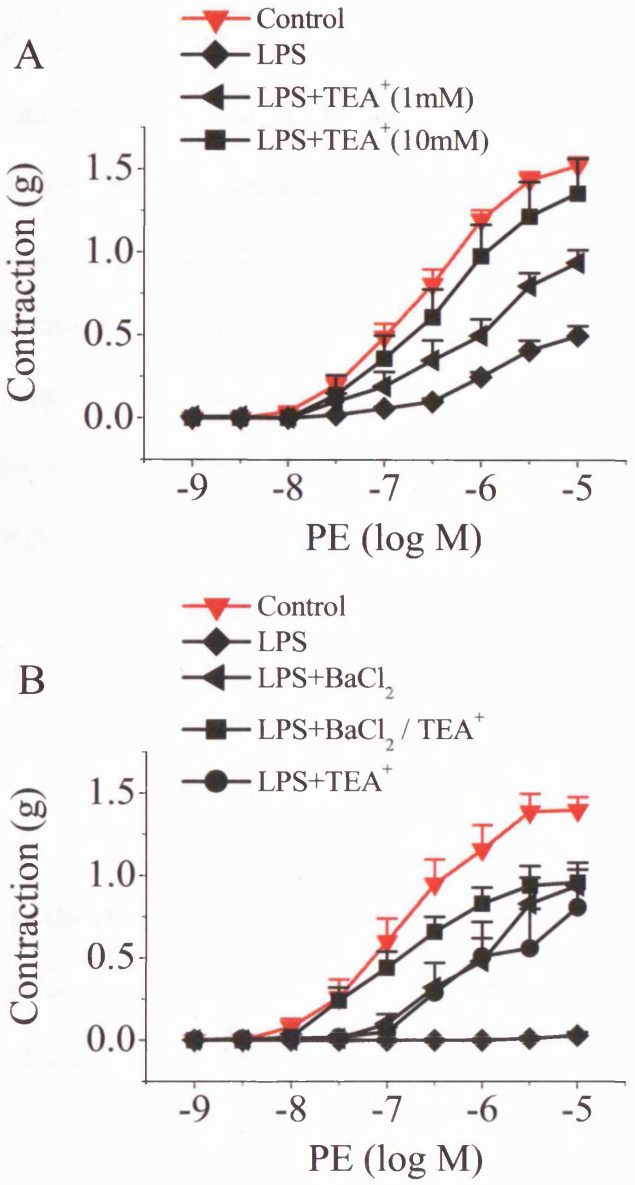


Figure 4.4 Effect of TEA^+ (1& 10mM) upon LPS-induced hyporeactivity at (A) 6 and (B) 20 h and a combination of $BaCl_2$ (300μM) and TEA^+ (1mM) at 20 h. At 6 h, 1 mM TEA^+ produced partial ($P = 0.029$) while TEA^+ at 10 mM produced almost complete reversal. At 20h TEA^+ produced some reversal but contractions were non-sustained. The combination of $BaCl_2$ / TEA^+ produced similar reversal to $BaCl_2$ alone. Data are expressed as the mean ± s.e.m. of 6-8 observations from 8-10 rats.

4.5.1.5 Effect of LPS on contractions to 60 mM KCl

The degree of reversal of LPS-induced hyporeactivity with the K^+ channel inhibitors suggests that the mechanism of relaxation predominately involves opening of K^+ channels at 6 h, but additional pathways at 20 h. To test this, we investigated the effect of LPS on contractions induced by 60 mM KCl, where relaxation cannot be promoted by K^+ channel opening due to a lack of transmembrane driving force on K^+ ions (Gurney & Clapp, 1994). Whereas a 6 h incubation with LPS did not significantly reduce contractions to 60 mM KCl (P=0.102, n=16), the contractile response was significantly depressed (~40%) at 20 h (P<0.001, n=10; Fig. 4.5). The lack of effect of LPS on KCl contractions at 6 h is consistent with a predominately K^+ channel mechanism at this time point, while additional mechanisms are likely to contribute at 20 h.

4.5.1.6 Effect of the thromboxane A_2 mimetic U-46619 on contractile responses

Sepsis up-regulates the production of thromboxane (Bernard *et al.*, 1997) although LPS only weakly affected contractile responses to thromboxane agonists in our organ bath model (O'Brien *et al.*, 2001; see chapter 2). Competitive antagonism of thromboxane receptors by the SUR inhibitors glibenclamide and tolbutamide (Cocks *et al.*, 1990) may underlie their inability to reverse hyporeactivity. No data exist for the potent pinacidil-derived inhibitor, PNU-99963. We observed a significant contraction following addition of the thromboxane-A_2 mimetic, U-46619 (10^{-7} M) when applied to PE-hyporeactive tissues previously incubated with LPS for 20 h (Fig. 4.6A & B). While tissues pre-treated with either glibenclamide (10 μM) or tolbutamide (1 mM) failed to

contract to U-46619, a large contraction was observed in tissues pretreated with PNU-99963 (1 μM). In rings not exposed to LPS, U-46619 only caused a small contraction (from 1.48 ± 0.06g to 1.78 ± 0.17g; n=5), presumably because the contractile response to PE at 10^{-5} M was close to the maximum tension the tissue could generate.

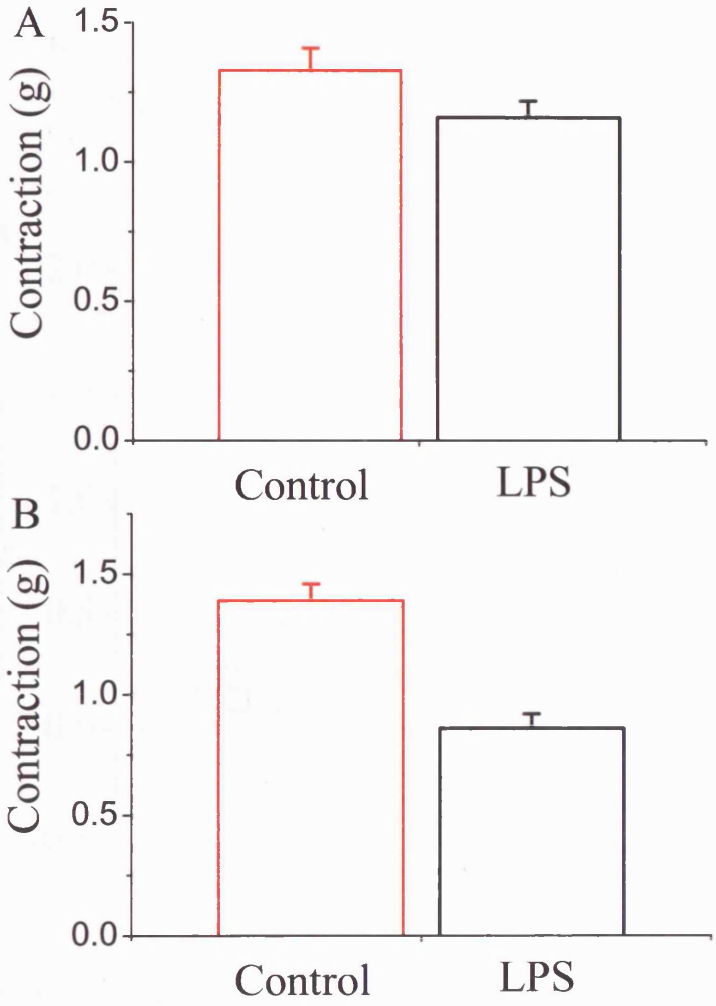


Figure 4.5 LPS (1μg ml^{-1}) induces hyporeactivity to contractions with 60 mM KCL following (B) 20 h incubation ($P < 0.001$) but not (A) 6 h. Data are expressed as the mean ± s.e.m of 10-16 observations from 6-8 animals

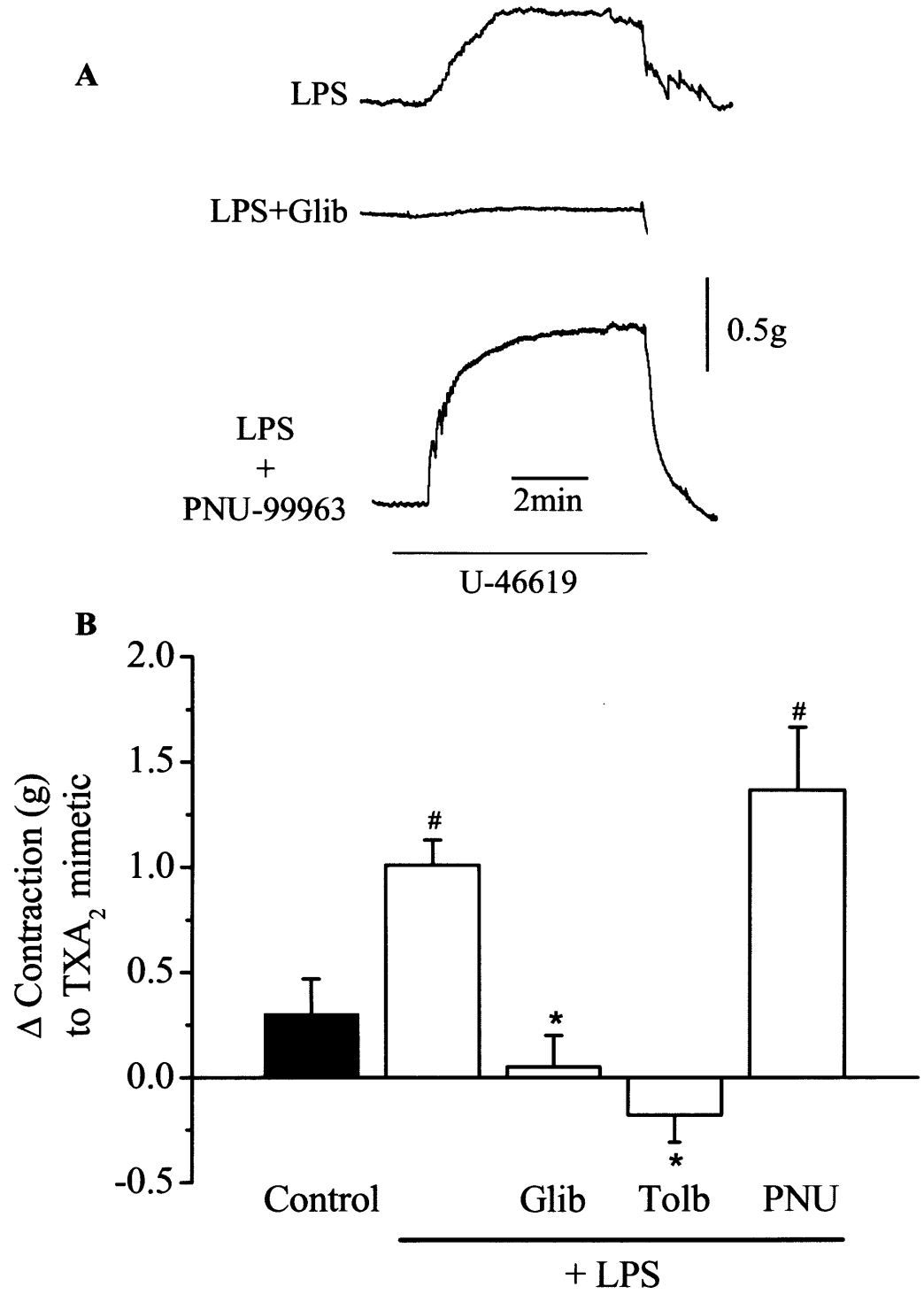


Figure 4.6 Addition of the thromboxane agonist U-44619 (10^{-7} M) at the end of concentration response curves to PE (A). LPS treated tissues incubated with tolbutamide (Tolb, 1mM) & glibenclamide (Glib, 10 μM) failed to contract compared to LPS treated tissues with PNU99963 (1 μM). *=$P < 0.001$ compared to LPS alone and #= $P<0.05$ compared to control). Data are expressed as mean ± s.e.m of 6 observations from 8 rats.

4.5.1.7 Effect of K_{ATP} channel inhibitors on LPS-induced nitrite accumulation

I demonstrated in chapter 2 the contribution of the iNOS / NO pathway to LPS induced hyporeactivity in an organ culture model. Having achieved reversal of vascular hyporeactivity using K^+ inhibitors, I needed to confirm that these agents had no effect upon NO production itself. I thus investigated whether the K_{ATP} inhibitors inhibited nitrite production in the culture medium supernatant. Nitrite (NO_2^-), a metabolite of NO, is a marker of NO production (Thiemermann, 1997). Compared to control supernatant of rat mesenteric rings, NO_2^- levels doubled in the presence of LPS (1 μg ml^{-1}) after a 20 h co-incubation ($P < 0.001$; Fig. 4.7). This rise was completely inhibited by the specific iNOS inhibitor, 1400W (10 μM) and by 70% with tolbutamide (1 mM) (Fig 4.7). Neither glibenclamide (10 μM), PNU-37883A (1 μM) nor $BaCl_2$ (300 μM) significantly affected LPS-induced nitrite production at the concentrations used in the tension studies (Fig. 4.7), although there was a trend to lower nitrite production in the presence of $BaCl_2$.

4.5.1.8 Effect of K_{ATP} inhibitors on relaxation to the nitric oxide donor, SNAP

I performed these experiments to investigate whether relaxation of vascular tone by NO donor is inhibited by K channel blockade. NO has been shown to activate BK_{Ca} channels (Hohn *et al.*, 1996). Vanheel & Van de Voorde (1997) showed that NO did not activate K_{ATP} channels, however, Davie and co-workers (1997) and Murphy & Brayden, 1995) demonstrated that NO activates K_{ATP} channels in aorta and mesenteric artery. The effect of the pore-forming subunit inhibitors PNU-37883A (1 μM) and $BaCl_2$ (300 μM) were investigated in mesenteric rings mounted within an hour of being taken out of the

animal. Neither agent had a significant effect on the concentration-dependent relaxation of PE contractions (1 µM) induced by the NO donor, SNAP (Fig. 4.8). In contrast, the guanylyl cyclase inhibitor, ODQ (3 µM) fully inhibited relaxation to SNAP. In addition, TEA^+ (1 and 10 mM) significantly inhibited concentration-relaxation curves to SNAP ($P < 0.001$, Fig. 4.8) shifting the pEC_{50} to the right (see Table 4.2).

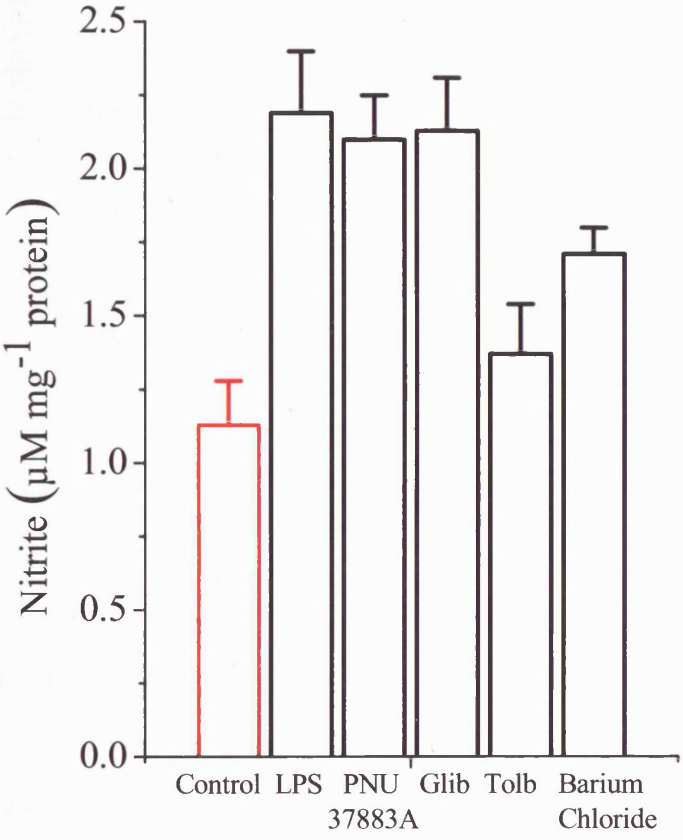


Figure 4.7 Rat mesenteric artery incubated with LPS (1µg ml^{-1}) for 20 h induces nitrite accumulation within culture media compared to control tissues ($P < 0.001$). This was inhibited by tolbutamide (Tolb 1mM, $P < 0.05$) but not by the other K_{ATP} channel inhibitors (PNU-37738A 1 µM, Glibenclamide – Glib 10 µM and $BaCl_2$ 300 µM). Data are expressed as the mean ± s.e.m of 6 observations from 5 animals.

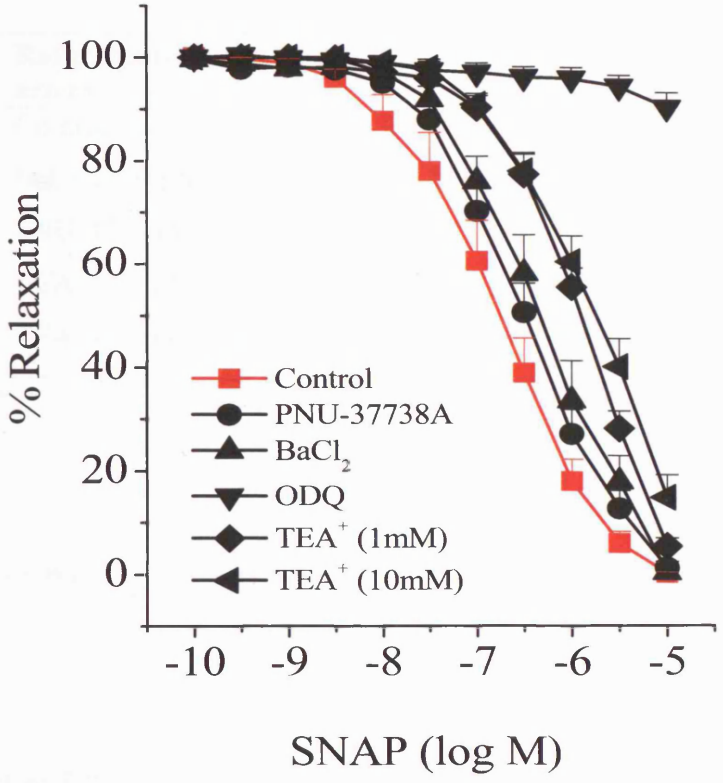


Figure 4.8 Effect of K_{ATP} inhibitors on concentration-relaxation curves to SNAP compared to the guanylyl cyclase inhibitor, ODQ (3μM). TEA^+ at 1 or 10 mM significantly inhibited relaxation to SNAP ($P < 0.001$ when compared to control). PNU-37738A (1μM), and $BaCl_2$ (300μM) had no effect. Data are expressed as mean ± s.e.m. of 6 observations from 6-8 animals.

Table 4.2 Effect of K^+ channel inhibitors on relaxation responses to SNAP in fresh rat mesenteric arteries precontracted with phenylephrine (10^{-6} M).

Data are expressed as mean ± s.e.m. of ≥ 6 observations.

Rat mesenteric artery	pEC_{50}	*n*
Control	6.76 ± 0.18	10
$BaCl_2$ (300 μM)	6.15 ± 0.23	6
PNU-37883A (1 μM)	6.42 ± 0.14	6
TEA^+ (1 mM)	5.73 ± 0.10*	6
TEA^+ (10mM)	5.41 ± 0.33*	7

* $P < 0.001$ compared to control

4.5.2 Effect of K_{ATP} channel inhibition in an *in vivo* rat model of endotoxaemic sepsis

4.5.2.1 Effect of LPS upon MAP, aortic and renal blood flow and microvascular oxygen tension

The baseline parameters and the effect of LPS are described fully in chapter 3. Briefly, in sham animals MAP, RBF, hepatic microvascular oxygenation ($HmvO_2$) and arterial base deficit did not change significantly throughout the experiment (Fig. 3.1). However, there was a tendency for in MAP and RBF to increase; a rise followed by a fall in ABF and $HmvO_2$ was also observed. These changes, particularly in ABF, were probably related to the fluid resuscitation. Following infusion of LPS, there was a rapid fall in all measured variables within 0-30 min (see Fig. 3.1). Recovery in MAP was observed

within 30 min (before fluid resuscitation was commenced), although aortic and renal blood flow took 2-3 h to return to baseline values. However, $HmvO_2$ failed to show any significant recovery after the initial abrupt fall. After 120 min the MAP fell significantly in LPS treated rats ($P < 0.001$, n=6, ANOVA) and as such this was the time at which I chose to administer the K_{ATP} inhibitors.

4.5.2.2 Effect of K_{ATP} channel inhibitors in LPS treated and sham-operated animals

I chose to use both a low (1 mg kg^{-1}) and high (10 mg kg^{-1} bolus and 5mg kg^{-1} h^{-1} infusion) dose of glibenclamide. Previous studies in rats have used 1mg kg^{-1} bolus (Wu *et al.*, 1995) and I found in the sham rats that a 1 mg kg^{-1} bolus of glibenclamide reversed hypotension induced by the K_{ATP} channel opener levcromakalim (3 µg kg^{-1}) by 70%. I chose to use a high dose of glibenclamide in addition as 10 mg kg^{-1} glibenclamide was required to produce complete reversal of levcromakalim-induced hypotension.

Low dose glibenclamide administered at 120 min had no discernible effect upon the measured parameters in either LPS-treated or sham rats (data not shown). In contrast, high dose glibenclamide resulted in a significant rise in MAP in both sham and LPS-treated rats ($P = 0.007$ and $P < 0.001$, n=6, ANOVA for shams and LPS respectively; Fig. 4.9 & 10). The intial δ rise in MAP seen (from 120 to 135 min) is similar in the LPS and sham treated tissues (≈20 mmHg compared to ≈18 mmHg) but the effect is more sustained in shams – until the end of the experiment (240 min) compared to 200

min with LPS rats. In LPS rats the RBF, ABF and arterial base deficit were unaffected by high dose glibenclamide treatment but there was a tendency to a reduction in $HmvO_2$ compared to untreated rats. In sham rats high dose glibenclamide worsened ABF and $HmvO_2$ compared to controls; the RBF and arterial base deficit appeared unaffected.

PNU-37883A (1.5mg kg^{-1} and 1mg kg^{-1} h^{-1} infusion) also resulted in a significant rise in MAP in both sham and LPS-treated rats ($P = 0.004$ and $P < 0.001$, n=6, ANOVA for shams and LPS respectively; Fig. 4.9 & 10). Here the rise in MAP is greater in shams (≈30 mmHg) compared to LPS-treated rats (≈15 mmHg). Again the effect is more sustained in shams where it lasts until 240 min compared to 200 min with LPS. In LPS-treated rats the RBF and $HmvO_2$ were unaffected by PNU-37883A, however there was a significant fall in the ABF and increase in acidaemia compared to LPS alone (arterial base excess: LPS -9.64 ± 0.41 and LPS + PNU-37738A -14.15 ± 1.11, $P = 0.031$, n=6, ANOVA). In shams PNU-37883A had no effect on the RBF and $HmvO_2$; however there was a significant fall in the ABF, similar to that observed in LPS treated animals and there was an increase in acidaemia.

4.5.3 Effect of an intravenous bolus dose of glibenclamide in a septic shock patient

An intravenous bolus dose of 10 mg of glibenclamide over 5 min had no effect on MAP or norepinephrine dose in 1 patient with streptococcal septicaemia and liver failure. No effect was seen on cardiac output, arterial base excess or blood glucose.

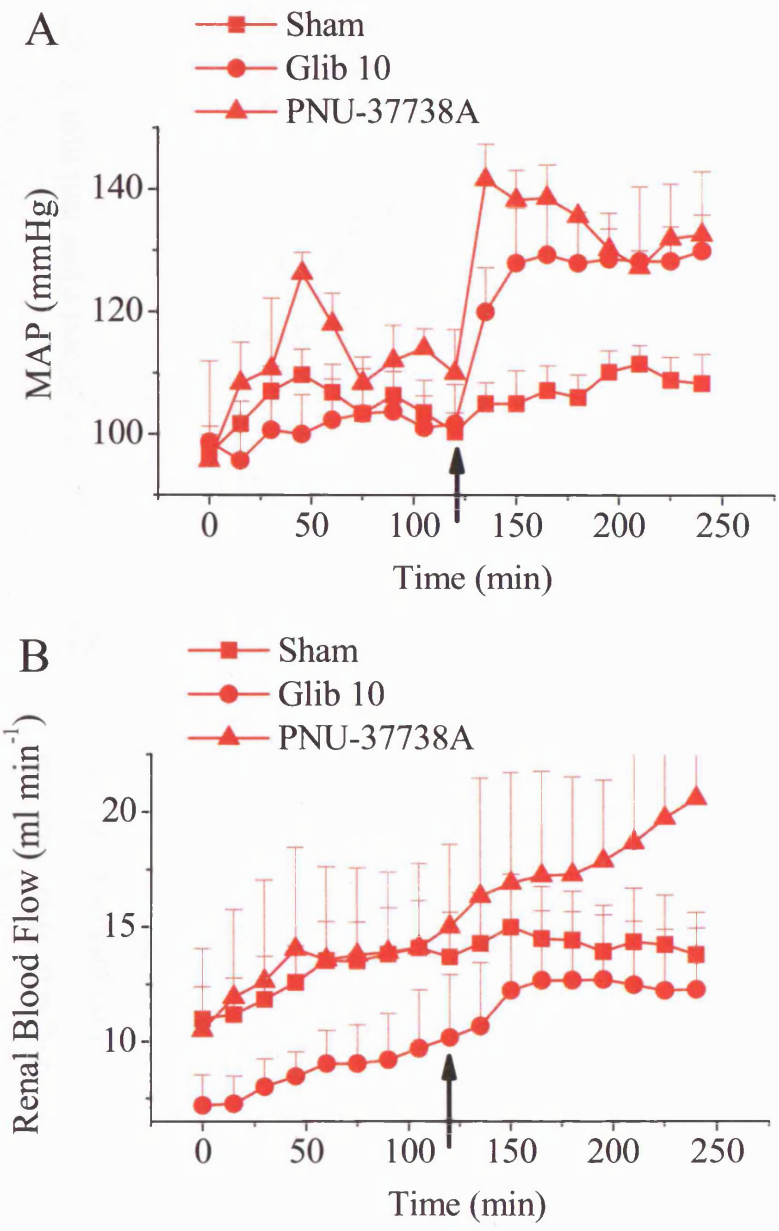


Figure 4.9 Effect of the K_{ATP} inhibitors glibenclamide (10 mg kg^{-1} bolus + 5 mg kg^{-1} infusion) and PNU-37883A (1.5 mg kg^{-1} bolus + 1 mg kg^{-1} infusion) given at 120 min (see arrow) on (A) mean arterial blood pressure and (B) renal blood flow in shams. High dose glib and PNU-37883A raised MAP (P<0.05, ANOVA). PNU-37883A did not raise renal blood flow significantly. Low dose glib had no effect (not shown). Data are expressed as mean ± s.e.m. from 3-6 rats.

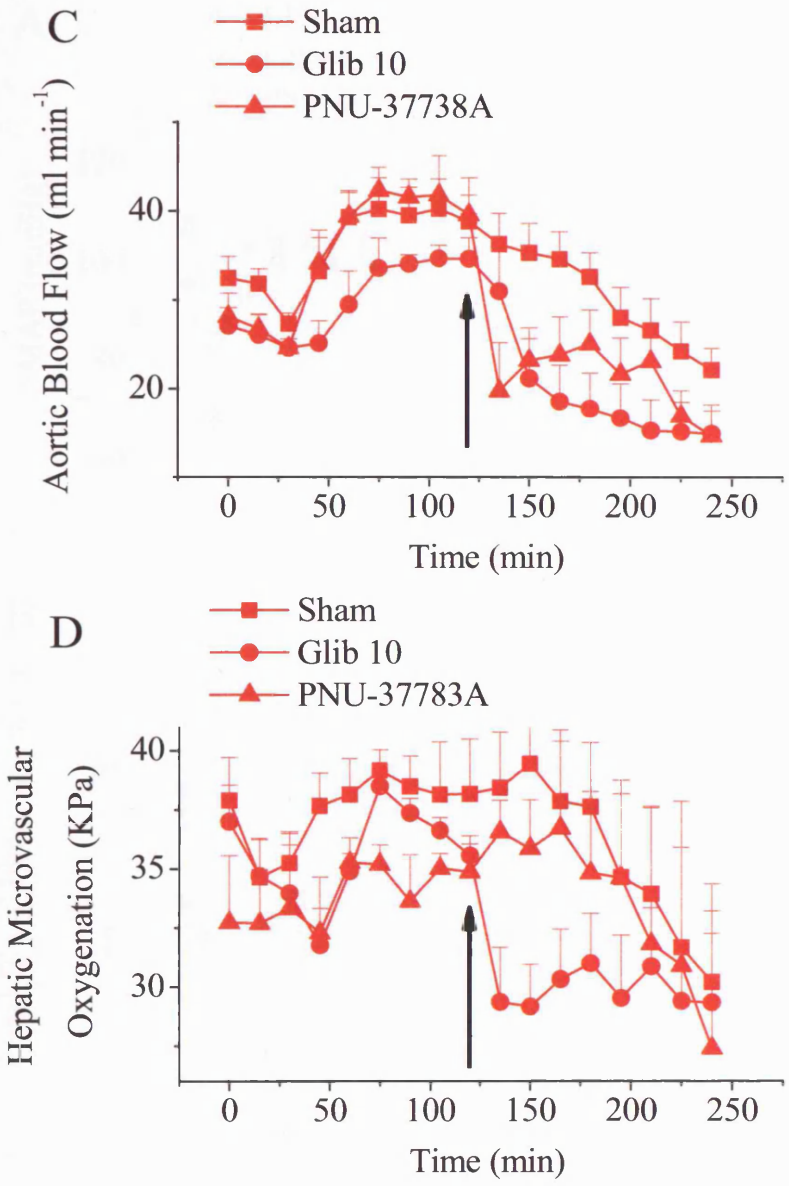


Figure 4.9 Effect of the K_{ATP} inhibitors glibenclamide (10 mg kg^{-1} bolus + 5 mg kg^{-1} infusion) and PNU-37883A (1.5 mg kg^{-1} bolus + 1 mg kg^{-1} infusion) given at 120 min (see arrow) on (C) aortic blood flow and (D) micro vascular oxygenation in sham rats. PNU-37883A and high dose glib reduced ABF significantly ($P<0.05$, ANOVA).Glib also reduced $HmvO_2$ significantly ($P<0.05$). Low dose glib had no effect (not shown). Data are expressed as mean ± s.e.m. from 3-6 rats.

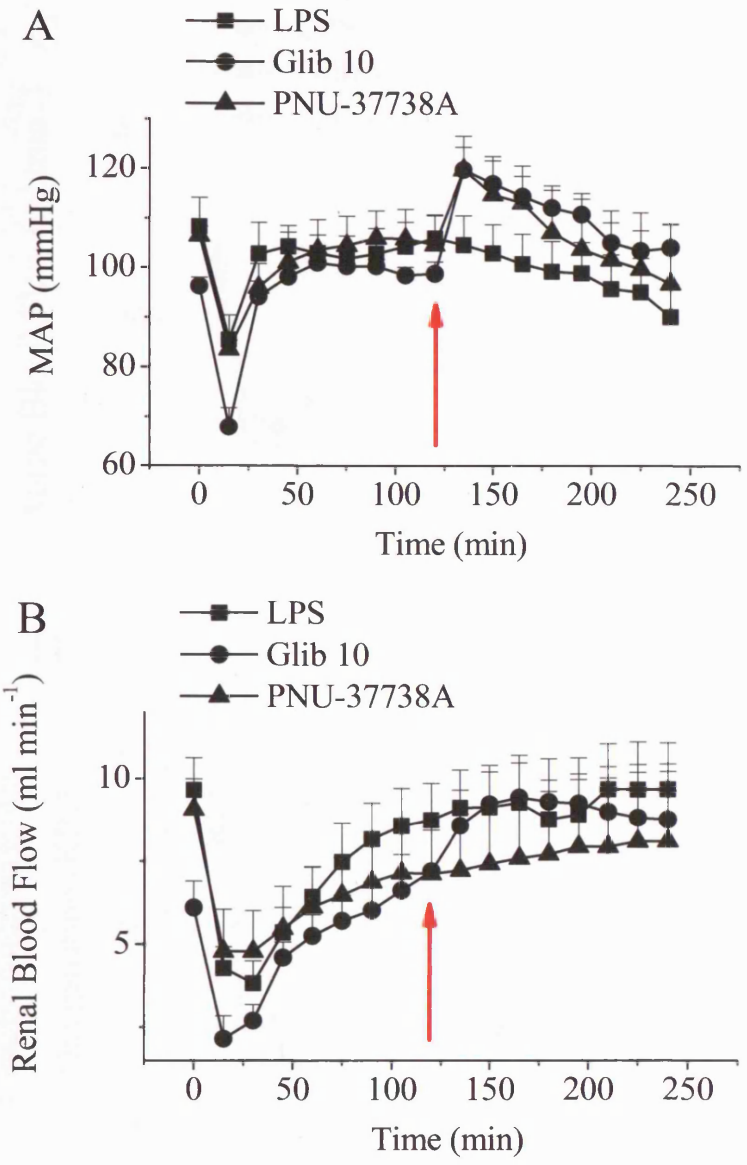


Figure 4.10 Effect of the K_{ATP} inhibitors glibenclamide (10 mg kg^{-1} bolus + 5 mg kg^{-1} infusion) and PNU-37883A (1.5 mg kg^{-1} + 1mg kg^{-1} infusion) given at 120 min (see arrow) on (A) mean arterial blood pressure and (B) renal blood flow in LPS-treated rats. High dose glibenclamide and PNU-37883A raised MAP ($P < 0.001$, ANOVA) without affecting RBF. Low dose glibenclamide had no effect (data not shown). LPS was infused over the first 30 min. Data are expressed as mean ± s.e.m. from 6 rats.

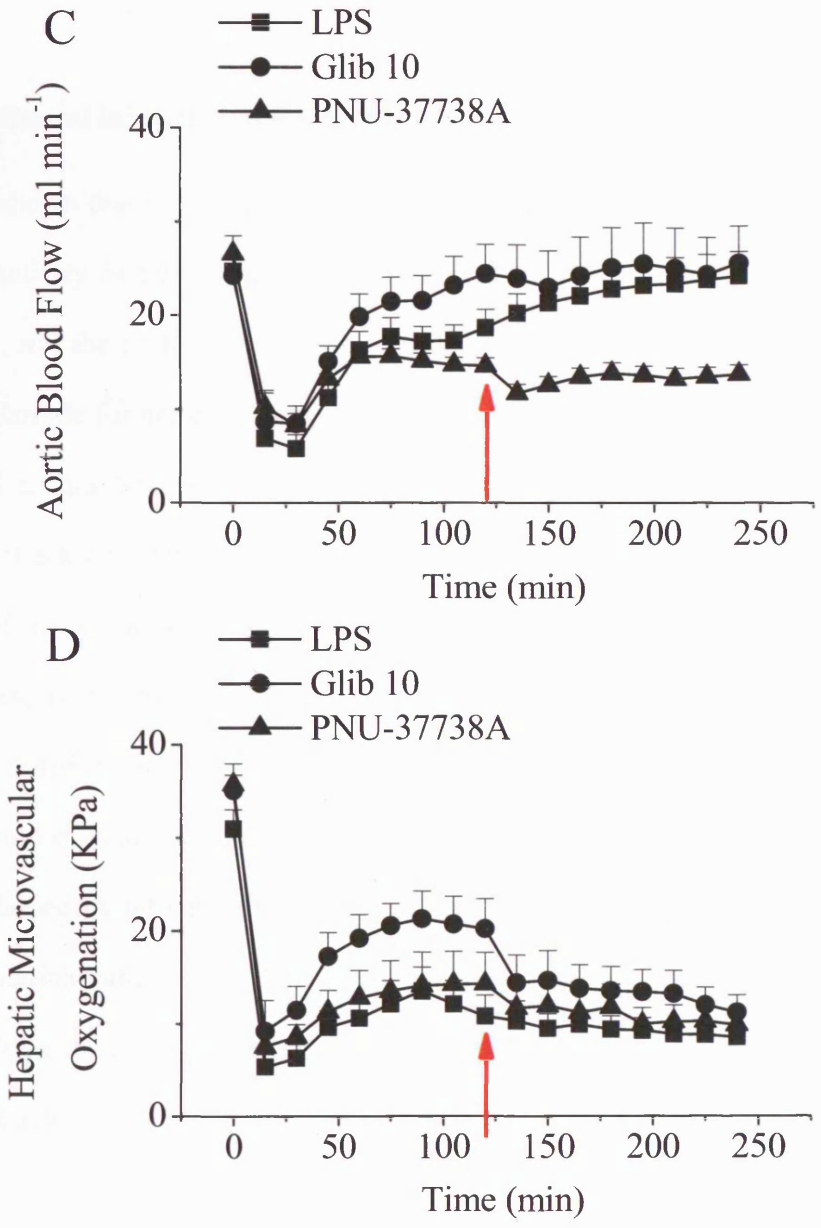


Figure 4.10 Effect of the K_{ATP} inhibitors glibenclamide (10 mg kg^{-1} bolus + 5 mg kg^{-1} infusion) and PNU-37883A (1.5 mg kg^{-1} bolus + 1 mg kg^{-1} infusion) given at 120 min (see arrow) on (C) aortic blood flow and (D) microvascular oxygenation in LPS-treated rats. PNU-37883A reduced ABF at later time points ($P<0.05$, ANOVA). Neither agent restored $HmvO_2$. LPS was infused over the first 30 min. Data are expressed as mean ± s.e.m. from 6 rats.

4.6 Discussion

4.6.1 Effect of inhibition of K+ channels in an *in vitro* model of sepsis

I have shown that the K_{ATP} channel is an important mediator of LPS-induced vascular hyporeactivity *in vitro*. Importantly, I found that only inhibitors of the pore-forming subunit, not the SUR subunit, were effective in reversing this hyporeactivity. Indeed, glibenclamide further depressed contractile responses to phenylephrine in the presence of LPS as has been shown previously in endothelium-intact vessels (Preiser *et al.*, 2003). Lack of effectiveness of either glibenclamide or tolbutamide on LPS-induced vascular hyporeactivity, could not have resulted from inhibition of thromboxane receptors, since PNU-99963, the most potent SUR2 inhibitor available (Cui *et al.*, 2003), failed to inhibit contractions to the thromboxane A_2 mimetic. In addition, restoration of vascular reactivity observed with the K_{ATP} channel pore-blockers cannot be explained by inhibition of NO production, since these agents did not reduce nitrite accumulation induced by LPS. The suppressed contractile response to 60 mM KCl seen after 20 h exposure to LPS, but not at 6 h, implies that mechanisms independent of K^+ channel activation also contribute to hyporeactivity at this later timepoint.

The interpretation of my *in vitro* experimental data relies heavily upon the specificity of the K_{ATP} inhibitors used at the stated concentrations *i.e.* that $BaCl_2$ (300 μM) and PNU-37883A (1 μM) are specific inhibitors of the pore-forming subunit of the K_{ATP} channel. In vascular smooth muscle, $BaCl_2$ inhibits the K_{ATP} channel with an IC_{50} of 100 μM and Kir channels with an IC_{50} of ~ 2-5 μM (Nelson & Quayle, 1995 and Bradley *et al.*,

1999). As I observed no effect at 30 μM $BaCl_2$, a concentration that effectively inhibits vascular relaxation associated with Kir channels (Quayle *et al.*, 1997 and Edwards *et al.*, 1998), this would rule out a contribution from these channels. Furthermore, mRNA for Kir1.1 and Kir3.1 has been identified in rat arteries (Michelakis *et al.*, 2001), subunits which encode weak (ROMK1) and G-protein gated (GIRK) inwardly rectifying K^+ channels. Both channels types would be expected to be blocked by Ba^{2+} in the high micromolar range (>100 μM) and thus it is conceivable that GIRK or ROMK1 channels may be activated by LPS treatment. However, the PNU-37883A data leads me to conclude that hyporeactivity does indeed result from activation of K_{ATP} channels. PNU-37883A is considered to be a highly selective inhibitor of the K_{ATP} channel (Humphrey, 1999). It antagonises the vasorelaxant effects of pinacidil, levcromakalim and minoxidil sulphate in rabbit mesenteric artery (IC_{50} ~1 μM), while not significantly affecting the vasorelaxant responses to forskolin, nitroglycerin or the Ca^{2+} channel blocker, D-600 (Meisheri *et al.*, 1993). Likewise in patch-clamp studies, PNU-37883A blocks pinacidil- or levcromakalim-activated K_{ATP} currents (IC_{50} =3.5 μM) in isolated rat arterial smooth muscle cells, while only marginally inhibiting (> 10 %) voltage-activated (Kv) or Kir currents at 10 μM (Wellman *et al.*, 1999). I have thus provided good pharmacological evidence for the involvement of K_{ATP} channels in mediating LPS-induced vascular hyporeactivity. Additional evidence comes from the observation that relaxations to K_{ATP} channel openers such as levcromakalim are potentiated in both *in vitro* and *ex-vivo* models of LPS-induced hyporeactivity (Sorrentino *et al.*, 1999; Chen *et al.*, 2000 and Wilson & Clapp, 2002). The iNOS pathway probably mediates this since both 1400W and, to a lesser extent ODQ, reversed potentiation (Wilson &

Clapp, 2002). I can exclude an effect upon the generation of nitric oxide, as PNU-37883A did not reduce nitrite production in the culture media following LPS treatment. Likewise, I have previously shown that inhibition of the inducible cyclo-oxygenase pathway did not reverse vascular hyporeactivity in our organ culture model (O'Brien *et al.*, 2001), excluding a contribution from the prostaglandin pathway in mediating K_{ATP} channel activation.

I have previously shown that both iNOS and guanylate cyclase inhibitors fully reverse hyporeactivity in mesenteric arteries treated with LPS for 6 h *in vitro* (O'Brien *et al.*, 2001, chapter 2). Thus the NO / cyclic GMP pathway is likely to be responsible for K_{ATP} channel activation at this time-point. Indeed, other studies have demonstrated activation of K_{ATP} channels in response to NO in mesenteric artery (Garland & McPherson, 1992; Murphy & Brayden, 1995). In my work, neither $BaCl_2$ nor PNU-37883A inhibited relaxations to the NO donor, SNAP in fresh control tissues. Similarly, relaxation to NO in endothelium-denuded aortic tissue was only weakly affected by either Ba^{2+} or 4-aminopyridine, suggesting activation of K^+ channels does not appear to be the primary mechanism of relaxation for authentic NO (Wilson & Clapp, 2002). Thus, additional factors associated with incubation of LPS may aid NO activation of K_{ATP} channels in this model. Consistent with this notion, relaxation to SNAP (or forskolin) in rat aorta was only inhibited by PNU-37883A in the presence of LPS (Wilson & Clapp, 2002). However, the degree of reversibility achieved by the K_{ATP} channel inhibitors declined at 20 h. This mirrors my previous finding of decreased responsiveness to iNOS inhibitors over time (O'Brien *et al.*, 2001, chapter 2).

Is the K^+ channel an important mediator of LPS-induced vascular hyporeactivity or hypotension

Bioenergetic failure and/or disruption of the actin cytoskeleton have been demonstrated in sepsis and perhaps either of these contributes to vascular hyporeactivity (Williams *et al.*, 1999 and Brealey *et al.*, 2003). Such a mechanism may also account for the reduced contractile response to 60 mM KCl at 20 h.

Different mechanisms of K_{ATP} channel activation may underlie the hypotension and vascular hyporeactivity induced by LPS, perhaps due in part to circulating hormones and mediators not present *in vitro*. For example, calcitonin gene-related peptide, a known activator of vascular K_{ATP} channels (Quayle *et al.*, 1997), is increased in septic shock (Arden *et al.*, 1994). Alternatively, K_{ATP} channels may open secondary to a fall in tissue ATP, oxygen tension or pH, conditions associated with severe forms of shock (Clapp & Tinker, 1998). However, the extent to which these metabolic defects occur in sepsis remain controversial (Singer & Brealey, 1999) and has not been investigated *in vitro*. Nonetheless, over-production of NO *via* iNOS is an integral part of both hypotension and hyporeactivity in sepsis (Vincent *et al.*, 2001).

The fact that glibenclamide had no effect *ex vivo* may be related to the use of aorta in previous studies (Wu *et al.*, 1995; Taguchi *et al.*, 1996 and Sorrentino *et al.*, 1999), which is a conduit vessel that makes little contribution to blood pressure. Results from this laboratory reported that glibenclamide was also ineffective at preventing iNOS-mediated relaxation induced by L-arginine in rat aorta (Wilson & Clapp, 2002). However, in the present study, I found that glibenclamide also failed to reverse hyporeactivity in the mesenteric artery. Another consideration may be the counter-

balancing effect of glibenclamide on the contractile effects of thromboxane (Cocks *et al.*, 1990). Thus, inhibiting thromboxane-induced vasoconstriction could negate any reversal of vascular hyporeactivity that might have occurred through blocking the K_{ATP} channel. It is worth noting that cytokine-induced hyporesponsiveness of aorta to phenylephrine was significantly augmented in TXA_2 receptor knockout mice ($TP^{-/-}$), suggesting a protective role for TXA_2 against the development of vascular hyporesponsiveness (Yamada *et al.*, 2003). Although I demonstrated that glibenclamide and tolbutamide antagonised thromboxane-induced contractions in mesenteric artery, PNU-99963 did not. I thus conclude that lack of reversal of SUR inhibitors on LPS-hyporeactivity is unrelated to thromboxane inhibition.

The striking difference between the effectiveness of glibenclamide and PNU-37883A *in vitro* could relate to their different sites of action (SUR *versus* pore). It has previously been reported that actin filament disruption with cytochalasin D abolishes high-affinity glibenclamide binding to the SUR subunit in rat aortic rings whereas KCO binding is unaffected (Loffler-Walz & Quast, 1998). Similar effects on glibenclamide binding can be achieved with metabolic inhibition or hypoxia (Loffler & Quast, 1997). Furthermore, NO can cause cytoskeletal disassembly either through actin nitration (Banan *et al.*, 2001) or through inhibition of the RhoA kinase pathway by cyclic GMP (Sauzeau *et al.*, 2000). Thus, LPS may cause NO- or ATP-dependent disruption of the cytoskeleton, rendering SUR agents ineffective, while leaving pore-blockers relatively unaffected. Indeed, we have previously shown that glibenclamide is significantly less effective than

PNU-37883A at inhibiting levcromakalim responses in the presence of LPS (Wilson & Clapp, 2002).

The greater degree of reversal obtained with $BaCl_2$ compared to PNU-37883A at 6 h suggests K_{ATP} channel independent mechanisms. This is consistent with the small increase in potency to PE in control tissues pre-incubated with Ba^{2+} but not PNU-37883A. Using the non-selective K^+ channel inhibitor, TEA^+ a similar reversal of LPS-induced hyporeactivity to that observed with PNU-37883A or $BaCl_2$ was seen at 10 mM. At this concentration TEA^+ is likely to inhibit K_{ATP} channels as well as other K^+ channels. Some reversal of LPS-induced hyporeactivity was also seen at concentrations as low as 1mM TEA, suggesting a contribution from BK_{Ca} channels. The role of additional K^+ channels is also consistent with a small, but significant leftward shift of the PE dose-response curve elicited by TEA over and above that observed with $BaCl_2$ alone. Indeed, a role for the BK_{Ca} channel in mediating some of the effects of LPS has previously been described in aortic smooth muscle (Hall *et al.*, 1996; Taguchi *et al.*, 1996; Chen *et al.*, 1999, and Yakubovich *et al.*, 2001). Furthermore, activation of BK_{Ca} channels in rat mesenteric artery has been shown to be involved in the development of vascular hyporeactivity following haemorrhagic shock (Zhou *et al.*, 2005). To be certain that BK_{Ca} channels were involved in LPS-induced hyporeactivity, I should have repeated experiments using iberiotoxin, a highly specific and potent inhibitor of this channel (Nelson and Quayle, 1995). At 20h however, there was a clear difference in the nature of the reversal of LPS-induced hyporeactivity. Contractions to PE were transient in the presence of 1mM TEA^+ whereas those obtained in the presence of PNU-37883A

or $BaCl_2$ were sustained. Depolarisation by whatever means may boost contractions to PE and the transient contractions observed with TEA^+ may represent just a smaller depolarisation with this agent. This may have the consequence of allowing significant calcium release from IP_3-senstive stores (see Quast *et al.*, 1994), which would give rise to a transient contraction, while not providing a greater enough depolarisation to promote Ca^{2+} entry through voltage-gated Ca^{2+} channels.

4.6.2 Effect of inhibition of the K_{ATP} channel in an *in vivo* model of sepsis

In the *in vivo* model administration at 120 min of the K_{ATP} channel inhibitors PNU-37883A and glibenclamide at high dose (10 mg kg^{-1} bolus and 5 mg kg^{-1} h^{-1}) infusion elevated blood pressure significantly in the LPS-treated rats and shams. However, low dose glibenclamide (1mg kg^{-1} bolus) had no effect in shams or LPS treated rats. Despite the improvement in blood pressure, neither agent improved the macro nor microcirculation. Indeed there were adverse effects in LPS-treated rats with high dose glibenclamide worsening the $HmvO_2$ while PNU-37883A reduced ABF and worsened the metabolic acidosis.

This is the first *in vivo* study to demonstrate that the pore-forming subunit inhibitor, PNU-37883A raises blood pressure in septic rats. Glibenclamide has previously been shown to reverse LPS-induced hypotension in several *in vivo* models (*e.g.* Landry and Oliver, 1992 and Vanelli *et al.*, 1995). However the difference between high and low dose glibenclamide despite both inhibiting levcromakalim relaxations has never been described. Unlike previous work, however, in my model, the effects of both K_{ATP}

inhibitors on blood pressure were greater in the shams than LPS-treated rats (this is in contrast to the iNOS inhibitor GW273629A that has a significantly greater effect upon LPS-treated rats, see chapter 2). It is tempting to suggest that the differing effects seen with low and high dose glibenclamide might be due to the higher dose acting on the pore-forming subunit of the K_{ATP} channel as the effect seen is very similar to that of PNU-37883A. Although the IC_{50} for inhibition of the SUR by glibenclamide is ~10-200 nM (Fujita & Kurachi, 2000), at a 1000-fold higher concentration, glibenclamide appears to interact with the pore directly (Gribble *et al.*, 1998; Bryan & Aguilar-Bryan, 1999). The calculated peak plasma concentration of glibenclamide for a dose of 1 mg kg^{-1} equates to approximately 60 µg ml^{-1} (or 120 µM) (Wu *et al.*, 1995). At this concentration (low dose) glibenclamide would be expected to inhibit the pore directly. Thus my assumption that both high dose glibenclamide is acting on the pore-forming subunit of the K_{ATP} channel, as PNU-37883A, may not be accurate. Interestingly many investigators have used much higher doses in *in vivo* rat models of sepsis. The *in vivo* studies that have demonstrated an increase in blood pressure in endotoxic animals with glibenclamide have used doses that range from 1mg kg^{-1} to 20 mg kg^{-1} intravenously and 40 mg kg^{-1} intraperitoneally (Landry and Oliver, 1992; Vanelli *et al.*, 1995; Wu *et al.*, 1995; Vanelli *et al.*, 1997; Gardiner *et al.*, 1999 and Sorrentino *et al.*, 1999). In addition, almost all *in vivo* studies use doses of 5 mg kg^{-1} or above to inhibit the potassium channel opener levcromakalim (Pratz *et al.*, 1991, Saito & Sakai, 1998 and Zhang *et al.*, 1999). Smits *et al.*, (1997) found that using concentrations of glibenclamide as low as 0.3 mg kg^{-1} significantly attenuated levcromakalim responses but that 10 mg kg^{-1} was required for full inhibition. Indeed in the sham rats I found that

glibenclamide 1 mg kg^{-1} reversed the potassium channel opener levcromakalim induced hypotension by 70% and 10 mg kg^{-1} by 100%. i.e. higher concentrations of glibenclamide were required to raise blood pressure in sham or LPS-treated rats than inhibit levcromakalim-induced hypotension. The specific vascular K_{ATP} inhibitor PNU-37883A (Humphrey, 1999) has never been used before in sepsis models. 3 mg kg^{-1} will prevent and reverse the fall in blood pressure induced by the potassium channel opener pinacidil in an anaesthetised rat (Smith *et al.*, 1994). I found in shams that PNU-37883A (1.5 mg kg^{-1}) fully reversed the hypotensive effects of levcromakalim.

Previous studies have not shown a pressor effect of glibenclamide upon shams (e.g Landry and Oliver, 1992; Vanelli *et al.*, 1995 and Wu *et al.*, 1995). Indeed one study described a reduction in blood pressure in shams that was considered to be related to an increase in cardiac afterload secondary to vasoconstriction (Presier *et al.*, 2003). As mentioned above, in stark contrast to these studies, the effects seen in shams in my model is superior to that seen in LPS-treated rats. Clearly K_{ATP} channels are activated in the absence of LPS in this model. Interestingly, SUR2 knockout mice are hypertensive and therefore one might predict an effect of K_{ATP} channel inhibitors upon blood pressure in the shams (Chutkow *et al.*, 2002). Although *in vitro*, glibenclamide and PNU-37883A had no effect upon contractile responses to PE in control tissues. Glibenclamide has been shown to inhibit the production of iNOS (Wu *et al.*, 1995) but I found it to have no effect upon nitrite production *in vitro*, although tolbutamide did reduce nitrite production. The laparotomy and instrumentation of the rat would be extremely likely to induce iNOS and certainly the iNOS inhibitor GW273629A had a

haemodynamic effect in shams (though non-significant) and iNOS was identified by immunohistochemistry in sham animals (see chapter 2). In addition the anaesthetic agent used, isoflurane is a K_{ATP} channel opener (Ida *et al.*, 1998 and Sumikawa *et al.*, 1998). Thus it is not surprising that the K_{ATP} channel is activated in shams, however, as levcromakalim still caused hypotension in shams, the channel was clearly not fully activated. Perhaps the superior effect seen in shams is because sepsis reduces the ability of the pore-forming subunit of the K_{ATP} channel to bind to PNU-37883A and glibenclamide; possibly due to metabolic acidosis. Why this has not been demonstrated in previous septic *in vivo* models I cannot readily explain.

A possible confounding factor when using glibenclamide is that it is difficult to dissolve and so the solvent dimethyl sulfoxide (DMSO) is often used (e.g Wu *et al.*, 1995 and Sorrentino *et al.*, 1999). DMSO has been shown to inhibit NO synthesis (Liang and Akaike, 1997) and I confirmed its pressor effect in a separate experiment – with 0.7 ml of a 100% solution of DMSO raising MAP from 76 to 116 mmHg in an LPS treated rat (not shown). 0.7 ml was the volume used for the bolus of all drugs *in vivo*. In these experiments, we use a lyophilised preparation of glibenclamide that was originally made for use in humans (and kindly donated by Aventis Pharma). The preparation contained mannitol and sodium hydroxide but not DMSO and has previously been used by Presier *et al.*, (2003) in dogs. Given that low dose glibenclamide had no significant effect on MAP in shams or LPS treated animals, would argue against non specific effects on this parameter. There should be no solvent problem for PNU-37883A, which was dissolved in water.

There was no response to glibenclamide in the one patient with septic shock. Obviously I would like to have recruited more patients, to see if this was a consistent finding. I gave this patient 1 mg kg^{-1} and perhaps had I used a higher dose as in my *in vivo* model there may have been a response. It has been shown that dexamethasone reduces vascular hyporeactivity induced by LPS *in vivo*, at least in part, by modulating K_{ATP} channel activity (D'Emmanuel di Villa *et al.*, 2003). It was suggested that steroids might work by reducing K_{ATP} channel expression. Such an effect may underpin the observation that the administration of hydrocortisone to septic shock patients improves the response to PE i.v. infusion (Bellissant & Annane, 2000). Indeed hydrocortisone has been shown to improve survival in septic shock patients (Annane *et al.*, 2002). Steroids are known to have a multitude of effects that may account for these effects including inhibition of iNOS and COX-2 expression, complement activation and PAF release (Imai *et al.*, 1982; Han *et al.*, 1997; Thiemermann, 1997; Leach *et al.*, 1998). Thus it remains to be determined if the beneficial effect of steroids is partially due to inhibition of the K_{ATP} channel expression.

In summary, I have shown that the K_{ATP} channel is an important mediator of LPS-induced vascular hyporeactivity in an *in vitro* model. Moreover, I have demonstrated for the first time that inhibitors binding to the K_{ATP} channel pore rather than the SUR are effective at reversing vascular hyporeactivity. Whether this relates to a structural change or functional uncoupling between K_{ATP} channel subunits remains to be determined. These findings were complimented by my *in vivo* study in which glibenclamide and

PNU-37883A both raised blood pressure, however, the effect seen in shams was superior to that seen in LPS-treated rats. As seen with the iNOS inhibitor GW273629A *in vivo* (chapter 4), an improvement in MAP did not correspond to an improvement in microvascular or renal and aortic blood flow.

Chapter 5

Is vasopressin an important mediator of sepsis-induced vascular hyporeactivity or hypotension?

5.1 Introduction

Thus far, I have been able to thoroughly characterise an *in vitro* and an *in vivo* model of septic shock. I have demonstrated evidence for activation of both the NO pathway and vascular potassium channels (especially K_{ATP}). In addition, I have shown that inhibition of either pathway reverses hypotension and vascular hyporeactivity induced by endotoxin. As a clinician, I wished to see whether my animal studies could be translated to the clinical environment. I had given intravenous glibenclamide to one patient with septic shock to no effect (see chapter 4). Moreover, the iNOS inhibitors that I had used in my experimental models were not considered safe to use in humans.

Several studies in patients have reported that infusions of vasopressin (VP) can increase blood pressure and lead to reduction in vasopressor requirements (e.g. Landry *et al.*, 1997 and see introduction). However, mechanisms underlying increased pressor sensitivity to VP and its relative deficiency in septic shock remain unknown.

I chose to study VP and its long acting analogue terlipressin (TP) in my two rat models of sepsis. I initially compared the vasoconstrictor properties of VP with phenylephrine (PE) in the *in vitro* rat mesenteric artery model of LPS-induced vascular hyporeactivity. I then examined the effect of TP upon LPS-induced hypotension in the *in vivo* rat model of sepsis, measuring its effects upon aortic and renal blood flow and arterial acid-base status. I chose TP in preference to VP as only the former was available for use in patients. I then conducted a short clinical study examining the effect of TP upon

hypotension, norepinephrine (NE) requirements and outcome in humans (O'Brien *et al.*, 2002).

5.2 Methods

5.2.1 Effect of LPS on responses to vasopressin *in vitro*

The methods for the organ bath experiments are fully described in chapter 2. Rings were incubated with and without LPS (1 μg ml^{-1}) for 20 h. Endothelial function was assessed by monitoring relaxation to acetylcholine (5 μM) in rings precontracted with 1 μM PE. Cumulative concentration-response curves were constructed to PE (10^{-9} to 10^{-5} M) and to VP (10^{-12} to 10^{-7} M) with increasing doses added at 5 min intervals at which time the contraction had reached a plateau. Although I used TP for the *in vivo* model and the human study, it is a long acting drug with a half-life of 4 hours. I thus used the short acting agent, VP for constructing the concentration-response curves. The NOS inhibitor, aminoguanidine (300 μM) was added to some LPS-treated tissues 25 min prior to the VP concentration-response curve being commenced.

5.2.2 Effect of treatment with terlipressin and norepinephrine *in vivo* rat sepsis

The method and model are described in detail in chapter 3. Four experimental groups were used:

(a) Group A (n=6) was given 40 mg kg^{-1} LPS *(Klebsiella Pneumoniae)* in 0.9 % saline by infusion over 30 min at a rate of 20 ml kg^{-1} h^{-1}. For the remainder of the experiment, all groups were fluid resuscitated with 25 ml kg^{-1} h^{-1} of the synthetic gelatin colloid, Gelofusin®. This was supplemented with 1 ml 10% glucose solution per 25 ml to keep blood sugars > 5 mM.

(b) Group B (n=6) received isotonic saline rather than LPS over the first 30 min (sham-operated controls).

(c) Group C comprised either LPS-treated rats (n=6) or sham-operated controls (n=3). These rats received the α-1-agonist NE as a 1 mg kg^{-1} bolus at 120 min (approx. 0.7ml volume) followed by a 0.5 mg kg^{-1} h^{-1} infusion (administered with gelofusin® in a similar volume to controls). This was to simulate the management of septic shock in which fluid resuscitation and catecholamines, such as NE, are the mainstays of treatment.

(d) Group D comprised either LPS-treated rats (n=6) or sham-operated controls (n=3). These rats received the long-acting vasopressin analogue, TP, as a 0.02 mg kg^{-1} bolus at 120 min.

5.2.3 Effect of terlipressin in norepinephrine-resistant septic shock in humans

This was an open-label pilot study performed in critically ill patients in severe septic shock requiring high doses of the catecholamine vasopressor NE, and in whom hypotension had not previously responded to corticosteroids or methylene blue (a guanylyl cyclase & iNOS inhibitor; Geng *et al.*, 1998).

TP was given to eight patients with vasodilatory shock and proven or suspected bacteraemia on the intensive care unit (ICU) at University College London Hospitals

(UCLH). Prior to the study approval had been obtained from the UCLH Ethics Committee and agreement sought from the patient's next-of-kin. Patients were unselected but were enrolled between January 1999 and June 2001 when at least one of three doctors involved in the study was present. All were intubated and ventilated, with high cardiac outputs and severe hypotension despite appropriate fluid loading to maximal stroke volume and high doses of NE. Cardiac output and fluid loading were assessed by transoesophageal Doppler ultrasound. In all patients, hypotension had previously failed to respond to both dexamethasone (16mg intravenously (iv)) and methylene blue (2mg kg^{-1} (iv) over 15 min) – the standard "rescue therapy" for catecholamine-resistant septic shock on the intensive care unit at UCLH. All patients were given an intravenous bolus dose of TP (0.5–2 mg) over 5 min which could be repeated at 30-min intervals to a maximum of 2 mg. As the 2mg bolus (a standard dose routinely given to liver failure patients with variceal bleeding or hepatorenal syndrome with a small haemodynamic effect ≈ 10 mmHg - Therapondos *et al.*, 2004) produced such a rise in blood pressure in the first patient (to a mean of 80 mmHg), such that she needed glyceryl trinitrate to lower this pressure (!), reduced doses were given to subsequent patients. A further dose of TP was administered six hours later to two patients. Mean arterial blood pressure (MAP), norepinephrine requirements, central venous pressure, oxygen saturations, heart rate, urine output and cardiac output were monitored continuously. Arterial blood gases were sampled regularly. The target MAP was between 60-70 mmHg. The NE infusion was reduced if this was exceeded.

5.3 Statistical analysis

As for chapters 2

5.4 Reagents and solutions

As for chapters 2 & 3. TP was purchased from Ferring Pharmaceuticals Ltd (Berkshire, U.K.) and had its own diluent. NE was purchased from Abbott (Maidenhead, U.K.) and was dissolved in 0.9% saline.

5.5 Results

5.5.1 Effect of LPS on contractile responses to vasopressin *in vitro*

In fresh control endothelial-intact tissues there was a triphasic response to increasing concentrations of VP (Fig. 5.1A). The maximum contraction was 0.65 ± 0.18 g at 10^{-7} M while at 10^{-5} M there was no contractile response. When the endothelium was denuded there was a biphasic response with an increased maximal contraction of 1.88 g (n=1 at this concentration of VP only). The effects of LPS upon contractions to PE in RMA have been described in chapter 2. Following incubation with LPS for 20 h there was virtually no contractile response to all concentrations of vasopressin, even in control tissues incubated in culture media without LPS (Fig. 5.1B). This hyporeactivity was not reversed by the iNOS inhibitor aminoguanidine.

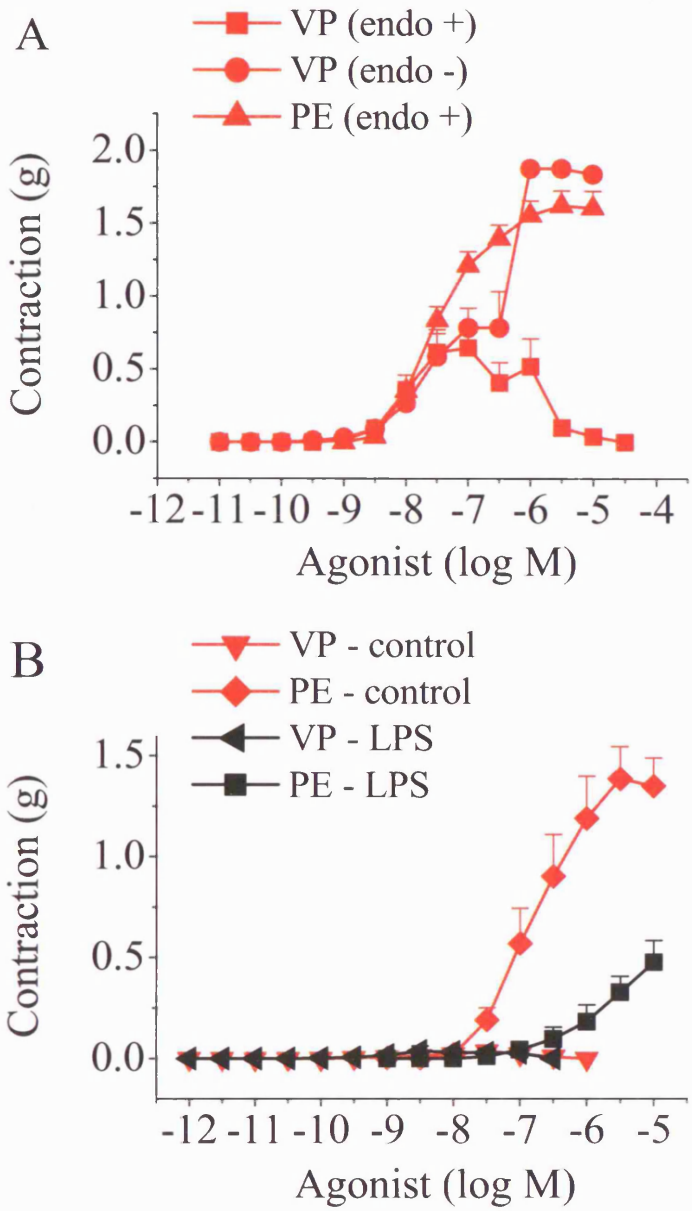


Figure 5.1 Comparison of contractile responses to vasopressin - VP (10^{-12} to10^{-5} M) and phenylephrine - PE (10^{-9} to 10^{-6} M) in (A) fresh tissue and (B) tissue incubated ± LPS for 20 h. Data are expressed as the mean ± s.e.m of 6-8 observations from 6 animals.

5.5.2 A comparison of norepinephrine and terlipressin in an *in vivo* model of sepsis

In sham animals MAP, RBF, hepatic microvascular oxygenation ($HmvO_2$) and arterial base deficit did not change significantly throughout the experiment (see Fig. 3.1 A-D). There was a tendency, albeit non-significant, for MAP and RBF to increase. A rise followed by a fall in ABF and $HmvO_2$ was also observed. These changes, particularly in ABF, were likely related to the fluid resuscitation. Following infusion of LPS, there was a rapid fall in all measured variables within 0-30 min (see Fig. 3.1 A-D). Recovery in MAP was observed within 30 min (before fluid resuscitation was commenced), although aortic and renal blood flow took 2-3 h to return to baseline values. However, $HmvO_2$ failed to show any significant recovery after the initial abrupt fall. After 120 min the MAP fell significantly in LPS treated rats ($P < 0.001$, n=6, ANOVA). I thus chose this time point to administer either NE or TP.

NE (0.5mg kg^{-1} bolus and 1mg kg^{-1} h^{-1} infusion) caused a large sustained rise in the blood pressure in sham rats that was significantly greater than that achieved in LPS-treated rats ($P = 0.007$; Fig. 5.2 A). In LPS-treated rats, there were small, non-significant falls in RBF, ABF and $HmvO_2$ while the arterial base deficit was unaffected (Fig. 5.2 B-D). In sham animals, RBF and arterial base deficit were unaffected, however there was a significant fall in ABF and $HmvO_2$ ($P < 0.05$; Fig. 5.2 B-D).

Conversely, a 0.02 mg kg^{-1} (equivalent to 1.5mg for a 75 mg man) bolus of TP caused a significantly greater rise in the MAP in LPS-treated rats than shams ($P < 0.001$; Fig. 5.2 A). The effect of this bolus appeared to last for approximately 75 min. In LPS-treated

rats, there were non-significant falls in the RBF, ABF and $HmvO_2$ following terlipressin, however the acidaemia significantly worsened ($P < 0.001$, see Table 5.1). In sham animals, the RBF, $HmvO_2$ and arterial base deficit were unaffected by TP, however there was a significant fall in ABF ($P < 0.05$; Fig 5.2B-D).

Thus the pressor effect of NE was attenuated significantly by LPS, whereas this was emphatically not the case with TP (Fig. 5.2 A). However, in spite of its superior pressor effect, TP was unable to improve renal or aortic blood flow or $HmvO_2$ (Fig 5.2 B-D). Neither did TP attenuate the metabolic acidosis seen in septic animals (Table 5.1); indeed at 180 min it worsened the acidosis.

Table 5.1 Effect of terlipressin and norepinephrine (given at 120 min) upon the arterial base deficit.

Data are expressed as mean ± s.e.m. of 3-6 observations from 3-6 animals.

	Time (min)			
	0	120	180	240
Sham	-4.4 ± 0.9	-7.5 ± 0.9	-7.7 ± 0.9	-6.73 ± 1.13
LPS	-3.4 ± 0.5	-11.1 ± 0.6	-9.64 ± 0.4*	-11 ± 1.45
Sham + TP	-4.2 ± 1.95	-6.2 ± 1.94	-8.9 ± 0.57	-8.5 ± 0.95
Sham + NE	-3.8 ± 0.45	-5.9 ± 0.71	-5.57 ± 0.35	-6.57 ± 0.2
LPS + TP	-5.2 ± 0.6	-13.5 ± 0.9	-17.1 ± 1.2*	-15.8 ± 1.9
LPS + NE	-3.17 ± 0.45	-11.9 ± 0.6	-10.4 ± 0.4	-10.3 ± 0.3

*$P<0.001$ when directly compared with each other

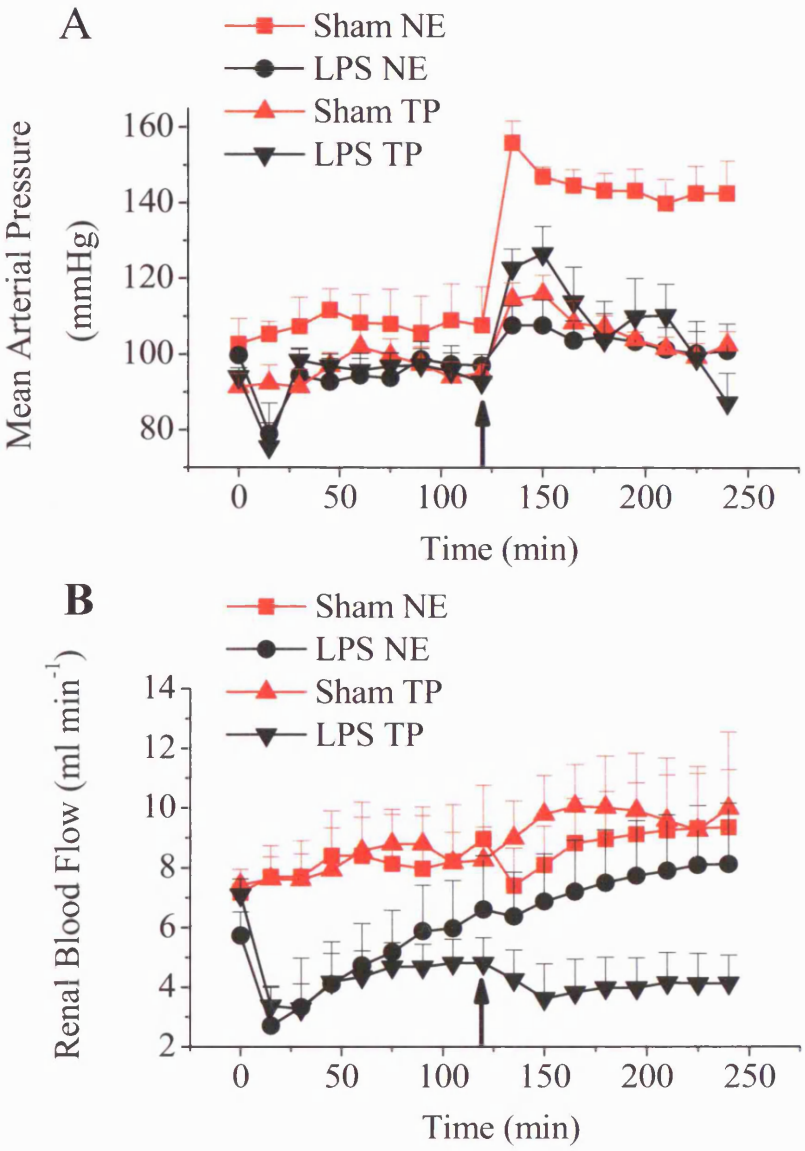


Figure 5.2 Comparison of a bolus of TP (0.02 mg kg^{-1}) with a bolus + infusion of NE (0.5 mg kg^{-1} & 1 mg kg^{-1} h^{-1}) given at 120 min (see arrow) upon (A) MAP & (B) RBF in LPS and sham treated rats. Data are expressed as mean ± s.e.m. of 3-6 observations from 3-6 animals. NE induced a rise in MAP (P = 0.007, ANOVA) in shams compared to LPS. Conversely, TP induced a rise in MAP in LPS-treated rats compared to shams (P < 0.001, ANOVA).

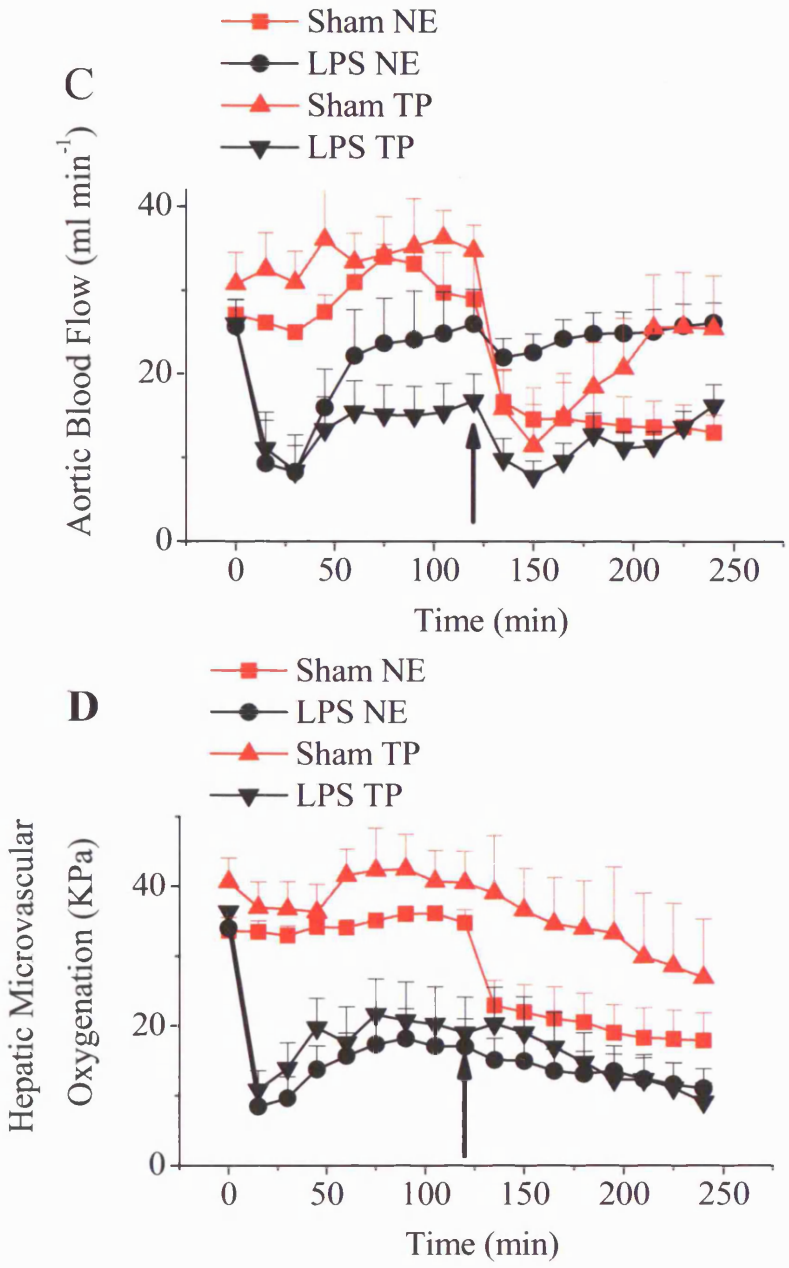


Figure 5.2 Comparison of a bolus of TP (0.02 mg kg^{-1}) with a bolus + infusion of NE (0.5 mg kg^{-1} & 1 mg kg^{-1} h^{-1}) given at 120 min (see arrow) upon (C) aortic blood flow (ABF) and (D) microvascular oxygenation ($HmvO_2$) in LPS and sham treated rats. Data are expressed as mean ± s.e.m. of 3-6 observations from 3-6 animals. ABF fell significantly in shams with both NE and TP (P <0.05, ANOVA). $HmvO_2$ also fell in shams treated with NE ($P < 0.05$ after 135 min).

5.5.3 Effect of terlipressin in norepinephrine-resistant septic shock in humans

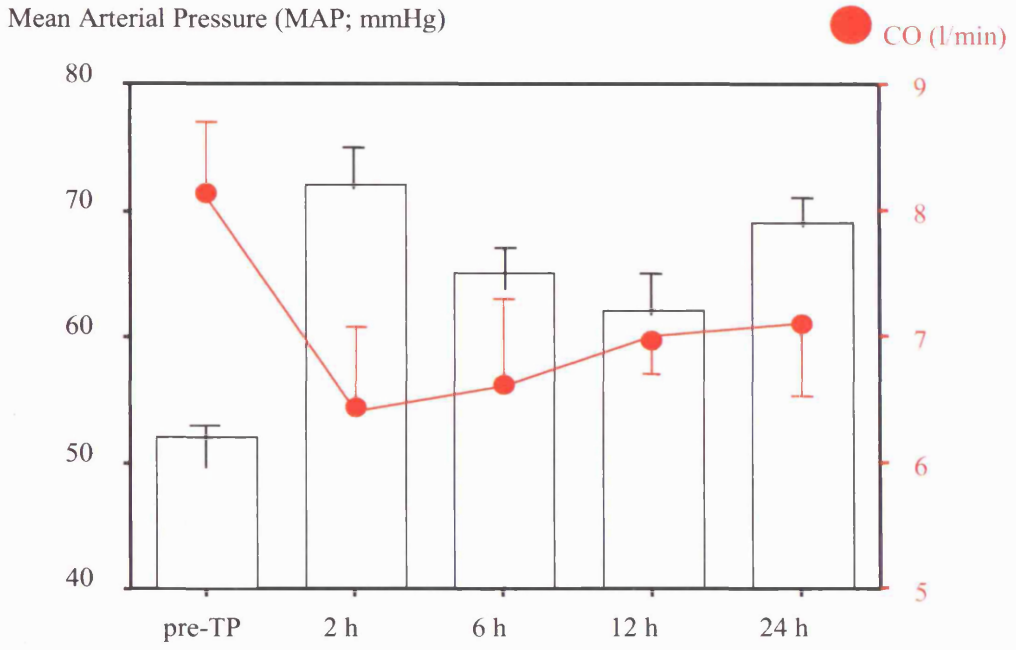
The 8 patients to whom TP was given were all extremely ill, with APACHE II scores ranging from 25-37 (Knaus *et al.*, 1985) and persisting low mean arterial pressures, despite high dose NE and previous administration of corticosteroids and methylene blue (see Tables 5.2 A and B). In all patients, the bolus of TP (ranging from 1-2 mg) produced, within minutes, a progressive rise in MAP (Fig. 5.3). The peak effect was, however, not seen until 20-30 min after administration. This enabled major reductions or cessation of the high-dose NE in 7 of the 8 patients (Fig 5.4). The elevation in MAP was sustained for at least 6 hours. Four patients required no subsequent increase in NE and survived the septic episode to be discharged from the ICU; three were subsequently discharged alive from hospital. No major chronotrophic effects and no deleterious consequences were observed, e.g. digital, splanchnic or myocardial ischaemia. The fall in cardiac output was commensurate with the elevation of blood pressure (Fig. 5.3) and did not appear to compromise organ perfusion. Six patients were anuric and being haemofiltered. Urine output in the other two patients rose following administration of TP. In the patient who received a repeat bolus dose, the pressor effect was similar, though of shorter duration.

Table 5.2A Patient demographics, severity of illness and shock

Patient	Cause of septic shock	Age (yr)	Apache II	Baseline NE ($\mu g\ kg^{-1}min^{-1}$)
1	Dog bite	56	25	0.41
2	Unknown	68	25	0.34
3	Streptococcal pneumonia	50	25	0.85
4	Staphylococcal septicaemia	52	37	0.53
5	Streptococcal septicaemia & liver failure	40	30	0.30
6	Faecal peritonitis	68	31	0.73
7	Salmonella gastroenteritis	82	25	0.73
8	Meningococcal septicaemia	62	28	0.82

Table 5.2B Patient demographics, severity of illness and outcome

Cause of septic shock	TP (mg)	NE ($\mu g\ kg^{-1}min^{-1}$)	Further NE	Outcome
Dog bite	2	0.41	No, needed GTN to lower MAP	Discharged to ward after 1 month
Unknown	1	0.34	Unchanged dose, good pressor response	Died 10 days later
Streptococcal pneumonia	1	0.85	NE weaned over 24 h	Discharged to ward after 2 months
Staphylococcal septicaemia	2	0.53	Effect for 5 h & then steady increase in NE	Died within 24 h
Streptococcal septicaemia & liver failure	2	0.30	Effect for 5 h & then steady increase to 0.7 $\mu g\ kg^{-1}\ min^{-1}$ over 15 h	Died within 24 h
Faecal peritonitis	1	0.73	Weaned off over 24 h	Discharged to ward 14 days later
Salmonella gastroenteritis	1	0.73	Weaned off over 2 days	Discharged to ward 17 days later
Meningococcal septicaemia	1.5	0.82	Continued at low dose for 4 days & then gradual deterioration	Died after 6 days



P<0.0001 for MAP, *P*<0.07 for CO (ANOVA)

Figure 5.3 Terlipressin significantly raises mean arterial blood pressure in septic patients with a non-significant reduction in cardiac output (CO). Data are expressed as the mean ± s.e.m of 8 observations from 8 patients.

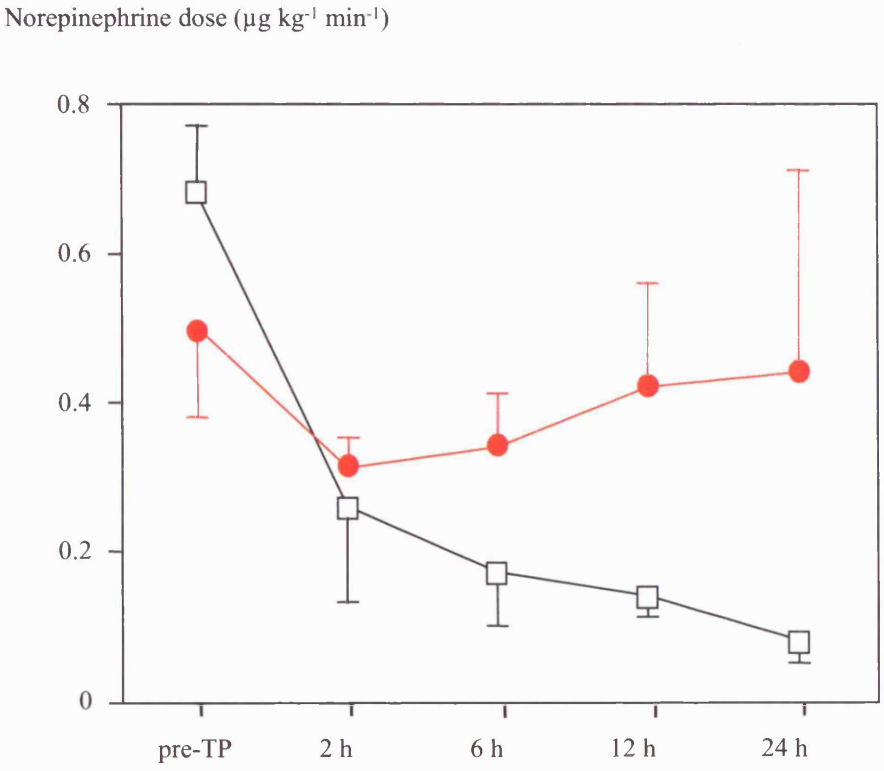


Figure 5.4 The effect of terlipressin on norepinephrine dose in 8 patients with septic shock. The survivors (white squares) had a dramatic, sustained (24 h) reduction in norepinephrine dose whereas the non-survivors (red circles) had only a short term reduction in dose. Data are expressed as the mean ± s.e.m of 8 observations from 8 patients.

5.6 Discussion

In these experiments I used the *in vitro* and *in vivo* models of endotoxin induced rat sepsis previously described in chapters 2 & 3 to investigate the effects of VP and its analogue TP during sepsis. In addition, I also investigated the effect of TP upon human patients with septic shock.

In the *in vitro* model VP was as hyporeactive as PE in LPS-incubated rat mesenteric artery. In addition, there was no contractile response in control tissues incubated for 20 h in culture media with serum, as opposed to the concentration response curves seen with PE. In fresh control tissues, however, there was a significant contractile response to VP. In fresh tissues denuded of endothelium, greater contractions were elicited by VP compared to endothelium intact tissues. Thus, in this *in vitro* LPS model, the vasculature appears highly hyporeactive to VP. LPS-induced hyporeactivity could also not be reversed with the NOS inhibitor aminoguanidine (chapter 2). Aminoguanidine was not added to control tissues incubated for 20 h.

On the other hand, TP did elicit a greater response than NE in the *in vivo* LPS model, whereas the pressor effect was greater for NE in the sham rats. As seen with both inhibition of iNOS and vascular K_{ATP} channels, there was no improvement in the macro and microcirculation despite the improvement in blood pressure in septic rats. Indeed, some adverse effects were noted with both drugs; TP administration resulted in a reduction in ABF and an increase in metabolic acidaemia whereas NE caused a fall in ABF and $HmvO_2$.

In patients the use of a bolus of TP was a success in that a critically low blood pressure, unresponsive to standard therapies, was elevated in all eight patients studied, and a large reduction (or cessation) in NE dosage was achieved in all but one. Four of these patients, who would be at high risk of dying, recovered from their shock and multi-organ failure to leave the ICU alive. The supersensitivity witnessed in septic rats was observed in humans. Though cardiac output initially fell, no obvious deleterious consequences were observed. We are unable to measure regional organ perfusion clinically, although there was no worsening of the metabolic acidosis (arterial base deficit).

Septic shock is associated in patients with a biphasic response in VP levels. In early shock appropriately high levels of VP are produced but, in established and prolonged shock, the levels are similar to those seen in healthy subjects (Landry *et al.*, 1997). The reason for this reduction in circulating concentration of VP is unclear. Depletion of neurohypophyseal stores has been demonstrated (Morales *et al.*, 1999) while NO has been shown to inhibit its release (Reid, 1994). Additionally, several studies have shown that a low-dose infusion of VP will restore blood pressure in septic patients (Landry *et al.*, 1997A; Malay *et al.*, 1999; Holmes *et al.*, 2001; Tsuneyoshi *et al.*, 2001; Patel *et al.*, 2002 and Dunser *et al.*, 2003). I showed, for the first time that the long-acting VP analogue, TP raised blood pressure and reduced NE requirements in septic patients (O'Brien *et al.*, 2002). A number of other studies demonstrating an increase in blood pressure and reduction in NE requirements with TP have been published subsequently (e.g. Leone *et al.*, 2004 and Morelli *et al.*, 2004), including paediatrics and neonates

(Matok *et al.*, 2004). Interestingly, the rebound hypotension requiring recommencement of catecholamines seen in studies when the VP infusion was stopped was not witnessed in half of my patients receiving TP. All of whom were able to be subsequently discharged from intensive care. Similar findings have been reported by Leone *et al.*, (2004) and Morelli *et al.* (2004). This rebound hypotensive effects reported on discontinuation of VP was even seen after infusion for 96 h (Tsuneyoshi *et al.*, 2001). Although TP has a plasma half-life of 6 h, an explanation for the sustained effect on blood pressure is still outstanding. I did not measure plasma levels to see whether its clearance is affected during sepsis. Potentially, it may be more effective at upregulating V1 receptors, thus increasing sensitivity to endogenous VP, or even have downstream intracellular effects. This question is currently undergoing further investigation in the host lab.

The reasons underlying the hypersensitivity of the septic vasculature to VP are unknown. Putative mechanisms (described in chapter 1) include autonomic insufficiency in sepsis unmasking the pressor effect of VP (Landry *et al.*, 1997), synergy with adrenergic agents (Noguera *et al.*, 1997), inhibition of K_{ATP} channels (Wakatsuki *et al.*, 1992) and modulation of NO inhibition (Umino *et al.*, 1999). I saw no evidence of baroreceptor-mediated bradycardia after the bolus of TP although this phenomenon has been considered as supporting evidence for autonomic insufficiency causing a relative deficiency of VP (Landry *et al.*, 1997). All my patients were being treated with NE thus any comment on synergy cannot be made. All patients had been given methylene blue, an inhibitor of both iNOS and guanylyl cyclase, which had not

increased blood pressure indicating that TP's effects were not secondary to modulation of NO. Likewise, corticosteroids, which are known to inhibit iNOS (Korhonen *et al.*, 2002) had had no effect on blood pressure when given pre-TP. Against a synergism between corticosteroids (and/or methlyene blue) and terlipressin was the fact that a similar and prolonged vasopressor effect of TP was seen without these drugs by both Leone *et al.*, (2004) and Morelli *et al.*, (2004),

Concern has been expressed over whether TP or VP may over-constrict some vascular beds causing serious adverse effects. Certainly, all studies using these agents have reported a corresponding reduction in cardiac output in line with the rise in blood pressure. Since I completed my series, there have been instances on the UCLH ICU where TP has been given (inappropriately) to some septic patients with low cardiac outputs. This resulted in considerable worsening of their metabolic acidosis and the clinical manifestations of digital ischaemia (with black fingers and toes) and generalised patchy skin blanching. The latter features have also been seen sporadically in patients with high cardiac outputs, but also in patients receiving high-dose norepinephrine (see Fig 5.5). Thus, current practice on the ICU is to only use the drug in patients with a high cardiac output circulation who have been well fluid resuscitated. Much smaller doses (0.25-0.5 mg) are used, but can be repeated at 30 min intervals to a maximum of 2 mg, watching carefully for any signs of digital, cutaneous, coronary or splanchnic ischaemia. If these should appear, no more TP is given and a prostacyclin infusion is commenced promptly, usually to good effect. This complication has also been shown

with VP. Ischaemic skin lesions were reported in 30 % of patients receiving VP infusions (Dunser *et al.*, 2003B).

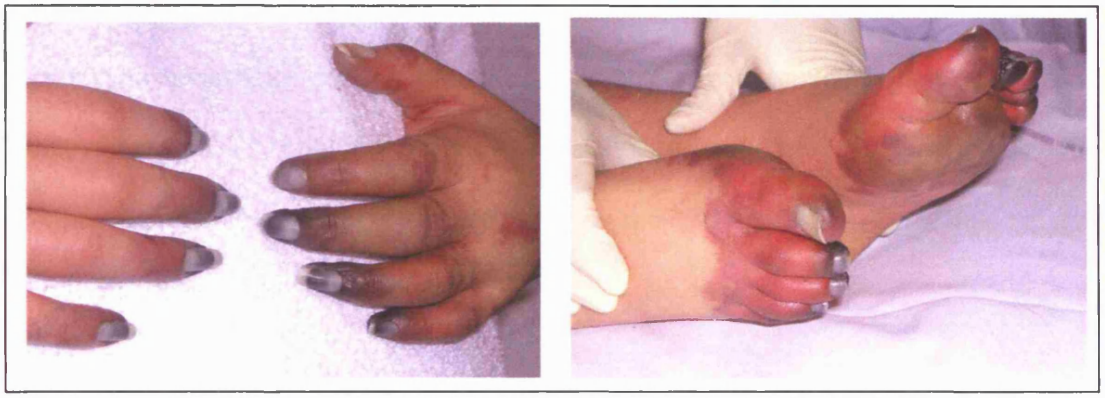


Figure 5.5 Digital ischaemia seen in patients treated with terlipressin (left) and norepinephrine (right).

Pre-existing peripheral vascular disease was found to be a risk factor. In addition, skin necrosis has been reported in one patient after extravasation of VP used to treat septic shock (Kahn *et al.*, 2002). Interestingly, TP (1mg) has been shown to increase gastric mucosal perfusion (Morelli *et al.*, 2003) whereas VP (0.04 units min^{-1}) caused a significant increase in gastric and arterial CO_2 partial pressure, indicating gastric hypoperfusion (van Haren *et al.*, 2003). Both VP and TP have been shown to increase urine output and improve renal function (e.g. Patel *et al.*, 2002 and Morelli *et al.*, 2003). The reasons for this are likely to be a combination of improving blood pressure and thus organ perfusion, an improvement in renal perfusion pressure due to renal efferent arteriolar constriction (see below), and improved renal blood flow by intrarenal vasopressin receptor stimulation. Pulmonary hypertension using either agent has not

been reported; indeed, low dose VP has been used to treat septic shock combined with chronic pulmonary hypertension (Wang *et al.*, 2003). Sublingual microcirculatory flow in a patient with catecholamine-resistant septic shock, as measured by orthogonal polarization spectral imaging, was significantly impaired by TP, although this patient appeared to be in a low cardiac output state (Boerma *et al.*, 2005). Finally, TP (0.5mg) was shown to increase the concentration of L-lactate in the rectal lumen of a patient with septic shock for 8 h (Perner *et al.*, 2004). It may have impaired metabolic dysfunction in the rectal mucosa either directly, via vasoconstriction of mucosal vessels, or through decreased cardiac output.

Clearly, some of the concerns about regional vasoconstriction and organ perfusion are not necessarily born out by the literature so far. However, in view of the serious nature of these potential adverse effects, I would advocate caution when using either VP or TP. At present, I would restrict its use to rescue therapy in patients not responding to conventional treatment with catecholamines and corticosteroids.

In view of the doubts outlined above regarding excessive constriction of vascular beds using TP, I studied its effects in the *in vivo* rat endotoxin model. Increased sensitivity to VP in microvascular beds has been previously demonstrated in rats given endotoxin (Baker *et al.*, 1990). In the rat model I used a 0.02 mg kg^{-1} bolus of TP, which is an equivalent weight-adjusted dose to that given to the septic patients. There was a significantly increased pressor effect in LPS-treated rats compared to sham animals, unlike the pressor effect of NE that was significantly reduced in LPS-treated rats. The

effects of a bolus of TP appeared to last for approximately 75 min (compared to a half-life in humans of approximately 4 h). This may reflect species differences in metabolism by arginases. Clearly, the rise in blood pressure is important in terms of management of septic shock, however, TP in the septic rats caused a reduction in aortic and renal blood flows and a worsening of the metabolic acidosis. In LPS rats treated with NE (the standard catecholamine used for septic shock) there were small non-significant falls in RBF, ABF and $HmvO_2$ while the arterial base deficit was unaffected. This is in contrast to an *in vivo* rabbit model that showed increasing concentrations of VP reduced renal and aortic blood flow in shams but increased renal blood flow velocity and maintained aortic blood flows in endotoxin-treated rabbits (Albert *et al.*, 2004). In a canine model of endotoxic shock, both NE and VP had comparable systemic and splanchnic effects though vasopressin improved renal blood flow and splanchnic oxygen delivery (Guzman *et al.*, 2003). In a sheep model of sepsis, VP alone or in combination with NE maintained blood pressure, mesenteric blood flow and cardiac output, limited increases in lactate and the gut mucosal-arterial pCO_2 gap, and prolonged survival time (Sun *et al.*, 2003). In LPS-treated rats, constriction in renal and mesenteric blood flows following VP have been observed (Bennett *et al.*, 2004) whereas, in another model, VP had no effect upon renal blood flow but there was an improvement in renal function in endotoxic rats. Indeed, VP actually blunted the increase in lactate (Levy *et al.*, 2004). However, these studies used either no fluid or just 5 ml kg^{-1} h^{-1} of crystalloid, unlike my model where 25 ml kg^{-1} h^{-1} of the synthetic gelatin colloid, Gelofusin® was infused throughout the experiment. Fluid resuscitation, usually with colloid, is an integral part of the treatment of septic shock in humans. The

renal effects of VP / TP are complicated and not simply related to renal blood flow. There is an antidiuretic effect through stimulation of V2 receptors but an increase of diuresis / natriuresis through V1 receptors via efferent arteriolar vasoconstriction and oxytocin receptor-mediated stimulation (Rudichenko *et al.*, 1995). I did not study urine output or creatinine clearance and thus am unable to comment on renal function in my model. In addition, there was no improvement of microvascular oxygenation in the septic rat despite the significant improvement in blood pressure; this indicates persistence of microcirculatory defects, possibly related to local shunting. Neither inhibition of iNOS nor K_{ATP} improved microvascular oxygenation despite a significant improvement in blood pressure (see chapters 2 & 3). Arginine vasopressin has been shown to impair tissue oxygenation and the microcirculation in hamsters (Friesenecker *et al.*, 2004) and to decrease intestinal oxygen supply and mucosal tissue PO_2 by reducing microvascular blood flow in a dose-dependent manner in pigs (Knotzer *et al.*, 2005). However, neither of these experiments was performed in fluid resuscitated or septic animals. Reasons for the differences reported between the different models may thus be related to duration of study, dose of endotoxin/sepsis, dosage of VP, and the degree of fluid resuscitation. There is a paucity of experimental models using TP.

My animal model thus confirms the super-sensitive pressor effect of TP in sepsis. In terms of regional vasoconstriction it appears no more dangerous than NE, although at one time point (180 min) the metabolic acidosis was significantly higher in LPS-treated rats given TP compared to untreated endotoxic animals. Microcirculatory oxygenation did not improve despite the restoration of blood pressure. In future studies I would

examine the effects of lower doses and also assess the combination of TP and NE which would complement my human study.

Contrary to my findings in both patients and the *in vivo* model, in the *in vitro* LPS model the contractile response to VP was decreased, and even less than that seen for PE. This hyporeactivity to VP was not reversed by inhibition of NOS. As addition of VP was able to produce contractions in fresh control tissue, possible explanations for the disparity in results include activation of nitric oxide synthase; the *in vitro* absence of a necessary plasma factor; the lack of an intact autonomic nervous system; and an alteration in VP receptor expression or activity, or in downstream pathways leading to calcium release.

In chapter 2, I described the production of iNOS in controls incubated for 20 h and the production of nitrite within culture media supernatant in which these tissues had been incubated. Thus, sufficient NO may be produced via iNOS in control tissues at 20 h to induce hyporeactivity to VP. In future studies, I would use the iNOS inhibitor 1400W rather than aminoguanidine as it had a greater effect at 20 h. As inhibition of either iNOS or the K_{ATP} channel (via the pore-forming subunit) reversed LPS-induced hyporeactivity in this same model (see chapters 2 & 4), a direct effect of VP on either of these pathways is unlikely (Wakatsuki *et al.*, 1992 and Umino *et al.*, 1999).

In contrast to my results, VP has been shown in other septic models to have an effect *in vitro*. In human gastroepiploic arteries, VP potentiated responses to NE in both normal

and endotoxin-treated tissues via action on V_1 receptors, (Hamu *et al.*, 1999). Similarly, studies in rats showed that VP strongly potentiated the vasoconstrictor effects of NE, electrical field stimulation and potassium chloride depolarization, effects mediated *via* an increase in intracellular calcium through voltage-dependent calcium channels (Norguera *et al.*, 1997). A further possible explanation for the lack of an effect *in vitro* is that VP may have differing vasoconstrictor properties according to the vascular bed examined. However, against this hypothesis was the fact that VP produced significant contractions in fresh control tissue in my studies. The differences in contractile responses observed in endothelium-intact or denuded fresh mesenteric artery tissue may be explained by the presence of V_2 and/or oxytocin receptors on vascular endothelium. V_2 receptor activation has been demonstrated to cause vasodilatation (Bichet *et al.*, 1988, see introduction).

In conclusion, TP produced a profound rise in blood pressure in septic patients, allowing reduction and/or cessation of NE. This drug offers a potentially important and inexpensive therapeutic alternative in hypotensive septic patients not responding to high-dose catecholamine therapy that appears easy and generally safe to administer. Its mechanisms of action have not been entirely clarified and concern remains that it may cause severe vasoconstriction in certain vascular beds. In my *in vivo* animal model its supersensitive pressor effects on blood pressure in septic rats compared to NE was confirmed. However, no improvement was seen in macro or micro-circulatory organ perfusion and there was some worsening in metabolic acidosis. The vasoconstrictive effects of VP in the mesenteric, pulmonary, renal and coronary beds are dose-

dependent; had I used a lower dose of TP there may have been a similar pressor effect associated with improved flows. I would thus advocate that it be used in human patients with great care and only when conventional treatment with fluid, catecholamines and steroids has failed. I would also use low incremental doses of 0.25-0.5 mg. Ideally, large multi-centre outcome studies should be performed with TP, as is currently ongoing with VP (Cooper *et al.*, 1993). In view of its frequent ability to reverse hypotension with a single dose, a comparison of TP against VP would also be of interest.

Chapter 6

Summary and Discussion

6.1 Introduction

The dramatic collapse of the cardiovascular system that occurs during vasodilatory septic shock is both fascinating and frightening. When I began my research, the management of septic shock had changed little over several decades, with the mainstays being fluid resuscitation, antibiotic therapy and the use of catecholamine vasopressors (reviewed in Deitch, 1998). These are still critical components of patient care however recent years have seen significant advances in management such as the use of hydrocortisone and recombinant activated protein C (Bernard *et al.*, 2001 and Annane *et al.*, 2002). Yet septic shock still carries a high attendant mortality, both in the short term - 56% in one recent study (Annane *et al.*, 2003) - and in the longer term – for example, Annane *et al.*, reported 20% of survivors dying within a year of hospital discharge (Annane *et al.*, 2002).

The stated aims of my thesis were to investigate mechanisms underlying vascular hyporeactivity and hypotension (*i.e.* cardiovascular collapse) in sepsis. To achieve this I embarked upon the development of both *in vitro* and *in vivo* rat models. Although the rat's response to sepsis does not precisely mimic that seen in humans, I hoped that lessons learned from these animal experiments could be extended to the critically ill patient, and performed studies accordingly. I was specifically interested in the contribution of three putative mechanisms: the nitric oxide (NO) pathway, the ATP-gated potassium (K_{ATP}) channel, and vasopressin. I naturally wished to add to the existing knowledge on this complex subject but my great hope was that my studies would lead to meaningful advances in the clinical management of septic shock.

6.2 Summary of Results

These are described in greater detail in the discussions accompanying the individual chapters but are summarised here.

6.2.1 Development of Models

I was able to successfully develop a reproducible *in vitro* organ culture model of rat sepsis in which endotoxin (LPS) induced vascular hyporeactivity in the mesenteric artery to the α_1-agonist, phenylephrine (PE). This hyporeactivity occurred over a range of concentrations of LPS (0.01 ng ml^{-1} to 100 µg ml^{-1}) and was dependent upon time (up to 46 h) and the presence of serum. I found that an intact endothelial layer was not necessary to maintain hyporeactivity after incubation with LPS.

I created a similar model using the human marginal artery (a branch of the mesenteric circulation). I used a combination of LPS (at high concentration) and the pro-inflammatory cytokine interleukin 1-β that induced significant hyporeactivity to PE. Alas, the iNOS inhibitor 1400W was unable to reverse this hyporeactivity.

I was also able to develop a reproducible short-term *in vivo* rat model of septic shock with monitoring of both macro- and microcirculation by adapting the model used previously by our group (Rosser *et al.*, 1995). In this model, an LPS infusion induced an early myocardial depression with falls in blood pressure and organ perfusion. Although blood pressure was spontaneously restored within 15 min, macrovascular organ flow

took much longer to recover, and then only partially. This required major intravascular volume resuscitation with a colloid solution. As is characteristic of such models, the blood pressure eventually fell after several hours. In addition, the hepatic microvascular circulation, as assessed by the protoporphyrin technique, an indicator of the balance between local oxygen supply and demand, fell sharply after LPS injection and also failed to show significant improvement despite fluid resuscitation. This suggests an ongoing pathological mechanism involving microcirculatory flow.

6.2.2 Effects of nitric oxide inhibition

In the rat *in vitro* model, inhibition of either iNOS or soluble guanylyl cyclase reversed LPS-induced hyporeactivity, confirming the importance of the NO-cGMP pathway. This was more notable at earlier time points (6 h) with the effects subsequently diminishing over the next 46 h. I was able to demonstrate increased NO production by finding elevated nitrite levels within the culture media in which the artery had been incubated. I further demonstrated iNOS production within the artery using immunohistochemical analysis. This, too, was time-dependent with maximal iNOS seen at 46 h but also significant amounts were seen at 20 h. Although mostly localised to the adventitial layer, iNOS was also seen in the endothelial and smooth muscle layer.

iNOS inhibition reversed LPS-induced hypotension *in vivo*. Administration of the specific iNOS inhibitor GW273629 succeeded in elevating blood pressure in both the LPS- and sham-treated rats, but was more pronounced in LPS-treated rats. Immunohistochemistry revealed a greater production of iNOS in the LPS-treated rat

compared to the sham in rat mesenteric artery (RMA) removed at the end of the experiment and GW273629 inhibited this production of iNOS. This may reflect an unknown mechanism of action of this drug or it may be due to the drug interfering with the stain. As I only performed this experiment in 1 rat, I cannot make further comment upon this. In the human marginal artery model, the iNOS inhibitor 1400W had no effect upon contractile responses to PE. Although, on face value, this would suggest that iNOS-derived NO would have no part to play in human sepsis, the finding in studies of patients with septic shock that L-NMMA increased blood pressure, albeit at the expense of a higher mortality, does highlight the importance of nitric oxide in the vascular hyporeactivity affecting the human being.

6.2.3 Effects of K_{ATP} inhibition

I was able to demonstrate that the K_{ATP} channel is involved in mediating LPS-induced vascular hyporeactivity *in vitro*. Importantly, I showed that inhibitors of the pore-forming subunit of the K_{ATP} channel, but not the sulphonylurea (SUR) subunit, were able to reverse LPS-induced vascular hyporeactivity. This difference was unrelated to the SUR inhibitors' ability to inhibit thromboxane contractions. After 6 h incubation with LPS, the activation of BK_{Ca} channels was also involved in LPS-induced vascular hyporeactivity, but to a lesser extent. After 20 h although there was still reversal of hyporeactivity, though now incubation mechanisms independent of K^+ channel activation appeared to be important. Administration of the K_{ATP} channel inhibitors PNU-37883A and glibenclamide at high dose (10 mg bolus and 5 mg kg^{-1} h^{-1} infusion) *in vivo* succeeded in elevating blood pressure in both shams and to a lesser extent LPS-

treated rats (both agents probably acting on the pore). The rise in blood pressure in the LPS-treated rats did not correspond to an improvement in macro or micro-circulatory blood flow.

6.2.4 Effects of the vasoconstrictors U-46619, vasopressin terlipressin and norepinephrine

In contrast to PE, I found that contractions achieved in response to the thromboxane mimetic, U-46619 were only weakly affected by LPS *in vitro*. The differential effect of LPS upon PE and U-46619 did not appear to be a NO-mediated phenomenon, as the NO donor SNAP caused an equipotent relaxation to contractions with either agonist in fresh rat mesenteric vessels. Contractions to vasopressin (VP) *in vitro* were more sensitive in LPS-treated vessels compared to that seen with PE. *In vivo* administration of a bolus of the long-acting VP analogue, terlipressin (TP), or an infusion of the α_1 adrenergic agonist, norepinephrine (NE), succeeded in elevating blood pressure in the LPS-treated rats. However, the pressor effect induced by NE was greater in sham rats, while the converse was found with TP which elicited a hyper-sensitive response in LPS-treated rats.

In patients with septic shock refractory to fluid resuscitation, conventional vasopressor and steroid treatment, I also performed a novel study assessing the impact of bolus treatment of TP. In all patients, the TP bolus produced an immediate and sustained elevation in mean systemic arterial pressure, enabling major reductions or cessation of

the high-dose NE infusions. Though cardiac output fell from initial high levels, no effects were seen on heart rate and no obvious deleterious consequences were noted.

In summary, I have shown that LPS given to rats results in increased production of nitric oxide from iNOS (especially within the adventitial cell layer), and that selective inhibition of the nitric oxide pathway reverses the resulting hyporeactivity and hypotension. I have shown that *in vitro* vascular hyporeactivity can also be reversed by inhibition of the K_{ATP} channel via its pore-forming subunit but not via its SUR, suggesting an endotoxin-induced dissociation. I believe that this effect also occurred *in vivo*. However, endotoxin reduced the ability of glibenclamide and PNU-37883A to inhibit the K_{ATP} channel. In addition, I was able to demonstrate that TP could also reverse LPS-induced hypotension. Although manipulation of these three pathways were all successful in elevating blood pressure, no improvement was seen in either blood flow within the large vessels (renal and aortic blood flows), or the microvascular circulation (as assessed by $HmvO_2$), or in metabolic acidaemia. Indeed, the latter was worsened by TP.

Of the three mechanisms investigated in the laboratory, only TP was used therapeutically as a rescue therapy in patients with severe septic shock not responding to conventional treatment. That four of the eight treated patients subsequently survived is a highly encouraging finding that has been since reproduced by other investigators (Leone *et al.*, 2004 and Morelli *et al.*, 2004); in addition it has also been used in paediatrics and neonates (Matok *et al.*, 2004). Interestingly, there was no 'rebound'

hypotension observed in the patients that survived (as opposed to those treated with a VP infusion which is then discontinued e.g. Tsuneyoshi *et al.*, 2001). This difference clearly warrants further investigation; it may be related to specificity of the two drugs for the different VP receptors or, possibly, other mechanisms.

6.3 Critique of models used

The majority of my work was performed using animal models. Extrapolation of animal data to human patients is fraught with controversy and, clearly, not all of my findings may be relevant to the patient. However, it is important to study mechanisms and to evaluate putative treatments for both benefit and harm in animals prior to human testing. Leaving aside baboon models which cannot be undertaken in the U.K., the pig's circulation is considered the closest to humans but these animals are extremely expensive to buy and store, not to mention unwieldy. I thus chose the rat, aiming to take my investigations from this animal to a human experimental model, and thence to patients.

Most *in vitro* studies investigating sepsis in rats have used the aorta. This, however, is a conduit vessel while vascular resistance within the arteriolar circulation is predominantly responsible for determining blood pressure. I used mesenteric artery which, although considerably smaller than aorta, is also a conduit vessel. A potential future direction would be to study the mesenteric arteriolar circulation using myography.

How relevant is an *in vitro* model in the investigation of sepsis? The pathophysiology of sepsis is far more complicated than simply the effects of circulating endotoxin upon blood vessels. An *in vitro* model does not examine important mechanisms such as autonomic pathways and hormonal influences e.g. glucocorticoid deficiency and the renin- angiotensin system (reviewed by Annane, 2005). In addition, a crucial aspect of treatment is fluid resuscitation that cannot be mimicked *in vitro*.

Using my *in vivo* model I was able to fluid resuscitate the rat with synthetic gelatin colloid, Gelofusin® – the colloid most used in human patients at UCL Hospitals (UCLH). In addition, I was able to compare the effects of iNOS inhibition, K_{ATP} channel inhibition and terlipressin directly with norepinephrine –the catecholamine most commonly used to treat high output human septic shock at UCLH. I was also able to monitor the effects of LPS and the different pharmacological modulators upon mean arterial blood pressure, macro and microcirculatory blood flow in a highly reproducible fashion. However, it was not without weakness. The use of a bolus of LPS is less relevant than a caecal puncture model; perhaps this provides too 'toxic' an insult. Of note, despite the aggressive fluid resuscitation, the model was not truly hyperdynamic and the microcirculatory flow never recovered despite fluid and pressor treatment. As the incidence of Gram-positive infections has now overtaken that of Gram-negative infections (Greenberg *et al.*, 2005), it may have been interesting to compare responses generated by a Gram positive toxin such as lipoteichoic acid. The model I used was short term (4 h) and the rat was sacrificed at the end of the experiment thus no information is providing on any survival benefit that may have accrued. In addition, I

did not use antibiotics. Though clearly an important part of the treatment of sepsis, it is not particularly relevant in a 4-hour model. Furthermore, the rat was anaesthetised with isoflurane, which does have some K_{ATP} channel opening properties (Ida *et al.*, 1998 and Sumikawa *et al.*, 1998), and the instrumentation performed did induce some production of iNOS within the sham animals (as demonstrated by immunohistochemistry of the mesenteric artery). Overall, however, I do believe this to be a reasonable model to study mechanisms of vascular hyporeactivity and it is clearly more relevant to the clinical situation than an *in vitro* model.

Interpretation of my data also relies heavily upon the specificity of the inhibitors used. This is potentially a weakness; however there is currently good evidence that both 1400W and GW273629A are highly specific iNOS inhibitors (Garvey *et al.*, 1997; Young *et al.*, 2000 and Alderton *et al.*, 2005), and that PNU-37883A and PNU-99963 are highly specific inhibitors of the K_{ATP} pore-forming subunit and the SUR, respectively (Khan *et al.*, 1997 and Humphrey 1999). Future studies may nevertheless show these compounds to have additional effects on other pathways.

6.4 Discussion

As mentioned in my introduction, clinical trials of treatments for sepsis are difficult due to the heterogeneity of patients, highly variable comorbidities, and the high rates of culture negative sepsis. Thus, large scale multi-centre studies are often required to demonstrate benefit. I clearly did not have the resources to undertake such a project. However, I was able to conduct a brief non-randomised, uncontrolled study of

terlipressin in patients that yielded some important results that have been since adopted by the host ICU and many others with success. An adequately powered, randomised trial is clearly needed to confirm the anecdotal benefit seen, and to identify subsets in whom in could be harmful, such as those with low cardiac output states.

I was able to develop an *in vitro* model of LPS + cytokine-induced vascular hyporeactivity using human marginal artery, however I was unable to reverse this with iNOS inhibition. There is a relative paucity of human *in vitro* models reported in the literature. These tend to use cardiac vessels that have little role in vascular resistance in sepsis, unlike the mesenteric circulation which thus seemed a natural choice. I was unable to progress much beyond establishing hyporeactivity due to lack of tissue but this certainly warrants further study.

Norepinephrine is the most commonly used vasopressor to maintain blood pressure in high cardiac output septic shock. However, it increases production of the pro-inflammatory cytokine tumour necrosis factor via the α_2-adrenergic receptor (Splengler *et al.*, 1990). Perhaps epinephrine (a beta-adrenergic agonist) may prove a better choice, at least from a pro-inflammatory point of view, as it inhibits TNFα, enhances production of interleukin 8 and suppresses production of NO (Severn *et al.*, 1992; van der Poll *et al.*, 1996 & 1997 and Zinyama *et al.*, 2001). Interestingly, both NE and the beta-adrenergic agonist dobutamine have been shown to accelerate bacterial growth and biofilm formation (Lyte *et al.*, 2003) while catecholamines have profound, dose-related metabolic effects (reviewed in Annane *et al.*, 2005). Thus, even though NE is presently

recommended as a first line pressor agent, it may be causing surreptitious harm that may not be appreciated by the treating clinician. Small randomised trials in patients have yielded conflicting results as to which pressor agent is superior/less harmful (Hollenberg *et al.*, 2004 and Mullner *et al.*, 2004). There are currently a number of large, multicentre trials in septic patients. One is comparing epinephrine, dobutamine & NE (CATS), another dopamine and NE, and a third is comparing VP and NE (the VAST study).

Where does vasopressin, or its analogue terlipressin, fit in? There are good theoretical reasons for it to be an ideal vasoconstrictor. It potentiates the effects of NE (Norguera *et al.*, 1997 and Hamu, 1999), it is a K_{ATP} channel inhibitor (Wakatsuki *et al.*, 1992) and it can blunt the effects of NO (Umino *et al.*, 1999). The reasons for its increased potency as a vasoconstrictor in septic shock states have yet to be elucidated. Landry suggests restoration of altered baroreflex sensitivity (Landry *et al.*, 1997A), while others have suggested replacement of depleted plasma levels (Morales *et al.*, 1999). A further possibility, currently under investigation in the host lab, is that VP sensitivity may be enhanced by altered receptor binding and/or changes in downstream signalling or intracellular calcium release. In clinical trials of septic shock, vasopressin has been predominantly used by continuous infusion at concentrations ranging from 0.01-0.04 IU/min (Malay *et al.*, 1999; Tsuneyoshi et *al.*, 2001; Patel *et al.*, 2002 and Dunser *et al.*, 2003A). In these and other studies haemodynamics were improved and catecholamine requirements decreased. However, rebound hypotension has been observed when the drug is discontinued and this can last for several days, necessitating recommencement

of vasopressin or re-institution of a norepinephrine (Tsuneyoshi et *al.*, 2001). There also remains concern over myocardial, cutaneous or mesenteric vasoconstriction which are recognised side-effects of VP and its analogues in the treatment of oesophageal varices. Dunser reported ischaemic skin lesions in 30% of patients receiving VP infusions (Dunser *et al.*, 2003B). Holmes reported a high overall mortality rate (85%) in a retrospective study of septic patients given vasopressin, and these predominantly occurred in patients given higher doses (≥0.04 U/min), often due to cardiovascular complications (Holmes *et al.*, 2001). Results are eagerly awaited from the VAST trial, mentioned above, currently underway in Canada (and Australasia) before recommendations for use are made.

I used the long acting analogue TP as rescue therapy for refractory hypotension in septic patients not responding to high dose NE, steroids and the NO pathway inhibitor, methylene blue. I did not encounter rebound hypotension or evidence of myocardial, cutaneous or mesenteric vasoconstriction though this has been seen subsequently in patients whose baseline cardiac output was lower in both the host intensive care unit and others. In my experience, it had profound effects upon blood pressure and NE requirements, as described in more detail in chapter 5. However, given the concerns over vasoconstriction in vascular beds, I would not advocate its use as a first-line treatment at present and would exercise more caution over dosing, perhaps starting with smaller bolus doses, e.g. 0.25-0.5 mg. I nevertheless believe that I have shown it has an important role in the treatment of refractory hypotension as mortality in such patients is

extremely high. Albeit in a small series, I could demonstrate a disproportionately high survival rate.

Although terlipressin, nitric oxide synthase inhibition or K_{ATP} channel blockade can improve blood pressure in septic shock models, is this necessarily beneficial? In my *in vivo* model all three approaches successfully elevated mean arterial pressure in LPS-treated rats; by comparison, the pressor effect of norepinephrine was blunted. Yet none of these agents improved either macro or microcirculatory blood flow, nor attenuated the development of metabolic acidosis in the rat. It is perhaps a sad indictment that despite years of researching and treating septic shock that we still do not know what optimal blood pressures we should target in an individual patient (Bourgoin *et al.*, 2005). Clearly, there will be inter-patient variation depending on their baseline blood pressure, yet the tendency over the last decade has been to aim for lower values (e.g. mean arterial blood pressure of 60 rather than 80 mmHg). Perhaps this is in belated recognition of the potential harmful effects of these agents. The multicentre study comparing the non-specific NOS inhibitor L-NMMA against norepinephrine in septic patients that was prematurely abandoned because of increased harm showed that the L-NMMA group achieved consistently higher blood pressures (Lopez *et al.*, 2004). Whether this is causally related to the worse outcomes requires further study.

In my *in vivo* model, the specific iNOS inhibitor GW273629A did not cause any adverse effects in terms of reduction of macrocirculatory flow or worsening of metabolic acidosis. Indeed, across both *in vitro* and *in vivo* models, the specific iNOS

inhibitors had the best and most consistent effects against LPS-induced hyporeactivity and hypotension. Again, there are other reasons beyond its pressor effect why inhibiting the overproduction of NO might be beneficial in sepsis *e.g.* NO has an important role in mitochondrial dysfunction (reviewed by Singer & Brealey, 1999). Yet, as mentioned above, non-selective inhibition of nitric oxide synthase was associated with an increased mortality from septic shock in a large scale randomised clinical trial (Lopez *et al.*, 2004). Thus NO derived from constitutive NO synthases may offer a degree of protection and thus removing this may prove detrimental. Indeed, constitutive NO synthase is essential for homeostasis, cellular immunity, endothelial function (Thiemermann, 1997). I believe that my work supports the notion that an inhibitor of inducible NO synthase deserves further consideration. Whether a pharmaceutical company is willing to invest in the development of a selective iNOS inhibitor for use in humans following the failure of the Glaxo Wellcome-led phase III study of a non-specific NOS inhibitor (Lopez *et al.*, 2004) remains to be seen. Certainly, parallels may be drawn with the re-emergence of steroid therapy for sepsis. Doses of 200 mg/day of hydrocortisone have been shown to reduce the duration of shock, systemic inflammation and mortality (Annane *et al.*, 2002). This dose is considerably lower than the 30 mg kg^{-1} methylprednisolone used in earlier sepsis studies in which survival was not improved and outcome may even be worsened, possibly through increasing the frequency of secondary infections (Cronin *et al.*, 1995).

K_{ATP} channels may be activated in septic shock by a number of mechanisms. For example, decreases in cellular ATP concentration and/or increases in cellular

concentrations of hydrogen ion and lactate (Keung 1991 and Davies 1990); neurohormonal activators – atrial natruietic peptide, calcitonin-gene related peptide and adenosine (Quayle 1997, Arnalich *et al.*, 1996, and Martin, 2000); and via NO through a cyclic guanosine monophosphate (cGMP) – dependent mechanism (Murphy & Brayden, 1995). K_{ATP} inhibitors may also have benefits aside from raising blood pressure, for instance the large conductance calcium activated K^+ channel is essential for innate immunity (Ahwullia *et al.*, 2004). I have shown that inhibiting the K_{ATP} channel via its pore-forming subunit reverses LPS-induced hyporeactivity *in vitro*. *In vivo* the differential effect on the pore-forming subunit of the K_{ATP} channel seen *in vitro* was probably observed in that the effect upon blood pressure was only seen with PNU-37738A and high dose glibenclamide. However, the effects of glibenclamide and PNU-37738A on blood pressure were greater in shams than LPS-treated rats. It would appear that endotoxin *in vivo* reduces the ability of glibenclamide and PNU-37738A to inhibit the K_{ATP} channel. Among the K_{ATP} channel inhibitors available for use in man are glibenclamide, tolbutamide and gliclazide. Glibenclamide could potentially be trialled in humans; however an intravenous preparation is not available in the U.K. while gastric function is reduced in septic shock, thus making the absorption of oral medication unreliable. I did administer 1 mg kg^{-1} of intravenous glibenclamide over 5 min (obtained from Hoechst, Germany) to one septic patient (in whom ethics committee permission had been obtained) but this had no haemodynamic effect. Had I used a higher dose (as with my *in vivo* model), there may have an increase in the patient's blood pressure. As my supply of intravenous glibenclamide was limited and then

curtailed by the manufacturers no longer making this preparation, I was unable to test this hypothesis further.

I did observe adverse effects of these agents in the *in vivo* model. Terlipressin, norepinephrine and PNU-37883A all caused a reduction in aortic blood flow while terlipressin and PNU-37883A worsened metabolic acidaemia. The observed adverse effects with terlipressin further strengthen the need for caution with this agent. It may be that the rat is more sensitive (I used an equivalent weight-adjusted dose to that used in the patients) and that a lower dose would have been as effective with fewer adverse effects. However, I do not believe that these findings necessarily imply despondency for terlipressin and K_{ATP} inhibition. As mentioned above, the bolus of LPS may have been too toxic both for the microcirculation and to create a truly hyperdynamic model. It may be that fewer adverse effects are seen in a longer term model, e.g. using caecal ligation and puncture. In addition, a combination of treatments may be required (as described in chapter 3). A future approach may see terlipressin and/or a K_{ATP} inhibitor used in combination with a NO donor.

The mechanisms underlying hyporeactivity and hypotension may be more physiological than pathological. Perhaps such a blanket approach that inhibits selected factors contributing to the inflammatory cascade is unwise. Certainly, mediator directed therapy has thus far failed to improve survival in sepsis (Marshall, 2003). We are interfering with mechanisms that we do not fully understand. For example, the precise reasons why hydrocortisone and activated protein C have been successful treatments are

far from clear as both have multiple effects. A future direction might be a more specific strategy using lower, more 'physiological' concentrations of vasopressin, or inhibition of pathways downstream of nitric oxide or the K_{ATP} channel.

Treating hypotension in sepsis is considered crucial in improving organ perfusion. However, despite an increase in MAP in my *in vivo* model, there was no corresponding improvement in blood flow and some adverse effects were noted. Aggressively treating hypotension in septic shock might not actually represent optimum treatment. Other goals are also considered in patients within the intensive care unit but an 'adequate' blood pressure is a predominant target for vasopressor and fluid resuscitation requirements. The same criticism can be applied to *in vivo* animal studies that also largely examine the effects of agents upon blood pressure. While obviously important, perhaps a superior approach in the future will be to use probes that can assess flow and the adequacy of perfusion within various vascular beds so that we might titrate vasoconstriction, fluids and other vasoactive drugs accordingly.

6.5 Conclusion

In conclusion, I have provided evidence that nitric oxide, vascular K_{ATP} channels and vasopressin all play a role in the vascular hyporeactivity and hypotension of sepsis in rat models, and that terlipressin can be used to successfully treat refractory hypotension in humans. However, I remain unsure as the future role that manipulation of these mechanisms will play in the treatment of septic shock. Do these mechanisms represent a purely pathological process or a degree of physiological adaptation developed over millennia that we would be well advised to study further, rather than to ignore, or to impatiently and blindly manipulate, albeit for altruistic motives? I would certainly advocate the former. I believe that potential use of agents manipulating these mechanisms should be restricted at present to rescue therapy in humans not responding to conventional treatments until we better understand their beneficial and detrimental effects. This is perfectly illustrated by the Lopez *et al.,* (2004) multicentre study using a non-specific NOS inhibitor in patients with septic shock. Only a minority of patients in the treatment group actually had high cardiac output vasodilatory septic shock, precisely the group that the vast amount of previously published work in animals and humans would predict to benefit. Secondly, post-hoc analysis revealed survival benefit from low doses but increasing harm from higher doses. Surely, this ill considered approach should not be repeated.

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